

Molecular Analysis of *CPR* α , a *MAT* α -Specific Pheromone Receptor Gene of *Cryptococcus neoformans*

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The putative *Cryptococcus neoformans* pheromone receptor gene *CPR* α was isolated and studied for its role in mating and filamentation. *CPR* α is *MAT* α specific and located adjacent to *STE12* α at the *MAT* α locus. It encodes a protein which possesses high sequence similarity to the seven-transmembrane class of G-protein-coupled pheromone receptors reported for other basidiomycetous fungi. Strains containing a deletion of the *CPR* α gene exhibited drastic reductions in mating efficiency but were not completely sterile. Δ *cpr* α cells displayed wild-type mating efficiency when reconstituted with the wild-type *CPR* α gene. Hyphal production on filament agar was not affected in the Δ *cpr* α strain, indicating no significant role for *CPR* α in sensing environmental cues during haploid fruiting. The wild-type *MAT* α *CPR* α strain produced abundant hyphae in response to synthetic *MAT* α pheromone; however, the hyphal response to pheromone by Δ *cpr* α cells was significantly reduced. Exposure of wild-type cells to synthetic *MAT* α pheromone for 2 h induced *MF* α pheromone expression, whereas unexposed cells showed only basal levels of the *MF* α transcript. The Δ *cpr* α cells, however, exhibited only basal levels of *MF* α message with or without pheromone exposure, suggesting that *CPR* α and *MF* α are components of the same signaling pathway.

Cryptococcus neoformans is the etiologic agent of cryptococcosis, one of the most serious fungal diseases encountered by immunocompromised patients worldwide (26). The fungus is a bipolar heterothallic basidiomycete in which the meiotic cycle is dependent upon interactions between cells of the *MAT* α and *MAT* α types (24). The initial interaction between cells of the two compatible mating strains is believed to involve pheromone-receptor pairs of both mating types (34). In *Saccharomyces cerevisiae*, *MAT* α cells secrete an *a*-factor pheromone and express the α -factor receptor Ste2p whereas *MAT* α cells secrete an α -factor pheromone and express the *a*-factor receptor Ste3p. Both receptors belong to a large class of G-protein-linked seven-transmembrane-domain receptors (reviewed in references 23, 29, and 40) and are expressed mostly at the tips of schmoos, where cell fusions subsequently occur (23). Binding of pheromone to the receptor induces the pheromone response signal transduction pathway via the mitogen-activated protein (MAP) kinase cascade, which leads to activation of genes required for mating (19, 23). For *C. neoformans*, identification of the *STE3* homolog has been reported but detailed information is lacking (30).

The mating system of *C. neoformans* has received considerable attention due to the preponderance of *MAT* α strains among both environmental and clinical isolates (25, 44) and its increased virulence compared to the *MAT* α type (28). Recently, a physical map of the *MAT* α locus from the *C. neoformans*

strain B-4500 (JEC21), chosen for the *C. neoformans* genome project, was constructed (21). In contrast to other fungi, homologs of several *S. cerevisiae* pheromone response MAP kinase cascade genes such as *STE20* α , *STE11* α , and *STE12* α , as well as three copies of the *MAT* α pheromone gene, were found embedded in the *MAT* α locus (21). Partial sequencing of the *MAT* α locus also revealed a gene located at the 5' end of *STE12* α that had high degrees of homology with the pheromone receptors of basidiomycetous fungi such as *rcb3* of *Coprinus cinereus* (35), *bbr2* of *Schizophyllum commune* (16), and *pra1* of *Ustilago maydis* (4) and a lesser degree of homology to the yeast *STE3* sequence (18). The gene was named *CPR* α (cryptococcal pheromone receptor, α mating type). Interestingly, a similar genomic organization of the locus bearing the *STE3* homolog was recently reported for *Pneumocystis carinii*, a species phylogenetically unrelated to *C. neoformans* (38). The *P. carinii* ortholog of *STE3* was present in the midst of a cluster of pheromone response signal transduction cascade genes, including an *STE12* homolog. Although a sexual life cycle has been proposed for *P. carinii*, there has been no clear indication that this organism is heterothallic and it is unknown whether these genes are mating type specific.

The known pheromone receptors of basidiomycetous fungi also contain the seven potential membrane-spanning domains characteristic of receptors that couple to heterotrimeric G proteins. In homobasidiomycete species such as *Coprinus cinereus* and *Schizophyllum commune*, however, pheromones and receptors play no role in mate attraction and fusion but promote the formation and maintenance of a dikaryon after cell fusion. In *U. maydis*, a heterobasidiomycete species, pheromones and receptors are essential for mate attraction and

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haploid cell fusion, which lead to the formation of infectious dikaryons (6). For this reason, pheromone and receptor genes of *U. maydis* are considered pathogenicity genes. Although the *CPR α* gene was predicted to encode a functional receptor protein for the *MAT α* pheromone, it has not been characterized. In this paper, we report the isolation and characterization of the pheromone receptor gene *CPR α* , expressed by *MAT α* strains of *C. neoformans*.

MATERIALS AND METHODS

Strains and media. All strains used in this study were serotype D, *MAT α* or *MAT α* mating type, derived from the congenic set B-4500 (JEC21; *MAT α*) and B-4476 (JEC20; *MAT α*). YEPD medium contained 1% yeast extract, 2% Bacto Peptone, and 2% dextrose. YNB with glucose medium (pH 6.0) was used as a minimal medium (MIN) and contained 6.7 g of yeast nitrogen base without amino acids (Difco) and 20 g of glucose per liter. Synthetic low-ammonium dextrose (SLAD) agar (17) with slight modification (11) was used to test the morphological response of cultures to synthetic *C. neoformans MAT α* pheromone (Mfap) as well as the accumulation of *MF α* transcript in response to synthetic Mfap. To observe the formation of hyphae in B-4500 and the Δ *cpr α* mutant in response to the presence of the opposite mating type, SLAD agar (11) was used. Filament agar (45) was used for haploid fruiting, and V8 juice (27) agar was used for crossing.

Identification of the *CPR α* gene. A 20-kb *Bam*HI fragment containing the *STE12 α* gene was cloned from a cosmid library of B-4500 (21) and sequenced by standard methods (37). Databases were searched to identify genes homologous to those found on the fragment. A gene that encoded a putative protein with high sequence identity (2e-41) with several pheromone receptors of basidiomycetous fungi was identified and named *CPR α* (see Fig. 1). A partial cDNA clone lacking the 5' end of *CPR α* was initially isolated from a cDNA library of B-3501 (15). The complete cDNA sequence was determined by using the SMART RACE cDNA amplification kit (Clontech). The two primers used for the 5' rapid amplification of cDNA ends were 5'-G GCG GTA GAC GAT GCA ATC ATC AC-3' and 5'-GCT GAA ATC CCA ACA GGT ACT CCG-3'.

Deletion and reconstitution of *CPR α* . To generate the deletion construct pMK1, the 5' (nucleotides -873 to +127, where the first ATG is at +1) and 3' (nucleotides +3873 to +4885) flanking regions of *CPR α* were each amplified by PCR and cloned into pYCC76 so that they flanked the *ADE2* gene in the plasmid (8). Cells of strain LP1 (*MAT $\alpha ade2 ura5$*) derived from B-4500 were transformed by the biolistic method (41) and plated onto MIN agar supplemented with uracil. A PCR protocol was used to detect the putative deletion mutants resulting from homologous recombination as described previously (11). Deletions of *CPR α* were confirmed by Southern blot analysis (37). The strain with the deleted *cpr α* locus was subsequently reconstituted back to the wild type by biolistic cotransformation (11) with plasmids pYCC331 (9) and pMK2. Plasmid pMK2 contained the 3.0-kb *Bst*XI/*Sall* fragment of *CPR α* , which was cloned into the *Eco*RV/*Sa*II site of pBluescript II SK(+). Putative adenine auxotrophs (pink colonies) were isolated from the transformation plates and screened by Southern blot analysis to identify the reconstituted clones.

Quantitative assay for mating frequency. The mating frequencies of various strains were assayed by a method devised in our laboratory (11). All cultures used for this experiment were less than 24 h old. Briefly, about 5×10^6 cells of each of the *MAT α* (JEC30 and JEC32) and *MAT α* (Δ *cpr α* and Δ *cpr α* ::*CPR α*) strains expressing different auxotrophic markers were suspended in 1 ml of saline (0.9%) and mixed thoroughly by vortexing. The cells were collected on a 0.45- μ m-pore-size nitrocellulose filter membrane by suction, placed on V8 juice agar, and incubated for 6 h at room temperature. The cells were washed off the filters, plated onto MIN agar, and incubated for 4 days at 30°C before the number of hyphal colonies was counted. The mating frequency of each strain was determined as the total number of hypha-producing colonies divided by the total number of input cells from both mating type strains. The relative mating frequency was expressed as a percentage of the mating frequency of the *CPR α* wild-type strain LP1 (*MAT $\alpha ura5 ade2$*) crossed with JEC30 (*MAT $\alpha lys2$*) or LP1 crossed with JEC32 (*MAT $\alpha lys2$*). Data were the average of the results derived from mating each strain with both JEC30 and JEC32. The experiments were repeated three times to establish reproducibility.

Synthesis of *MAT α* pheromone (Mfap). The presumptive mature peptide portion of Mfap, Glu-Glu-Ala-Tyr-Gly-Ser-Gly-Gln-Gly-Pro-Thr-Tyr-Ser-Cys (32), was synthesized as follows: the 13-mer sequence fragment missing the C-terminal cysteine residue was assembled by standard 9-fluorenylmethoxy carbonyl (Fmoc)

peptide synthesis methodology (22) using Fmoc-based solid-phase peptide synthesis on a 2-chlorotrityl chloride resin. The fully protected 13-amino-acid peptide was then cleaved from the solid support and condensed with the suitably protected cysteine (22) as follows: the protected peptide fragment and 2 eq of *S*-trityl-cysteine methyl ester (22) were dissolved in dimethylformamide (~150 μ l/ μ mol of peptide), and 5 eq of *N*-methyl morpholine (NMM) was added to an ice bath with stirring and cooling. To the resulting solution, *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (1.5 eq) was added. After 1 h, the solution was allowed to warm to 25°C and stirred for 12 more hours. Most of the solvent was removed in vacuo. The residue was then dissolved in ethyl acetate and washed with a mixture of 2% citric acid, 2% sodium bicarbonate, and saturated aqueous NaCl. Drying of this solution with anhydrous MgSO₄, filtration, and solvent removal in vacuo yielded the crude product. This material was then treated with a mixture of 87.5% trifluoroacetic acid, 2.5% ethanedithiol (EDT), 5% phenol, and 5% thioanisole (by volume; ~50 μ l/ μ mol of peptide) for 3 h at room temperature. After removal of the bulk of the solvent in vacuo, the peptide was precipitated with methyl *tert*-butyl ether and the solid was filtered off, washed with methyl *tert*-butyl ether, and dried, yielding the crude peptide Fmoc-Glu-Ala-Tyr-Gly-Ser-Gly-Gln-Gly-Pro-Thr-Tyr-Ser-Cys(SH)-OMe.

In order to remove Fmoc and to *S*-farnesylate the cysteine residue, the following procedure was used. While the solution of the Fmoc-protected peptide in dimethylformamide was cooling with ice and stirring, 2 eq of farnesyl bromide (Sigma-Aldrich, St. Louis, Mo.) and 8 eq of diisopropylethylamine (Sigma-Aldrich) were added at a concentration of 1 μ mol/100 μ l. After 2 h, 4 eq of ethanedithiol was added to the reaction mixture, and the mixture was stirred for 30 min.

Next, neat piperidine (~36 μ l/ μ mol of peptide) was added to the reaction mixture and the Fmoc group removal was performed for an additional 20 min at room temperature. The reaction mixture was subsequently diluted with 3 volumes of water and acidified to pH 6 with acetic acid. The resulting solution was applied to a semipreparative high-performance liquid chromatograph column (DELTA PAK C₁₈ reverse phase; Waters) (10- μ m inside diameter, 300 by 19 mm), and the peptides were eluted with a linear acetonitrile gradient with 15 mM ammonium acetate in both solvents (solvent A, 15 mM ammonium acetate in water; solvent B, 15 mM ammonium acetate-80% acetonitrile-20% water). The fractions with the best high-performance liquid chromatograph analytical profile were pooled, frozen, and lyophilized to afford the final products a purity of >90%. The identity of the expected product was confirmed by matrix-assisted laser desorption ionization-time of flight and electrospray mass spectrometry analyses (molecular mass, 1,667 Da).

Response to *MAT α* pheromone. Three different methods were used to observe the response of a Δ *cpr α* strain to the *MAT α* pheromone. In the first method, the Δ *cpr α* and Δ *cpr α* ::*CPR α* strains, as well as the wild-type *MAT α* and *MAT α* strains, were streaked in parallel with the *MAT α* wild-type strain in close proximity on SLAD agar, incubated for 48 to 72 h in the dark, and then observed for hyphal formation (11). The second and third methods employed the putative synthetic Mfap. The synthetic pheromone (3.9 mg) was dissolved in 1 ml of acetonitrile solution (acetonitrile and distilled water; 1:1 [vol/vol]). Ten aliquots (100 μ l each) of Mfap were lyophilized and stored in a -20°C freezer until needed. To test the response of the wild-type strains and the Δ *cpr α* strain to Mfap, the previously described method (14) was used with some modifications. The contents of each lyophilized tube of pheromone were dissolved in 6 μ l of dimethyl sulfoxide for 10 min and diluted with 6 μ l of distilled water. SLAD agar plates were spotted with 20 μ l of the pheromone solution and left overnight to dry. A small loopful of cells from a 48-h YEPD culture of the Δ *cpr α* mutant and *MAT α* (B-4500) and *MAT α* (B-4476) wild-type strains were patched on the center of the Mfap spots on the agar. An identical culture plate of SLAD agar without pheromone was used as a control. The plates were incubated in the dark for 24 h at 25°C and examined under a microscope. The same method was used for detection of *MF α* transcripts in cells exposed to pheromone except that approximately 10^8 cells of a 24-h YEPD culture were spread on SLAD agar over the area of synthetic Mfap application. After 2 h of exposure to Mfap, RNAs were extracted and subjected to Northern analysis with the *MF α* gene as a probe. The quantity of RNA was assessed by hybridizing the same filter with the *ACTIN* gene.

RESULTS

Isolation of the *CPR α* gene. During mapping of the *MAT α* locus in B-4500, an open reading frame present only in *MAT α* strains (21) was observed approximately 1 kb upstream of the *STE12 α* gene and named *CPR α* . In this study, the 1.3-kb *CPR α*

gene present in the *MAT α* locus of strain B-4500 was isolated and characterized. *CPR α* cDNA encodes a 42-kDa protein that shows high degrees of homology with the pheromone receptor genes of *Coprinus cinereus* (*rcb3*), *Schizophyllum commune* (*bbr2*), and *U. maydis* (*pra1*) (4, 16, 35). As is the case with these pheromone receptors, the *Cpr α* sequence was suggestive of it being a seven-transmembrane G-protein-coupled receptor. Comparisons between the genomic and cDNA sequences revealed three introns (GenBank accession number AF259519). Alignment of the *Cpr α* amino acid sequence with pheromone receptors of other basidiomycetous fungi showed high similarities: 46% with the *Rcb3* protein of *Coprinus cinereus*, 43% with the *Bbr2* protein of *S. commune*, and 42% with the *Pra1* protein of *U. maydis* (Fig. 1).

Constructions of Δ *cpr α* and reconstituted *CPR α* strains. In order to study the function of *CPR α* , the gene was deleted in the *MAT α* strain LP1 (*MAT α ura5 ade2*) (7) by biolistic transformation (41) with the deletion plasmid construct pMK1. Putative transformants resulting from homologous recombination between the deletion construct and the genomic *CPR α* locus were first identified by PCR and subsequently confirmed by Southern blot hybridization. DNA was digested with *EcoRV*, blotted, and hybridized with a PCR probe of the deleted portion of *CPR α* in pMK1 (data not shown). Hybridization signals corresponding to the *CPR α* gene were observed for the wild-type *MAT α* strain (B-4500), whereas none were detected in either the *MAT α* strain (B-4476) or the Δ *cpr α* strain. The membrane was stripped and hybridized with the 5' and 3' flanking regions of *CPR α* in pMK1. The putative deletion mutant strain also showed signals corresponding to fragments of predicted size (data not shown).

To avoid the possible introduction of gene disruptions resulting from ectopic integrations, the wild-type gene was reintroduced into the homologous site by transforming the Δ *cpr α* strain with the reconstitution construct, pMK2, by the cotransformation method described previously (11). The putative transformants from this replacement event were isolated and analyzed by Southern hybridization. The putative reconstituted *CPR α* strain exhibited the same hybridization pattern as B-4500 (data not shown). This result indicated that the Δ *cpr α* locus was reconstituted to the *CPR α* allele.

Mating of the Δ *cpr α* strain. The Δ *cpr α* strain (*MAT α ura5*) and the reconstituted strains both produced hyphae and basidiospores when crossed with B-4476 on V8 juice agar supplemented with uracil. The mating frequency of the Δ *cpr α* strain, however, was drastically reduced relative to those of the *CPR α* or Δ *cpr α ::CPR α* strains (Fig. 2). To quantify the extent of the reduction in mating frequency in Δ *cpr α* , the strains were mated with JEC30 and JEC32, both *lys2* mutants derived from B-4476. The Δ *cpr α* strain showed 0.3% of the mating frequency exhibited by the *CPR α* wild-type strain, whereas the reconstituted strain exhibited an 89% mating frequency. To confirm that the rare mating observed in the Δ *cpr α* \times *MAT α* strains resulted only from the interactions between the two

opposite mating type cells, the Δ *cpr α* strain was also crossed with JEC31 (*MAT α lys2*). No mating was observed in this cross. In order to prove that the basidiospores were the product of meiosis, single basidiospores were isolated by micromanipulation from the cross of the Δ *cpr α* and JEC30 strains. Recombination of genetic markers was observed among the single-basidiospore cultures, and a prototrophic Δ *cpr α* strain was obtained (data not shown). Growth rates of the prototrophic Δ *cpr α* strains at both 30 and 37°C were the same as those for the wild-type strains.

Response of the Δ *cpr α* strain to *MAT α* pheromone. To observe the response of the Δ *cpr α* strain to the *MAT α* pheromone, the Δ *cpr α* strain, as well as the Δ *cpr α* strain reconstituted with the wild-type *CPR α* gene and the *MAT α* and *MAT α* strains, was streaked in parallel with the *MAT α* wild-type strain in close proximity on SLAD agar and incubated for 48 to 72 h in the dark (11). Hyphal formation was observed in the Δ *cpr α* , Δ *cpr α ::CPR α* , and wild-type *MAT α* strains within 72 h. As shown in Fig. 3A, the number of hyphae produced toward the streak of a *MAT α* strain by the Δ *cpr α* strain was considerably less than the number produced by the Δ *cpr α ::CPR α* strain. The abundance of hyphae produced by the wild-type *MAT α* strain was similar to that of the Δ *cpr α ::CPR α* strain, whereas no hyphal production was observed between the streaks of the *MAT α* and *MAT α* strains (data not shown).

The putative *Mfap* confirmed by matrix-assisted laser desorption ionization–time of flight and electrospray mass spectrometry analyses (molecular mass, 1,667 Da) (data not shown) was dissolved in dimethyl sulfoxide and used to test the induction of hyphae in both the wild-type strain and the Δ *cpr α* strain on SLAD agar (see Materials and Methods). Colonies of both mating types grown outside of the pheromone spot as well as *MAT α* colonies grown on the pheromone spot failed to produce any hyphae. However, *Mfap* induced short hyphal growth in colonies of the B-4500 and Δ *cpr α* strains. Predictably, the amount of hyphae induced in the Δ *cpr α* mutant strain was considerably less than that in the strain with the wild-type *CPR α* gene (Fig. 3B).

When exposed to *Mfap* for 2 h on SLAD agar, the cells of B-4500 accumulated abundant *MF α* message, whereas in the absence of pheromone, they exhibited only basal levels of *MF α* message (Fig. 4). Transcription of the *MF α* genes in the Δ *cpr α* strain remained at basal levels regardless of exposure to pheromone (Fig. 4).

Effect of the *CPR α* gene deletion in haploid fruiting. Haploid fruiting, a phenomenon believed to be *MAT α* strain specific (45), was tested in the Δ *cpr α* strain in order to evaluate the role of pheromone receptor protein-sensing environmental cues that induce haploid fruiting. This test was performed since haploid fruiting (induced on filament agar) and mating (induced on V8 agar) in *C. neoformans* share common environmental conditions: nitrogen starvation and dehydration (4% agar). The Δ *cpr α* strain produced hyphae almost as abundantly as the Δ *cpr α ::CPR α* (Fig. 5) or wild-type strain on filament

FIG. 1. Protein sequence alignment of pheromone receptors. CcRcb3, Rcb3 of *Coprinus cinereus* (GenBank accession number AF186385); ScBbr2, Bbr2 of *S. commune* (GenBank accession number AF148501); UmPra1, Pra1 of *U. maydis* (GenBank accession number U37795); CnCPR α , Cpr α of *C. neoformans* (GenBank accession number AF259519). Black boxes indicate the regions of identity, and gray boxes indicate the regions of similarity. Putative transmembrane domains 1 to 7 are indicated by dotted underlines.

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CcRcb3 1 MSSSRKSFDDPTYPAPFPVLSFIGFVLVLPWPWQAWNSGTCLYMIWTALCLNFFFIN
ScBbr2 1 -----MYSNDPTYPFPPTFAFLGFVLSVLPWPWQAWNSGTCAVMWTATACLISFVN
UmPra1 1 -----MLDHTTFFALVAFFLVLPFAWHIKSKNVGLIMLSLWMLNLNDNFVN
CnCpra 1 -----MHDLSLVFSGIGIILVLPPLHWRARNCTLLLTWLFANLIFEV
-----1-----2-----

CcRcb3 61 SIVWHG-NALDRAPWCDISTRFFVGLSVAIPAASLCINRRLYKIASCRFANIRSRKRI
ScBbr2 55 SVVWRG-NALNTAPIWCDISSRLILGVSIGIPASGLCISRRLYKISMRSVSITRODKIR
UmPra1 50 SVVWVK-TTADLAPAYCELSVRLRHLLFPAIPASNLAIRLESIASITROVRAGPGDIRR
CnCpra 50 GIVVWNSYDLPSPIWCDIASLFGMPVCHISAASLCISRRLVMIASITAVITQRQKRI
-----3-----

CcRcb3 120 AVVVDLAIAGLGIPLVQMPLOYVQVHREDIFQDGCYATVNTTPPAYPLVFLWPTIGL
ScBbr2 114 AICDITIAVGLPILVMILHVVQVHREDILEDCYAAIYITPAYLFFWPVLGAF
UmPra1 109 AVTDLILICLGIPIITYSLMIVNQSNRIGILEACCPIMVFSWLWVLLVAAPVIVSLC
CnCpra 110 AAVDLLIGTGMPLVMALHYIVQVHREDITEGYGCQPIWPGYPALFAVTWVSPILTI
-----4-----5-----

CcRcb3 180 SAVYCILTLREEMRRRAQFSQSSNSSSLTVNRYERLMSLALILELNLPIITTTGNYLS
ScBbr2 174 SFVTSALCLRAFYIRRLQFAQLAHN-KSLDTSRYRLMLLAILDCCCTIPGALSYLS
UmPra1 169 SAVYSALAFRFWVRRRQFQAVLASSASTNRSEYVRLILITADMLLFFPIYGTAAQ
CnCpra 170 TAGYGTALRFLHRRRLQFHTVLRSSRSNLDREYRLMALASVDLGLPATFTIIN
-----6-----

CcRcb3 240 IISRPIYPWTNWADHYENVIITYPRILWAHKTADATILELGRWSIVFCALFFAFFGF
ScBbr2 233 SHGKLSPLWISWADTHYDFGRVEQVPSITWRSEATYRSSVELTRWLPVYCAFFFALFGF
UmPra1 229 IASSISIPGWSVSVHTGFNOIQPYASLVLMENIFORNILRLCPLSAYFFAMFGL
CnCpra 230 IQQRQSYF--SWDWHLDNSRIELYPASSILSCAQTIAVLPRWLAPLLIFFLFEGV
-----7-----

CcRcb3 300 ABEARKNYPKAILAKKIG-----YNPFS--
ScBbr2 293 ANEAKKIFYSEKYAVAKKVAVPLPSSSEKLFKLAHPKWDNKDVKNVPGGLPVVMPKGG
UmPra1 289 GIEVRQGYKFAFRALLFCR-----
CnCpra 288 SIAAMGEYARWYNTIRAKAP-----

CcRcb3 325 ---PDEKAKATFVHVGKPAFLPFTPR-----
ScBbr2 353 DVFEARKMKDTFDSSTAADSSVSEPEKGLPLPSSPATTAPPDYDYPGNNDYSGDDIISH
UmPra1 309 -----LTKKPK-----
CnCpra 308 -----FLPFHREDIIP-----

CcRcb3 349 -----PLHASTATLITATQSDKSINFT-----ILSISISSGST
ScBbr2 413 YPGDGNSLPVHMIPSPQSGSGSSPSSRRSSTEIEHREFSFQRLPSSILAGPSTS
UmPra1 315 -----AALQHV-----
CnCpra 319 -----IAAPKLGST-----

CcRcb3 384 KVACSPPAFRLDDIKVKLSSPGVSDFPSSPISMT-----TRLDDSDNAHSEV
ScBbr2 473 VHSQTSFRFDVSSSLHAHTSSEFKTAPSSHSATSPAVPTLQNVPPRTSSRPQHSRS
UmPra1 322 VADIEVVFIRSHDTFDANTSTKSEKSDIMRGSEAA-----
CnCpra 328 IAKPSGADWGSTIANDDIMEGSEYFNDIRFDSIPG-----VIRGGIMIAVSVERA

CcRcb3 434 -----
ScBbr2 533 QSSLHSLATTLASTASGRDVIYIYDAYDSSPSFEHATYQLPEHETPPFSNLAPVADEQR
UmPra1 -----
CnCpra 379 VV-----

CcRcb3 -----
ScBbr2 593 RSRRPATPLPPLFTARRERSPSSVRVTVRTERDEEGS
UmPra1 -----
CnCpra -----

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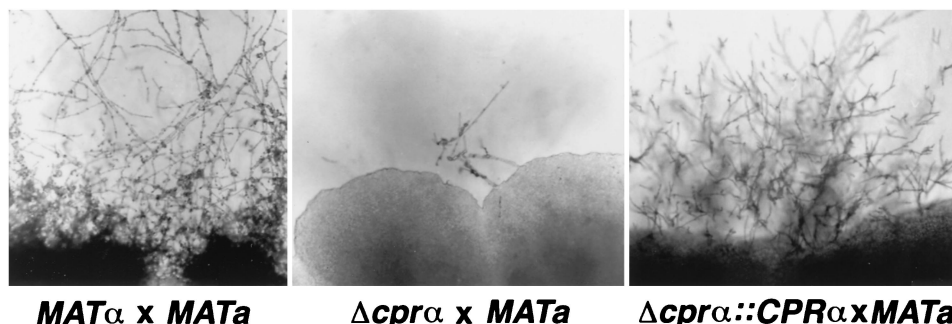


FIG. 2. Mating behavior of the wild type (B-4500), the $\Delta cpr\alpha$ mutant, and the $\Delta cpr\alpha::CPR\alpha$ mutant crossed with B-4476. $\Delta cpr\alpha \times B-4476$ shows drastic reduction in mating efficiency compared to B-4500 \times B-4476 or $\Delta cpr\alpha::CPR\alpha$.

agar (data not shown). These results indicated that the pheromone receptor does not play a significant role in sensing the environmental cues conducive to haploid fruiting in *C. neoformans*.

DISCUSSION

We have identified and characterized a $MAT\alpha$ -specific pheromone receptor gene embedded in the $MAT\alpha$ locus of *C. neoformans* (21). Our study suggests that the $CPR\alpha$ gene of *C. neoformans* plays an important role in mating, presumably by mediating the signal transduction response to pheromone. The drastic reduction in mating efficiency in the $\Delta cpr\alpha$ strain but not in the $\Delta cpr\alpha::CPR\alpha$ strain corroborates this conclusion. Furthermore, while the expression of the $MF\alpha$ genes in the wild-type strain was drastically upregulated upon exposure to synthetic $MATa$ pheromone (Mfap), $\Delta cpr\alpha$ cells exhibited only basal levels of $MF\alpha$ transcript in spite of exposure to the $MATa$ pheromone. These findings support the notion that $CPR\alpha$ and $MF\alpha$ are components of the same signaling pathway. Since the $\Delta cpr\alpha$ strain is able to mate, albeit poorly, and produce viable basidiospores, it indicates that the $\Delta cpr\alpha$ strain still responds to the presence of its mating partner and transmits the signals downstream to genes involved in sexual morphogenesis. This notion is supported by the failure of mating between $\Delta cpr\alpha$ and $CPR\alpha$ cells. It is further supported by the results of the SLAD agar test (11) and the test with synthesized Mfap. Although rare, $\Delta cpr\alpha$ cells streaked side by side and in close proximity to B-4476 ($MATa$) produced short hyphae toward B-4476 cells. Since such hyphae are produced by $MAT\alpha$ cells on SLAD agar only when they are streaked in proximity to $MATa$ strains (11), it was considered to be a pheromone response. Our tests with the synthetic putative Mfap ($MATa$ pheromone) supported this assumption. Though rare, Mfap did induce hyphal formation in a $\Delta cpr\alpha$ colony. It is not unusual that $\Delta cpr\alpha$ cells of *C. neoformans* are still able to mate since none of the other *S. cerevisiae* STE gene homologs of *C. neoformans* such as $STE20$ (31), $STE11$ (12), and $STE12$ (10) caused complete sterility upon deletion. Unlike *S. cerevisiae*, *C. neoformans* contains mating type-specific alleles of the STE gene homologs listed above. Of the three genes, the $STE11\alpha$ gene is the only one that caused a drastic reduction in mating efficiency upon deletion (12). Deletion of either alleles of $STE20$ (J. Heitman, personal communication) or $STE12$ (10, 11), however, showed no dramatic mating defects. Mating efficiency between $\Delta ste20a$

and $\Delta ste20a$ strains (J. Heitman, personal communication) or $\Delta ste12\alpha$ and $\Delta ste12a$ strains (10), on the other hand, was markedly reduced. These observations suggest that the signaling pathway for mating in *C. neoformans* is different from that in *S. cerevisiae*. *C. neoformans* may contain other sets of genes that supplement the defects caused by deletion of $STE20$ and $STE12$ but not $STE11$. It is also possible that $STE20$ and $STE12$ of *C. neoformans* belong to different pathways that cross talk with the pheromone response pathway, where $STE11$ presumably functions. The cumulative effect of deletions in both alleles of $STE20$ and $STE12$ upon mating also suggests that the mating type-specific alleles supplement each other in signaling. Our experience with the $STE12$ alleles supports this notion. When a $\Delta ste12\alpha$ strain is reconstituted with the wild-type $STE12a$ allele, the mutant phenotype of the $ste12\alpha$ strain is complemented (10).

A phenomenon similar to our observation on the mating ability of the $\Delta cpr\alpha$ strain has also been reported for another heterobasidiomycetous fungus, *Ustilago hordei* (2). Deletion of a receptor gene in the species produced occasional dikaryotic hyphae when crossed with wild-type strains of a compatible mating type. It would be interesting to know whether the efficiency of dikaryote formation between a pheromone receptor deletion mutant strain and the wild-type strain of *U. hordei* is as dramatically reduced as reported for *S. cerevisiae* or less severely reduced as is the case for *C. neoformans*.

Fungal cells sense pheromones through G-protein-coupled receptors (3). It is believed that these receptors anchor to the plasma membrane using seven-transmembrane helices and regulate the expression of second messengers through their interactions with heterotrimeric G protein. As is the case in *S. cerevisiae*, the MAP kinase pathway regulating hyphal formation in *C. neoformans* shares many signaling elements with the mating pathway (11, 30). In *C. neoformans*, the β subunit of a heterotrimeric G protein (Gpb1p) has been shown to play a critical role in mating and haploid fruiting, presumably through a pheromone-triggered MAP kinase cascade (42). Although the receptor-coupled α subunit of the $GPB1$ gene has not been characterized, a model depicted for signal transduction pathways by Lengeler et al. (30) suggests that it is $GPA3$. $STE20\alpha$ (J. Heitman, personal communication) and $STE11\alpha$ (MAP kinase kinase kinase) also appear to play crucial roles in haploid hyphal formation and mating (12) in *C. neoformans*. Our results indicated that haploid fruiting is not impeded in the $cpr\alpha$ deletion mutant strain, suggesting that the $Cpr\alpha$ does

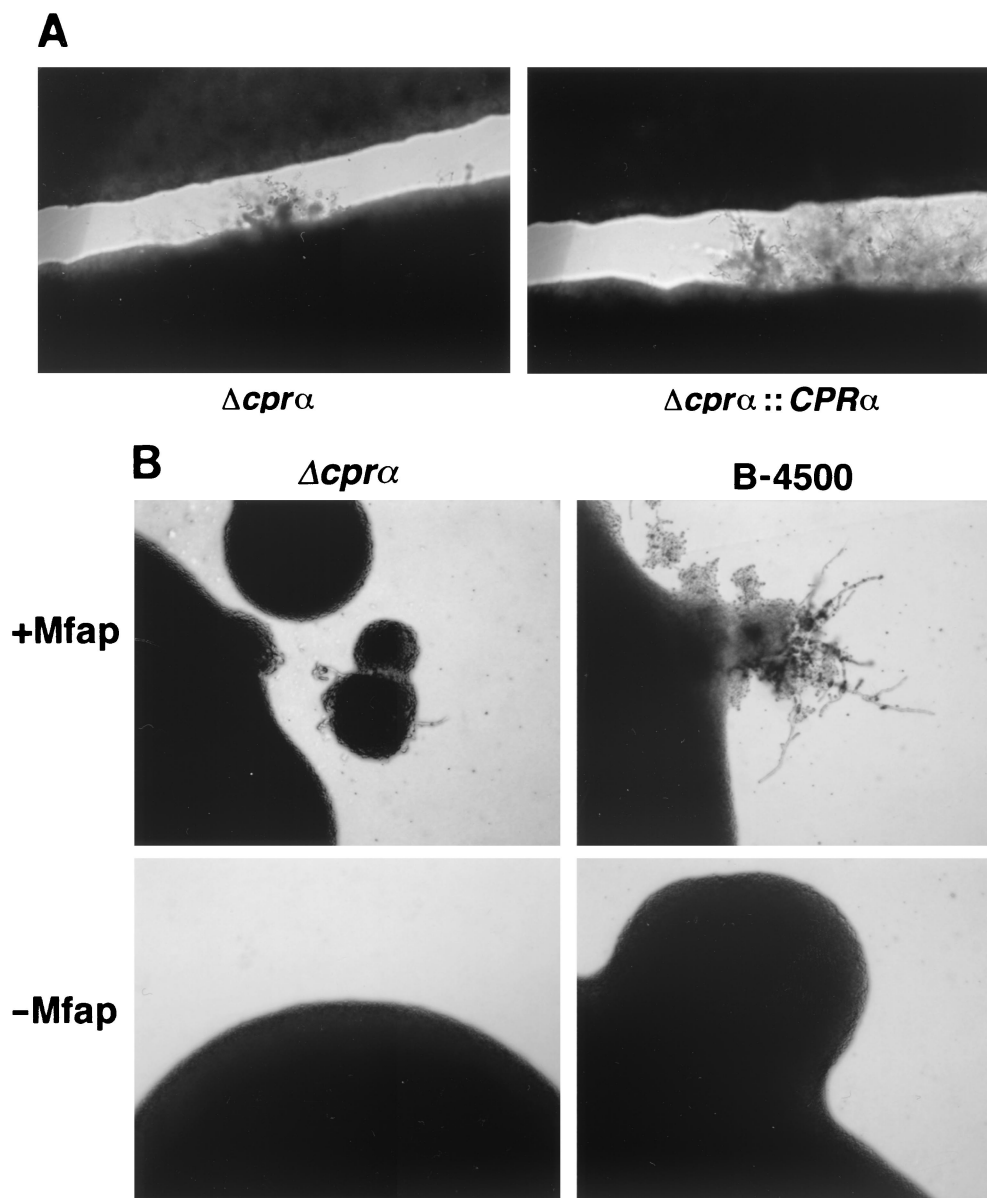


FIG. 3. Response of the $\Delta cpr\alpha$ mutant toward *MATa* culture or *MATa* pheromone (Mfap). (A) Formation of hyphae on SLAD agar. Left panel, $\Delta cpr\alpha$ strain (lower side) produced some hyphae toward *MATa* strain (upper side). Right panel, $\Delta cpr\alpha$ strain reconstituted with the wild-type *CPR* α gene (lower side) produced abundant hyphae toward *MATa* strain (upper side). (B) Synthetic Mfap triggered hyphal formation in *MAT* α wild-type cells and with a markedly reduced rate in the $\Delta cpr\alpha$ mutant. No hyphal formation was observed in the same strains cultured on the same plate where Mfap was not applied.

not play an important role in sensing the environmental cues conducive to haploid fruiting. This result concurs with previous observations that pheromone receptors do not sense the environmental stresses that induce filamentation in other yeasts (3). We, however, performed the test since the environmental conditions such as nitrogen starvation and dehydration that promote sexual reproduction also promote haploid fruiting in *C. neoformans*. This observation indicates that other molecular components are involved in the sensing and transduction of the signals that trigger haploid hyphal growth. It has been suggested that the MAP kinase cascade functions during mating in both *MAT* α and *MATa* cells (42). The involvement of Ras1 and

Gpa1 pathways in mating as well as filamentation has also been reported (1). The cross talk between these two pathways and *CPR*- and pheromone-initiated signal transduction requires further dissection in order to elucidate precise networks of signaling for mating versus haploid filamentation.

We synthesized the predicted mature pheromone peptide of mating type *a* (Mfap) based on the sequence of the *MFa* genes (32). When *MAT* α and *MATa* cells were exposed to synthetic Mfap, the pheromone triggered hyphal formation only in *MAT* α strains. This result suggests that the synthesized Mfap is an active form of *MATa* pheromone. Furthermore, the synthetic pheromone induced *MF* α gene expression in the *CPR* α

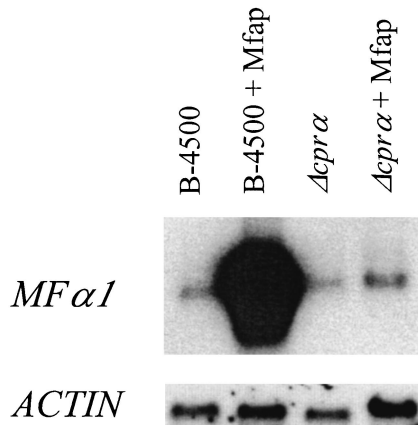


FIG. 4. Induction of *MFα* gene expression by synthetic *MATa* pheromone. (A) The wild-type (B-4500) and $\Delta cpr\alpha$ strains were grown on SLAD agar plates for 2 h with or without Mfap. Total RNAs were extracted, fractionated on an agarose gel, and transferred to a nitrocellulose membrane. The resulting blot was hybridized with the *MFα1* gene probe. (B) The membrane described for panel A was stripped and hybridized with the *ACTIN* gene.

wild-type strain, whereas the wild-type strain not exposed to pheromone and the $\Delta cpr\alpha$ strain exposed to pheromone showed only basal levels of *MFα* transcript. The drastic reduction of hyphal formation in the $\Delta cpr\alpha$ strain in response to *MATa* pheromone suggests that the decrease in mating efficiency of the $\Delta cpr\alpha$ strain is due to the loss of the Cpr α protein. This assumption was further supported by the recovery of mating efficiency in the $\Delta cpr\alpha::CPR\alpha$ strain. The *MATα* pheromone is reported to be an undecapeptide, and synthetic Mf α p induced hyphae in only the *MATa* strain (14). It was

noticed that fungal pheromones identified in ascomycetes and basidiomycetous fungi encode CAAX motifs at the carboxyl termini of the proteins (reviewed in reference 6). The carboxyl-terminal CAAX motif has been believed to direct posttranslational farnesylation and carboxymethylation. Without exception, pheromone receptors from these fungi have sequence similarity to Ste3p, which responds to the a-factor-containing CAAX motif. Though Cpr α p showed lower sequence similarity to Ste3p than to the pheromone receptor proteins of basidiomycetous fungi, it still showed 36% similarity. It is interesting to note that the mature *MATa* pheromone contains three more amino acids (32) than the *MATα* pheromone (14). The Mfap appears to be larger than most of the well-characterized fungal lipopeptide mating factors that are usually 9- to 13-amino-acid peptides (2, 4, 5, 13, 14, 20, 33, 36, 39, 43).

Since the $\Delta cpr\alpha$ strain still responded to synthetic *MATa* pheromone and mated with *MATa* wild-type strains, though with low efficiency, one may suspect genetic redundancy of pheromone-sensing proteins in *C. neoformans*. We searched the Cryptococcal Genome Database created by the Stanford Genome Technology Center (<http://www.sequence.Stanford.edu>) and found one more pheromone receptor-like gene, which we named *CPR2*. Unlike *CPRα*, the *CPR2* gene was present in strains of both mating types and was not associated with the *MAT* locus (Y. C. Chang and K. J. Kwon-Chung, Abstr. 101st Gen. Meet. Am. Soc. Microbiol. 2001, abstr. F-55, 2001). We are currently studying the role of *CPR2* in mating by constructing a $\Delta cpr2$ strain and a $\Delta cpr\alpha \Delta cpr2$ double deletion strain. Phenotypes of these strains would reveal the role of *CPR2* in mating and offer an explanation as to whether there is genetic redundancy in *C. neoformans* pheromone receptors.

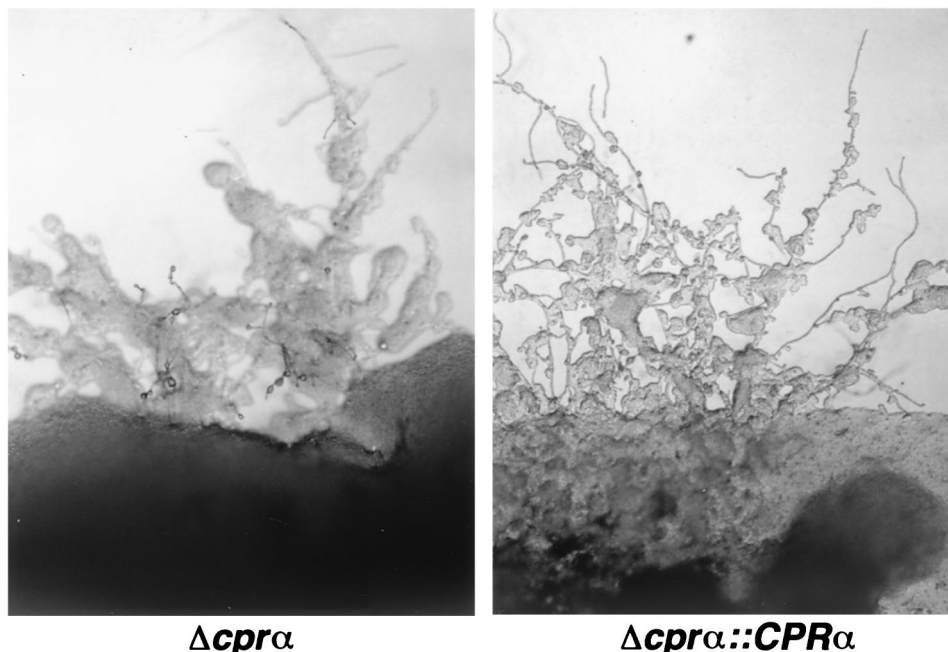


FIG. 5. Haploid fruiting in the $\Delta cpr\alpha$ strain and the $\Delta cpr\alpha::CPR\alpha$ strain on filament agar. No significant difference was found in the formation of haploid fruiting between the two strains.

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