The **RAM1** gene encoding a protein-farnesyltransferase β-subunit homologue is essential in *Cryptococcus neoformans*

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**INTRODUCTION**

*Cryptococcus neoformans* is an opportunistic human fungal pathogen. Several small G proteins have previously been demonstrated to regulate the growth and development of this fungus. For example, genetic studies demonstrated that the **RHO1** gene is likely essential, as it is in other fungal species, and that precise regulation of the **Rho1** protein is required for sustained growth of *C. neoformans* (Chang et al., 2000). Additionally, the small G protein **Ras1** is required for proper growth, development and pathogenicity of *C. neoformans*. A null mutation of the **RAS1** gene results in a mutant strain unable to grow at 37°C, rendering it avirulent in animal models of cryptococcal disease. Ras1 is also required for hyphal formation of this fungus; *ras1*-mutant strains are defective in mating and haploid fruiting, two types of differentiation characterized by a yeast–hyphal transition (Alspaugh et al., 2000).

Many small G proteins require post-translational modification to allow functional association to the cell membrane. This process often involves the enzymic addition of hydrophobic prenyl groups to a conserved cysteine residue near the C-terminus of the protein. The enzymes that catalyse these reactions include protein farnesyltransferase and protein geranylgeranyltransferases.

The human fungal pathogen *Cryptococcus neoformans* requires functional Ras and Rho proteins in order to undergo normal growth and differentiation. Since farnesylation and geranylgeranylation are likely required for the proper function of these small G proteins, we hypothesized that inhibition of these prenylation events would alter the growth and cellular morphogenesis of this fungus. We cloned the **RAM1** gene encoding the single protein-farnesyltransferase β-chain homologue in *C. neoformans*. Using a gene-disruption strategy in a diploid *C. neoformans* strain, we demonstrated that this gene encodes an essential function, in contrast to the case in *Saccharomyces cerevisiae*, in which the homologous **RAM1** gene is not essential for growth. Pharmacological inhibition of farnesyltransferase activity resulted in dose-dependent cytostasis of *C. neoformans*, as well as prevention of hyphal differentiation. Simultaneous inhibition of farnesylation and calcineurin signalling results in a synthetic effect on growth. Protein farnesylation is required for the growth and cellular differentiation of *C. neoformans* and may provide novel targets for antifungal therapy.

In order for small G proteins such as Ras and Rho to localize to the cell membrane and to function properly, they must undergo prenylation, a post-translational modification in which hydrophobic groups are added to the C-terminus of the protein (Casey et al., 1996). Three prenyltransferases have been described in eukaryotic cells that are responsible for these protein modifications: protein farnesyltransferase (FTase) and protein geranylgeranyltransferase (GGTase) types I and II. FTase catalyses the addition of 15-carbon (farnesyl) groups to proteins destined for cell membranes (Reiss et al., 1990), and GGTases catalyse a similar reaction with 20-carbon (geranylgeranyl) groups (Seabra et al., 1991). Since activating Ras mutations have been demonstrated in many human malignancies (Schafer et al., 1989), and since prenylation is required for Ras function (Casey et al., 1989; Hancock et al., 1989), these prenyltransferases have been extensively studied as potential targets for cancer chemotherapy (Hill et al., 2000; Lerner et al., 1995; Prendergast, 2000).

FTase and GGTase I are related heterodimeric proteins that consist of a common α subunit and distinct β subunits. In *Saccharomyces cerevisiae*, the **RAM2** gene encodes the common α subunit of FTase and GGTase I, and the **RAM1** and **CDC43** genes encode the β subunits of FTase and GGTase I.
GGTase I, respectively (He et al., 1991; Mayer et al., 1992). RAM2 and CDC43 are essential genes, in contrast to the FTase β-subunit gene RAM1, which is not essential for growth (He et al., 1991; Mayer et al., 1992). However, ram1 mutant strains are not able to grow at 37°C or survive other stressful conditions (He et al., 1991). Therefore, for optimal cell growth, both FTase and GGTase I activities are required for S. cerevisiae survival.

We hypothesized that cellular functions that depend on small G proteins in the pathogenic fungus C. neoformans, such as cell polarization, high-temperature growth, and differentiation, would be defective in strains with inhibited prenylation. Here we describe the identification of a gene encoding a homologue of a protein farnesyltransferase β subunit in C. neoformans. Using genetic and pharmacologic inhibition of FTase activity, we have investigated the role of this prenyltransferase in the growth and differentiation of this human fungal pathogen.

### METHODS

#### Strains, media and growth conditions. The C. neoformans strains used in this study included the serotype D congenic laboratory strains JEC21 (MATα) and JEC20 (MATα/a) (Kwon-Chung et al., 1992), the haploid serotype D strain JEC43 (MATα, ura5) (Wickes et al., 1997) and the diploid serotype D strain RAS10 (a/α, ade2Δ + , ura5Δ, lys1Δ + , lys2Δ + ) (Sia et al., 2000). The serotype A strains studied included the clinically derived wild-type strain H99 (Perfect Charge, Schleicher & Schuell). The probe comprised the region of the RAM2 gene (nucleotides 232–1531, corresponding to amino acid residues 77–440) was replaced by the URA5 gene by the PCR overlap-extension technique (Davidson et al., 2002). The primers for the PCR-based construct are shown in Table 1.

Primers AA19 and AA24 were used to generate the left fragment for PCR overlap extension; primers AA20 and AA25 generated the right fragment; primers AA23 and AA26 generated the URA5 marker. The three PCR fragments were assembled into one construct using all three initial fragments as template, and using the primers AA19 and AA20, with the following PCR conditions: 1 cycle of 95°C for 5 min; 35 cycles of 95°C for 20 s, 56°C for 20 s, and 72°C for 4 min; and 1 cycle of 72°C for 10 min. PCR reactions were performed using ExTaq PCR mix (Takara), according to the manufacturer’s instructions. The resulting ram1::URA5 disruption construct was transformed into the haploid strain JEC43 and the diploid strain RAS10 by biolistic transformation, as previously described (Davidson et al., 2000a).

#### C. neoformans colony PCR. Homologous construct integration was verified using one primer located within the RAM1 locus but outside of the ram1::URA5 disruption construct (primer AA21, 5’-GCGGATATCGTCTCTCGATAGAGGCG-3′, RAM1 position –1073 to –1053 bp) and one primer located within the URA5 sequence (AA16, 5’-CGGAGCTCAGGGAAGGTGAGTGAGGGG-3′, URA5 position –147 bp to –122 bp). This allowed an amplification of 1-2 kb to be generated only from a strain in which the endogenous RAM1 allele was precisely replaced by the ram1::URA5 mutant allele. One yeast colony was used as template for a PCR reaction as follows: 1 cycle of 95°C for 12 min; 35 cycles of 95°C for 20 s, 56°C for 20 s, and 72°C for 4 min; 1 cycle of 72°C for 10 min.

#### cDNA amplification and RACE. The 5′ and 3′ ends of the RAM1 cDNA were determined by the 5′, 3′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Gibco). Primer AA138 (5’-CGACATGGAGGCAGGCGGCGG-3′) is the gene-specific primer used to determine the 5′ end, and primer AA139 (5’-GGGACGAACCACCGGGTTGAGGTTG-3′) is the gene-specific primer used to determine the 3′ end. The RACE assays were performed according to the manufacturer’s instructions.

### Table 1. Primers for the RAM1 PCR-based gene-deletion construct

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Positions (bp)</th>
</tr>
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<tbody>
<tr>
<td>AA19</td>
<td>CGGACATGGCGACACAAAGGCG</td>
<td>–832/–812*</td>
</tr>
<tr>
<td>AA20</td>
<td>GCTAACAAGATGGCAACCATTGG</td>
<td>2772/2792*</td>
</tr>
<tr>
<td>AA23</td>
<td>CGGGGAAGTTTGGAGCCTCGGACCTTCTCCTCGATTCG</td>
<td>232/252* to –207/–185†</td>
</tr>
<tr>
<td>AA24</td>
<td>GCGATGGGAAGGAAGGATTGGCGGAGGCTCAAAACATTCCG</td>
<td>232/252*</td>
</tr>
<tr>
<td>AA25</td>
<td>GGAACCTTAACTACAGGCGGCCGGGTCCTCTTACGTCGTCG</td>
<td>1511/1531*</td>
</tr>
<tr>
<td>AA26</td>
<td>GCTGAGCGATAGAAAGACCCCGCGGCTTGACTCTTAAGGTC</td>
<td>1511/1531* to 859/879†</td>
</tr>
</tbody>
</table>

*Related to RAM2 gene.
†Related to URA5 gene (underlined).
MIC testing. MIC testing was performed according to the NCCLS standard assay, with slight modification (Galgiani et al., 1997). Briefly, five C. neoformans colonies grown on a YPD plate for 48 h at 37 °C were inoculated in 5 ml sterile saline (0.85% NaCl) to an OD$_{530}$ of 0.23 to 0.27 (about 1 x 10$^{6}$–5 x 10$^{6}$ cells ml$^{-1}$). A final cell suspension was made in RPMI 1640 plus 2% glucose that contained 1 x 10$^{6}$ cells ml$^{-1}$. Cells were inoculated in a final concentration of 100 cells per well. Serial twofold dilutions of the drugs tested for MIC were made from stock solutions in 96-well tissue-culture plates. Cell growth was analysed at 72 h. The stock solution of FPT Inhibitor III (Calbiochem) was diluted in RPMI 1640 to a final concentration of 5 mM. The FK506 (Fujisawa) stock solution was made by diluting the drug in RPMI 1640 to a final concentration of 25 μg ml$^{-1}$. Fluconazole (Pfizer) was diluted with saline (0-85% NaCl) to concentrations ranging from 800 μg ml$^{-1}$ to 25 μg ml$^{-1}$. The 96-well plates were incubated in an air incubator at 35 °C, or as otherwise stated.

Genetic analysis. Sporulation of the transformed diploid strains was performed on V8 mating medium in the dark at room temperature, as described by Sia et al. (2000). The basidiospores were separated by micromanipulation using a Nikon Eclipse 400 microscope. To test for specific auxotrophies, the basidiospores were incubated on YPD medium at 30 °C and transferred to synthetic media lacking uracil, adenine or lysine. Mating type was determined by crossing the basidiospores with either JEC21 (MATα) or JEC20 (MATα) on V8 mating medium at room temperature in the dark, and assessing for mating filaments at 3–5 days.

RAM1 overexpression construct. The RAM1 gene was PCR amplified with primer AA237 (5’-ACGTCCGCGGCGCGGCGG-GCTACGGTGGAGTGGG-3’, position -1389 bp to -1372 bp, NotI site underlined), and AA238 (5’-ACGTTCGACACTGTA-GGAGGACATGAACAATGTGGG-3’, position 2734 bp to 2754 bp, SphiI site underlined), which added NotI and Sphi restriction sites to the 5’ and 3’ ends, respectively. This gene was cloned into pHYG7-KB1, which contains the hph gene conferring resistance to hygromycin B as a selectable marker (Cox et al., 1996). The resulting plasmid was completely sequenced to confirm the absence of PCR-induced sequence errors. This plasmid was transformed by biolistic transformation into serotype A wild-type strain H99, and transformants were selected on YPD containing 300 μg hygromycin B ml$^{-1}$. In order to select for stable genomic integration of the construct, the transformants were passaged multiple times on a non-selective medium. Those that retained resistance to hygromycin B were analysed further. Two transformants demonstrated marked overexpression of RAM1 by Northern analysis, and were designated MVC72 and MVC73. The level of expression remained stable, despite repeated incubations on non-selective media.

RESULTS

Identification and characterization of a C. neoformans gene encoding a protein-farnesyltransferase β-subunit homologue

S. cerevisiae Ram1p is the β subunit of the heterodimeric protein farnesyltransferase. To identify a gene encoding a homologous protein in C. neoformans, we used the Ram1p sequence in a TBLASTN search (amino-acid sequence versus the translated nucleotide database) of the C. neoformans (serotype D) genome database at Stanford University (http://www-sequence.stanford.edu/group/C.neoformans/). One C. neoformans contig, sgct05.030912.C18, contained a predicted ORF with striking amino acid similarity to the Ram1p sequence. After amplifying the entire locus by PCR, we used the RACE procedure (see Methods) and comparison with the S. cerevisiae RAM1 gene sequence to confirm the 5’ transcription initiation and 3’ termination sites, as well as exon–intron junctions. We designated this gene C. neoformans RAM1. It contains six introns and encodes a predicted protein of 521 amino acids, with an estimated molecular mass of 56 kDa.

The predicted C. neoformans Ram1 protein shows homology to S. cerevisiae Ram1p (48% similarity), as well as to mammalian protein farnesyltransferase β subunits (46% similarity to rat fntb). The RAM1 gene sequence was submitted to GenBank under accession number AY162319.

Deletion of RAM1 gene in a diploid strain

Using the PCR overlap-extension technique (Davidson et al., 2002), we replaced the majority of the RAM1 coding region (nucleotides 232–1532, corresponding to amino acid residues 77–440) with the URA5 gene. The linear ram1::URA5 construct was biolistically transformed into two strains: JEC43 (haploid, MATα ura5) and RAS10 (diploid, MATα/MATα, ura5/ura5, ade2/ADE2, lys1/LYS1, lys2/LYS2). PCR and Southern blot analysis of 29 haploid JEC43 transformants revealed that no homologous recombination had occurred. In contrast, among 32 transformants for the diploid RAS10 background, by both colony PCR and Southern blot analysis, three demonstrated a precise replacement of one RAM1 allele with the ram1::URA5 mutant construct (Fig. 1a).

Genetic analysis

To determine whether the RAM1 gene was essential, we induced one of the diploid heterozygous transformants (RAM1/ram1::URA5) to undergo meiosis and sporulation by incubation on V8 mating medium at 25 °C. Twenty-four basidiospores were dissected by micromanipulation on YPD medium, and eight germinated after 7 days of incubation. None of these strains were self-filamentous on V8 medium supplemented with uracil, adenine or lysine, suggesting that all were haploid.

Genetic analysis was performed to confirm that the basidiospores were products of meiosis. The genetic markers segregated among these strains in a manner consistent with meiotic recombination. Mating type segregated 1 : 1, with 4 MATα and 4 MATa strains among the eight germinated spores. Exactly one half of the germinated spores were adenine auxotrophs, and one half were prototrophic for adenine. Only one of these strains was prototrophic for lysine, consistent with the presence of the lys1 and lys2 mutations in the original diploid strain.

In contrast, all eight strains failed to grow on synthetic medium lacking uracil, indicating that they likely lacked the ram1::URA5 mutant allele and were all RAM1 wild-type strains. Southern blot analysis confirmed that all eight basidiospores indeed contained only the wild-type RAM1
allele (Fig. 1a). Together, these data confirm that RAM1 is an essential gene in C. neoformans.

**MIC testing and RAM1 overexpression**

Since the gene encoding a homologue of the farnesyltransferase β subunit is essential in C. neoformans, we hypothesized that pharmacologic inhibition of farnesylation would result in growth arrest. A commercially available protein farnesyltransferase inhibitor III (FPT Inhibitor III, Calbiochem) had been previously investigated for its ability to inhibit the growth of malignant cells (Prendergast, 2000). Using standardized protocols for MIC testing (Galgiani et al., 1997), we tested FPT Inhibitor III for anti-cryptococcal activity. At 35°C in RPMI medium, FPT Inhibitor III completely inhibited growth of the clinically derived serotype A strain H99 at concentrations of 400–500 μM (Fig. 2). We tested other C. neoformans strains, including the serotype D congenic strains JEC20 and JEC21, and they were inhibited by similar concentrations of this compound at 72 h incubation.

We subsequently used a genetic approach to determine whether the Ram1 protein is a component of the cellular target of the FPT Inhibitor III. We hypothesized that if Ram1 was the target of FPT Inhibitor III, overexpressing the RAM1 gene would create a strain with a higher MIC than the wild-type as a consequence of competitive interaction with the drug. We specifically overexpressed RAM1,
first by cloning it into the pHYG-KB1 vector containing the *hph* gene, which encodes resistance to the aminoglycoside hygromycin B (Cox *et al*., 1996), and second by using biolistic transformation to integrate this construct into the genome. Since a single copy of *hph* is often insufficient to allow growth in the presence of hygromycin B, we had previously used this selectable marker to stably integrate genes into the *C. neoformans* genome in multiple copies, resulting in gene overexpression (Alspaugh *et al*., 2000). In this way, we isolated two strains in which the *RAM1* gene was markedly overexpressed, as confirmed by Northern blot analysis (Fig. 1b).

Compared to the wild-type strain, the *RAM1*-overexpression strain demonstrated a greater than fourfold increase in the MIC for FPT Inhibitor III in six independent experiments. Limited drug solubility in the growth medium at higher concentrations prevented the determination of an exact MIC for the overexpression strain. This result supports our hypothesis that the Ram1 protein is a limiting component of the cellular target for FPT Inhibitor III in *C. neoformans*. The identification of other FTase inhibitors with increased solubility and improved fungal-cell entry may allow a more precise delineation of the differences in MIC between the wild-type and *RAM1*-overexpressing strains.

**FPT Inhibitor III inhibits *C. neoformans* mating**

*C. neoformans* has a bipolar mating system in which α and α strains fuse and undergo meiosis under appropriate environmental conditions. After the initial fusion of the opposite mating partners, the heterokaryon forms mating hyphae. These filamentous structures typically undergo terminal differentiation to form a basidium, in which meiosis and sporulation occur (Kwon-Chung, 1975). The mating process and the associated steps of differentiation are dependent upon the pheromone response and Ras1 signal transduction pathways (Alspaugh *et al*., 2000; Davidson *et al*., 2003; Wang *et al*., 2000). Since components of each of these pathways, including the α mating factor and the small G protein Ras1, are presumed to require prenylation in order to be functional (He *et al*., 1991), we hypothesized that pharmacological inhibition of farnesylation would also inhibit mating.

The congenic serotype D strains JEC20 and JEC21 were co-incubated in a mating reaction on V8 mating medium containing various concentrations of FPT Inhibitor III. In the absence of drug, the co-incubated strains underwent a rapid filamentous mating response, with initial hyphae evident by 48 h, and mature mating structures visible by 4–7 days. However, the addition of FPT Inhibitor III inhibited this mating process (Fig. 3). Mating filamentation was almost completely inhibited by 100–200 μM FPT Inhibitor III, concentrations that are permissive for growth of these strains in a liquid medium. The mating mixture cells grew as well on V8 medium containing these concentrations of the drug as they did on V8 mating medium alone, suggesting that a general growth inhibition was not the cause of decreased mating. At lower concentrations of FPT Inhibitor III, a dose–response relationship was

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**Fig. 3.** Dose response to FPT Inhibitor III of *C. neoformans* mating. The congenic strains JEC21(α) and JEC20(α) were co-incubated on V8 mating medium containing different concentrations of FPT Inhibitor III. The edges of the mating cultures were assessed for mating hyphae and photographed after 7 days’ incubation at 25°C. Bar, 100 μm.
FPT Inhibitor III inhibits C. neoformans haploid fruiting

Haploid fruiting is an asexual form of filamentous differentiation in C. neoformans (Wickes et al., 1997). The expression of this phenotype is very strain-dependent and is inhibited by the presence of a nitrogen source and water. A more vigorous form of haploid fruiting is observed by overexpressing the STE12a transcription factor in C. neoformans strains. To test the effect of FPT Inhibitor III on Ste12a-induced hyphal formation, we transformed the pCGS-1 plasmid, containing the STE12a gene under control of the GAL7 promoter (Wickes et al., 1997), into strain JEC43 (MATa ura5) by biolistic transformation, resulting in a stable genomic integration of the galactose-regulatable STE12a gene. After 15 days of incubation on filament agar containing galactose, vigorous haploid fruiting was observed for the STE12a-overexpressing strain. In contrast, under non-inducing conditions (filament agar with glucose as the carbon source), the same strain only demonstrated limited haploid fruiting after prolonged incubation (>28 days).

To assess the effects of farnesylation and calcium inactivation on haploid fruiting, we supplemented the filament agar containing galactose with subinhibitory concentrations of FPT Inhibitor III (0–400 μM). The STE12a-overexpressing strain was incubated on this medium for 14 days. No hyphae were observed in the presence of 25 μM FPT Inhibitor III, a concentration of the drug that is 10-fold lower than the MIC (Fig. 4). At lower concentrations of FPT Inhibitor III, haploid fruiting was inhibited in a dose-dependent manner (Fig. 4). Therefore, this farnesylation inhibitor blocks both forms of hyphal differentiation in C. neoformans, mating and haploid fruiting.

Synergism between FK506 and FPT Inhibitor III

The Ras and calcineurin signal transduction pathways are both required for growth of C. neoformans at 37°C (Alspaugh et al., 2000; Odom et al., 1997). Additionally, genetic studies have demonstrated that simultaneous inhibition of both of these pathways results in a synthetic growth defect (Alspaugh et al., 2000). We therefore studied whether pharmacological inhibition of farnesylation and calcineurin signalling would demonstrate an additive/synergistic effect on the growth of C. neoformans.

The immunosuppressive drug FK506 pharmacologically inhibits calcineurin signalling, and C. neoformans cells treated with this drug are unable to grow at high temperatures. However, FK506 has little effect on cells incubated at 25–30°C (Odom et al., 1997). A ras1 mutant strain demonstrates increased susceptibility to FK506 at the permissive temperature of 30°C. The wild-type (H99), ras1 mutant (LCC1), and ras1 + RAS1 reconstituted (LCC2) strains were tested for growth inhibition by FK506. In contrast to the wild-type and LCC2 strains, which grew in the presence of 1000 ng FK506 ml⁻¹ at 30°C, the growth of the LCC1 strains (ras1) was inhibited by 60 ng FK506 ml⁻¹ at this temperature (Fig. 5).

In addition, mutant strains with genetic defects in the calcineurin-signalling pathway demonstrated an increased susceptibility to FPT Inhibitor III. C. neoformans strains with mutations in the calcineurin A gene (CNA1) grow poorly at temperatures above 30°C (Odom et al., 1997). At the permissive temperature of 25°C, this strain was four- to eightfold more susceptible to FPT Inhibitor III than an isogenic wild-type strain, further demonstrating that simultaneous inhibition of calcineurin signalling and farnesylation results in a synthetic inhibition on C. neoformans growth.

At various temperatures, we performed a checkerboard MIC test to compare the combined activity of FPT Inhibitor III and FK506 to inhibit the growth of C. neoformans. As demonstrated in Fig. 6, FK506 inhibits C. neoformans growth at 37°C at concentrations between 50 and 100 ng ml⁻¹. As noted previously, no growth inhibition by FK506 is observed at 30°C, and incubation at 35°C resulted in an intermediate effect. However, subinhibitory concentrations of FK506 dramatically decrease the MIC of FPT Inhibitor III at 37°C. Thus, there is a synthetic effect on C. neoformans growth with the combination of calcineurin inhibition and treatment with an FTase inhibitor. Slightly less growth inhibition by FK506 was observed at 35°C as compared to 37°C, both alone and in combination with FPT Inhibitor III. There was no change in the MIC to FPT Inhibitor III at these different temperatures.

DISCUSSION

Prenylation is a conserved mechanism by which proteins may be modified post-translationally to allow membrane association. In S. cerevisiae, three protein prenyltransferases have been described: FTase, GGTase type I and GGTase type II. FTase and GGTase I share a common α subunit, and both recognize and modify proteins with CAAX motifs at the C-terminus (He et al., 1991; Kinsella et al., 1991; Mayer et al., 1992). The GGTase II protein in S. cerevisiae is composed of distinct α and β subunits encoded by the BET2 and MAD2 genes. GGTase II is responsible for adding geranylgeranyl groups onto the Rab proteins Sec4 and Ypt1, and it recognizes C-terminal consensus sequences distinct from the other prenyltransferases (Jiang et al., 1993). Protein farnesylation in S. cerevisiae is specifically required for the proper functioning of Ras proteins as well as for the a factor mating pheromone (He et al., 1991). In contrast, GGTase I demonstrates specificity for other small G proteins, such as Rho1 and Cdc42 (Ohya et al., 1993). The
S. cerevisiae RAM2 gene, encoding the α subunit common to FTase and GGTase I, and the CDC43 gene, encoding the β subunit of GGTase I, are both essential (Mayer et al., 1992). Strains with null mutations of the RAM1 gene, which encodes the β subunit of FTase, are viable, but display a temperature-sensitive lethality and grow poorly at a permissive temperature of 30 °C (He et al., 1991). These results suggest that S. cerevisiae proteins that are typically farnesylated may undergo alternative prenylation and retain partial function in the absence of FTase, but that alternative prenylation cannot compensate for defective protein-geranylgeranyltransferase activity in this organism.

The closely related yeast Candida albicans is a prominent human pathogen. In contrast to S. cerevisiae, GGTase-I activity in Ca. albicans is not required for viability. Ca.
albicans strains with mutations in the GGTase-I β-subunit gene CDC43 are viable, although lacking GGTase-I activity (Kelly et al., 2000). These mutant strains demonstrate similar growth rates to wild-type strains in early- and mid-exponential-phase cultures, but they arrest in later-phase cultures. Ca. albicans cdc43 mutants also exhibit altered cellular morphology. Interestingly, the levels of two Ca. albicans proteins that undergo geranylgeranylation under normal circumstances, Rho1 and Cdc42, are increased in GGTase-I-null (cdc43 mutant) strains. These two proteins are also mislocalized in the cdc43 mutant, being present in cytosolic rather than membrane fractions. This observation led the investigators to hypothesize that Rho1 and Cdc42 may undergo alternative prenylation (farnesylation) in the absence of GGTase I in Ca. albicans (Kelly et al., 2000).

However, prenylation is required for viability and pathogenicity in Ca. albicans, since the RAM2 gene, encoding the α subunit of FTase and GGTase I, is essential (Song & White, 2003).

In these studies, we demonstrated that the RAM1 gene, encoding a homologue of an FTase β subunit of C. neoformans, encodes an essential function. Even though

![Fig. 5. Effect of the the calcineurin-signalling inhibitor FK506 on wild-type and mutant C. neoformans strains. Wild-type H99 (wt), ras1 mutant LCC1 (ras1), and ras1+RAS1 reconstituted LCC1-2 (ras1+RAS1) strains were incubated in RPMI medium with varying concentrations of FK506 at 30°C. The OD₆₂₀ of the cell suspensions was assessed to determine cell growth after 72 h incubation.](image)

![Fig. 6. Synthetic inhibition of C. neoformans growth by FK506 and FPT Inhibitor III. The wild-type strain H99 was tested in a 'checkerboard' MIC assay to determine its susceptibility to the calcineurin inhibitor FK506 and the protein farnesyltransferase inhibitor FPT Inhibitor III, individually and in combination. Serial dilutions of each drug were made in RPMI medium as indicated, and each well was inoculated with 100 C. neoformans cells. +, wells containing no drug. After 72 h incubation at 30, 35 and 37°C, the MIC for each drug or drug combination was determined (indicated with a box).](image)
the *S. cerevisiae raml* mutant is viable, it grows poorly even under optimal conditions, and does not grow at all at 37 °C. Therefore, farnesylation is clearly required for *S. cerevisiae* to survive under stressful conditions such as elevated temperatures. Together, these observations suggest that intact microbial protein farnesylation may be required for the pathogenesis of human fungal infections.

The question remains why GGTase I and FTase appear to be able to partially compensate for each other’s absence in the ascomycetous yeasts *S. cerevisiae* and *Ca. albicans*, while FTase activity is absolutely required for viability in the distantly related basidiomycetous fungus *C. neoformans*. Perhaps the *C. neoformans* FTase and GGTase I proteins have differentiated sufficiently that they are no longer able to serve redundant functions. Interestingly, analysis of the completed *C. neoformans* genome databases using a BLAST search with the *S. cerevisiae* Cdc43p reveals no close match for genes encoding GGTase-I β subunits. Therefore, it is also possible that no GGTase-I activity is present in *C. neoformans*, explaining why the FTase β-chain gene is essential. Biochemical analysis of *C. neoformans* protein-prenyltransferase activities will help to address whether GGTase-I activity is indeed absent in *C. neoformans*. Additionally, as genome projects from other basidiomycetes are completed, it will be interesting to determine whether both FTase and GGTase-I β-subunit genes are present in these organisms.

The possibility that *C. neoformans* possesses FTase activity without GGTase-I activity might also explain the C-terminal amino-acid sequences of the Ras1, Ras2 and Rho1 proteins. All of these proteins have a C–V–V–L amino-acid sequence at the C-terminus, a typical consensus sequence associated with farnesylation (Zhang et al., 1996). This is in contrast to Ras proteins in the ascomycete group of fungi, such as *S. cerevisiae*, *Ca. albicans*, *Aspergillus fumigatus* and *Neurospora crassa*, whose Ras proteins possess C-terminus CAAX motifs with terminal amino-acid residues such as cysteine, serine or methionine, sequences typically associated with farnesylation (Zhang et al., 1996). Interestingly, similar to *C. neoformans*, two other basidiomycetes, *Ustilago maydis* and *Lentinula edodes*, display leucine as the terminal amino acid of the CAAX motif of their Ras proteins (*U. maydis* Ras1, –CNIL; *L. edodes* LE ras, –CVVL). Therefore, the prenylation process of small G proteins may differ substantially between the ascomycete and basidiomycete groups of fungi.

**Pharmacological inhibition of prenylation**

As discussed above, the *S. cerevisiae* and *Ca. albicans* RAM2 genes, encoding the α subunit of both GGTase I and FTase, are essential. This indicates that simultaneously abolishing both FTase and GGTase-I activity results in lethality. Also, FTase activity is required for viability in *C. neoformans* and for growth of *S. cerevisiae* at human physiological temperature. Therefore, protein prenylation remains an interesting target for further antimicrobial drug development.

Compounds that inhibit protein prenylation have been developed and studied as potential agents in treating human malignancies. This field was motivated by the observation that activated Ras mutations were associated with a significant number of human cancers. Initial trials indicated that inhibiting prenylation could result in a reduction in the growth rate of some tumour lines (Kohler et al., 1995).

In limited initial experiments, these agents have also been considered for antifungal effects. *Ca. albicans* was treated with the protein farnesyltransferase inhibitor FPT Inhibitor III and a related compound, FTase inhibitor II, but no antifungal activity was observed at FPT Inhibitor III concentrations as high as 400 μM (McGeady et al., 2002; Song et al., 2003). However, slightly lower concentrations of FTase inhibitor II were able to inhibit prenyltransferase enzymatic activity in cell extracts: *Ca. albicans* FTase activity was inhibited *in vitro* by 100 μM FTase Inhibitor II (Song et al., 2003). Similarly, in our studies, high concentrations of FPT Inhibitor III were required for growth inhibition of *C. neoformans*, precluding its use in clinical studies. Therefore, this drug is clearly not a sufficiently potent inhibitor of fungal FTase, and other compounds may demonstrate significantly lower inhibitory concentrations. Related farnesyl diphosphate-based compounds inhibit the mammalian FTase enzyme at concentrations ranging from 75 nM to 200 μM (Patel et al., 1995), suggesting that chemical modification of these compounds may yield enhanced antifungal activity. Additionally, the compound may be degraded by cellular factors in fungi, or perhaps this drug does not efficiently enter the fungal cell. The identification of prenylation inhibitors with enhanced specificity for fungal protein prenyltransferases and optimized for entry into fungal cells might offer novel antimicrobial therapeutic options.

We also explored the possibility of simultaneously inhibiting two distinct signalling pathways that control the growth of *C. neoformans* at human physiological temperature. We observed that co-inhibition of the calcineurin and Ras1 pathways results in a synthetic effect on the growth of this fungus. Additionally, the simultaneous inhibition of calcineurin signalling and farnesylation also resulted in a synthetic growth arrest. Such strategies may offer new possibilities for novel targets for antifungal therapy.

Although the farnesylation inhibitor FPT Inhibitor III does not impair growth of *Ca. albicans* at low concentrations, this compound is able to inhibit hyphal development of this pathogenic yeast (McGeady et al., 2002). This observation is clinically relevant, since the yeast–hyphal transition in this organism has been closely associated with pathogenicity. Defined signalling and structural molecules are required for hyphal formation in *Ca. albicans*, including Ras proteins and components of the MAP-kinase and cAMP-signalling cascades (Feng et al., 1999; Kohler et al., 1996; Rocha et al., 2001). Several of these proteins, including small G proteins, would be expected to require prenylation in...
order to function properly, providing a rational, hypothet-
cial mechanism for the ability of FPT Inhibitor III to inhibit
hyphal development.

Our studies similarly demonstrated that FPT Inhibitor III
inhibited hyphal differentiation in the pathogenic fungus
Cryptococcus neoformans. We demonstrated that two hyphal processes,
mating and haploid fruiting, were inhibited by FPT Inhi-
bor III. Several proteins in C. neoformans are known to be
required for mating and haploid fruiting. The pheromone
response pathway is activated by peptide pheromones to
initiate the mating process (Moore et al., 1993), and the
Mfx1 pheromone contains consensuses farnesylation sequences.
Moreover, Mfx1 synthesized heterologously in bacterial cells
has been demonstrated to require both farnesylation and
carboxy-methylation in order to be functional and induce
hyphal differentiation in MATa cells (Davidson et al., 2000b).
The pheromone response pathway is also activated by the
Ras1 protein, which also likely requires prenylation to be
membrane-associated and functional (Waugh et al., 2003).
It is likely that one or several of these proteins is affected
by FPT Inhibitor III, resulting in the failure of C. neofo-
mans to undergo hyphal differentiation when treated with
this drug. Thus, this prenylation inhibitor blocks steps
required for hyphal development in both ascomycetes and
basidiomycetes.

In conclusion, these studies have explored the role of pro-
tein farnesylation in the pathogenic fungus Cryptococcus neoformans.
Although individual prenyltransferases may demonstrate
varying substrate specificity in different fungal species,
these proteins regulate central processes controlling growth
and differentiation in these micro-organisms. Novel
therapeutic targets can be identified by understanding the
differences between prenylation in microbial pathogens
and in the human host.

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