

RAM2: an essential gene in the prenylation pathway of *Candida albicans*

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Prenylation is a post-translational protein modification process that results in correct protein localization to membranes in the cell. It is mediated by at least three prenyltransferases (PTFs) in eukaryotic cells. The *RAM2* gene encodes the common α -subunit of two PTFs, farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I). In this study, the *RAM2* gene of the prenylation pathway in the opportunistic pathogen *Candida albicans* was analysed. The heterozygote trisomy test was used to demonstrate that *RAM2* is essential to the viability of *C. albicans*. *RAM2* mRNA in the yeast and hyphae growth states was not detected by Northern blot analysis, but was detected by RT-PCR. Drugs that inhibit mammalian PTFs do not alter *Candida* cell growth, but they do inhibit FTase and GGTase I activities in cell-free enzyme assays. The data from genetic studies and cell-free enzyme assays suggest that the drugs may not have access to the *Candida* cytoplasm. The regulation of PTF subunits was also examined in a strain in which *RAM2* was under the control of a regulable promoter. Overall, this study demonstrated that *RAM2* is essential to *C. albicans*, indicating that protein prenylation is an indispensable cellular process in this yeast.

INTRODUCTION

Candida albicans is an opportunistic pathogen that causes oral, oesophageal and systemic infections in immunocompromised populations (Odds, 1988). Over the past decade, *Candida* infections have increased dramatically with the rise in AIDS, cancer and bone marrow transplant patients (Georgopapadakou & Walsh, 1994). The common use of azoles in the prophylaxis and treatment of candidiasis has led to the emergence of azole-resistant isolates. A recent study indicated that azole-resistant isolates were found in 20–30% of HIV patients suffering from oral candidiasis (Perea *et al.*, 2001). Therefore, the discovery and development of new drug targets and compounds against fungal pathogens are greatly needed.

Approximately 0.5% of mammalian cellular proteins are prenylated (Epstein *et al.*, 1991). Protein prenylation is an important and essential cellular process, which adds farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) derived from the ergosterol pathway to the C terminus of proteins (reviewed by Omer & Gibbs, 1994; Schafer & Rine, 1992; Sinensky & Lutz, 1992). These isoprenoid groups anchor the proteins to the plasma membrane at the correct

intracellular location. Prenylated proteins participate in a variety of cellular functions, such as the control of cell growth, differentiation, cytokinesis, membrane trafficking and signal transduction. Proteins that are prenylated include small GTP-binding proteins, lipopeptide pheromones, nuclear lamins and trimeric G-proteins (Schafer *et al.*, 1989). Many of these GTPases play crucial roles in cell-signalling pathways, which are likely to be involved in the pathogenesis of *C. albicans*.

To date, there are at least three prenyltransferases (PTFs) that have been identified in eukaryotic cells: farnesyltransferases (FTases), geranylgeranyltransferase I (GGTase I) and geranylgeranyltransferase II (GGTase II). All three PTFs are heterodimeric, magnesium-dependent zinc metalloenzymes, with the exception of mammalian GGTase I, which has been reported to require only zinc (Zhang & Casey, 1996). PTFs catalyse prenyl group transfer from either FPP or GGPP onto the carboxyl terminus of proteins containing CAAX (C, cysteine; A, aliphatic amino acid; X, any amino acid), the recognition sequence for FTase and GGTase I, and CC and CAC, the recognition sequence for GGTase II (Casey & Seabra, 1996). GGTase I and FTase in *C. albicans* have preferences for substrates that end in a leucine residue and methionine residue, respectively (Mazur *et al.*, 1999). GGTase II has not been characterized in *C. albicans*; however, the CC and CAC sequences have been demonstrated to be substrates for GGTase II in *Saccharomyces cerevisiae* (Farnsworth *et al.*, 1994).

Abbreviations: FPP, farnesyl diphosphate; FTase, farnesyltransferase; GGPP, geranylgeranyl diphosphate; GGTase, geranylgeranyltransferase; HT, heterozygote trisomy; PTF, prenyltransferase.

The GenBank accession number for the α -subunit farnesyltransferase I gene (*RAM2*) sequence reported in this paper is AF548361.

All three PTFs in yeasts and mammals consist of α - and β -subunits. Previous studies on FTases have indicated that the β -subunit contains the binding sites for the FPP, peptide substrate and Zn^{2+} , which is required for binding of the peptide substrate and enzyme activity (Reiss *et al.*, 1991, 1992). The α -subunit of FTase contains the catalytic function and is required for stabilization of the β -subunit (Andres *et al.*, 1993). The α -subunit of both FTase and GGTase I is encoded by *RAM2*. The β -subunits of FTase and GGTase I are encoded by *RAM1* and *CDC43*, respectively. The GGTase II α - and β -subunits are encoded by *BET4* and *BET2*, respectively.

The ability to alter cell types between the yeast and hyphal form is an important virulence trait in *C. albicans* (Cutler, 1991; Odds, 1988). An important prenylated protein, Ras1p, has been found to be involved in serum-induced hyphal formation in *C. albicans* (Feng *et al.*, 1999; Leberer *et al.*, 2001). Ras1p is one of the many prenylated proteins that have an important cellular role. A *C. albicans* strain that had both copies of *ras1* deleted was viable, but its virulence was greatly reduced in the mouse model (Leberer *et al.*, 2001). Failure to prenylate Ras1p may lead to the inability of *Candida* to undergo the yeast-to-hyphal transition, as inhibition of prenylation blocks the membrane association and biological activity of both human and *S. cerevisiae* Ras proteins (Schafer *et al.*, 1989). Therefore, a disruption of *RAM2* may result in disturbances of various cellular functions, including the yeast-to-hyphal transition.

Previous prenylation studies in fungi have focused on GGTase I, because its substrates, Rho1p and Cdc42p, are essential in important roles of morphogenesis in both *S. cerevisiae* and *C. albicans* (Drgonova *et al.*, 1996; Kondoh *et al.*, 1997; Mazur & Baginsky, 1996; Qadota *et al.*, 1996). In *C. albicans*, a gene deletion of *CDC43* and the resulting absence of GGTase I activity did not affect the viability of the cells, while the same gene in *S. cerevisiae* could not be deleted, indicating that the gene is essential in *S. cerevisiae* (He *et al.*, 1991; Kelly *et al.*, 2000). It was demonstrated that *Candida* FTase can add FPP onto GGTase I substrates and that this cross-farnesylation activity was greater in *Candida* than in *Saccharomyces*; thus, GGTase I activity is not essential in *Candida* (Caplin *et al.*, 1994; Mazur *et al.*, 1999). GGTase II stands apart from the other PTFs both structurally and functionally (Casey & Seabra, 1996; Farnsworth *et al.*, 1994). Therefore, it would be unlikely that GGTase II would compensate for the loss of FTase and GGTase I activity (Kelly *et al.*, 2000). A disruption of the *RAM2* gene would be expected to abolish both the GGTase I and the FTase activity. Since both activities would be affected, the *RAM2* disruption would be expected to be detrimental to cell growth. Furthermore, the *Candida* Ram2p protein is only 30% identical to human Ram2p, suggesting that it may be possible to identify fungal-specific inhibitors (Mazur *et al.*, 1999).

The heterozygote trisomy (HT) test was developed to identify essential genes in *C. albicans*, using PCR tests for

homozygosity and triplication (Enloe *et al.*, 2000). In this study, the HT test results demonstrated that *RAM2* is most likely essential for the viability of *C. albicans*. Minimum inhibitory concentration (MIC) drug-susceptibility tests and cell-free enzyme assays suggest that a PTF inhibitor is active against *Candida* FTase and GGTase I, but this drug may not access the *C. albicans* cytoplasm. In addition, an initial characterization of PTF subunits indicated that an overexpression of the α -subunit did not upregulate the β -subunits of FTase and GGTase I, suggesting that these genes may be regulated independently of each other.

METHODS

Cell growth and maintenance. The *C. albicans* strain BWP17 (*ura3 Δ :: λ imm434/ura3 Δ :: λ imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*) was generously provided by A. P. Mitchell (Wilson *et al.*, 1999). Cultures were routinely grown at 30 °C in YEPD medium (10 g yeast extract, 20 g peptone and 20 g glucose per litre) or on YEPD plates (YEPD and 10 g Bactoagar per 500 ml). Another lab strain, SS (Blagoyevich, 1979), was also used in this study. The *PCK1* promoter (see below) was induced in YEPC medium (10 g yeast extract, 20 g peptone and 20 g Casamino acids per litre). Transformants for the HT test were grown in synthetic defined media and plates, SD-Ura, SD-Arg and SD-Ura-Arg, prepared as described (Bio101). To induce hyphal formation, cells were grown in M199 at 37 °C for 3 h. Cells were subcultured routinely or stored at -80 °C in 10% (v/v) glycerol.

Chemicals, reagents and supplies. Ras-CVLL, Ras-WT (CVLS), GGTI-2147, GGTI-286 and FTase Inhibitor II were purchased from Calbiochem. α -Hydroxyfarnesylphosphonic acid and FTase Inhibitor III were generously provided by Michael Gelb. Chemicals, amino acids and protease inhibitors were purchased from Sigma. Casamino acids and all restriction enzymes and reagents used in RT-PCR were purchased from Fisher Scientific. M199 medium and TRIZol were purchased from Gibco-BRL. [³H]GGPP (22 Ci mmol⁻¹; 814 GBq mmol⁻¹), [³H]FPP (16.1 Ci mmol⁻¹; 595.7 GBq mmol⁻¹) and Kodak BioMAX MS films were purchased from Perkin-Elmer Life Sciences. *Pfu* DNA polymerases, PCR-Script Amp SK(+) vector and XL-1 Blue cells were purchased from Stratagene.

Cloning of *RAM2* by inverted-PCR. Genomic DNA from strain SS (Blagoyevich, 1979) was digested with *Hind*III and electrophoresed in a 1% low-melting-temperature gel in TAE buffer (40 mM Tris/HCl; 5.71%, v/v, glacial acetic acid; 1 mM EDTA, pH 8.0). A 3 kb band, containing the *RAM2* gene, was excised and purified using GeneClean (Bio 101) according to manufacturer's instructions. The DNA fragments were circularized by ligation at low concentrations in a reaction mix consisting of 10 μ g gel-purified DNA, 100 μ l of 10 \times ligase buffer and 5 μ l T4 DNA ligase in a 1 ml volume. The ligation reaction was incubated at 15 °C overnight, then phenol/chloroform extracted and ethanol precipitated at -80 °C for 1 h. The reaction mix was subsequently centrifuged, dried and resuspended.

Circularized DNA fragments were used as DNA template for PCR with oligonucleotide primers *RAM2AR* and *RAM2CR*, which were designed based on a partial 3' end of the *RAM2* sequence available at that time (Scherer & Ran, 1996) (Table 1). PCR products of approximately 1.5 to 3.5 kb in size were gel-purified, blunt-ended with *Pfu* DNA polymerases, ligated to PCR-Script Amp SK(+) cloning vectors and transformed into XL-1 Blue cells. Plasmids containing PCR inserts were isolated by using the Qiagen Plasmid Maxi Kit (Qiagen). Oligonucleotides were designed to sequence the 5' and 3' ends of the *RAM2* gene in both orientations using an ABI automated DNA

Table 1. DNA primer sequences and plasmids used in this study

Primer/plasmid	Gene	Positions*	Reference†	Sequence (5'→3')‡
Primer				
ACT-50	<i>ACT1</i>	2 478–2 527	D	GATTTAGGTTTGAAGCTGCTGGTATTGACCAAACCACTTTCAACTCC
ACTIN R	<i>ACT1</i>	2 517–2 497	D	GTGGAAAGGTCAATACCAGCA
ACT INTRN	<i>ACT1</i>	1 017–1 034	D	CATTCAAAATGGACGGTG
ARG 1282	<i>ARG4</i>	2 135–2 115	C	TTGAAAAAGACGTTATGGAA
CDC43-50	<i>CDC43</i>	781–831	A	TATCAATAAAGGTGGGTTGTGCGCGACTCTATTATAATGAAGAAAATG
HT 1133	<i>RAM2</i>	25 593–25 570	B	AGCAGATACACACACACTC
HT3'RAM	<i>RAM2</i>	1 413–1 352	A	TGGTAATATCGGTGGTATAACAATGATTTACCTAGTTAAACAT ATTGAAAACAAGAAAGTATATGTGGAATTGTGAGCGGATA
Ppck-crRAM	<i>RAM2</i>	394–444	A	AGTGTTTATATCGACAGGAGTAATGTCAGAATAGTCATATTG GAGTCTGTCATGATTGATAATTATTTGAAT
PRAM-PL	<i>RAM2</i>	23 265–23 316	B	CAATTACTCGATAATTGGAAGCAGATGTTGTACTTGCATTTTTCTTTTC TGTTGGAATTGTGAGCGG
RAMBamComp	<i>RAM2</i>	24 247–24 270	B	CCGGGATCCGACTCACTCAATACGTACTATTC (<i>Bam</i> HI site underlined)
RAMHindUra	<i>RAM2</i>	2 603–22 620	B	GCGAAGCTTGTGCTGTGGTATGGGA (<i>Hind</i> III site underlined)
RAM1-50	<i>RAM1</i>	942–892	B	TTAGTAATATACCAAATACTGAAGCTCATGGAGTTTATACATATTGTGCC
RAM2A	<i>RAM2</i>	1 322–1 303	A	TACCTTGGAATTACACCG
RAM2AR	<i>RAM2</i>	1 303–1 322	A	CGGTGTAATTACCAAGGT
RAM2CR	<i>RAM2</i>	1 054–102	A	TACTTGGATTCTGTGGACAT
RAM2NA-D3	<i>RAM2</i>	272–290	A	CAAACAAACCAATACCATC
5'RAM80	<i>RAM2</i>	391–451	A	ATGACAGACTCCAAATATGACTATTCTGACATTACTCCTGTGCATATAA ACACTGAAGAGGTTTTCCAGTCACGACGTT
Plasmid				
pRAM1.65	<i>RAM2</i>	23 897–25 547	B	
pRAM-288	<i>RAM2</i>	1 033–1 321	A	

*Nucleotide positions according to the published papers.

†A, (<http://alces.med.umn.edu/Candida.html/>) GenBank accession no. AF110691; B, Scherer & Ran (1996), unpublished; C, Hoyer *et al.* (1994), GenBank accession no. L25051; D, Losberger & Ernst (1989), GenBank accession no. X16377.

‡The *RAM2* sequence is from contig 6-2394 and the *RAM1* sequence is from contig 6-2230.

sequencer with *Taq* dye-primer and dye-terminator chemistries (Applied Biosystems). The *RAM2* gene was sequenced together with 999 bp upstream and 180 bp downstream of the gene. The resulting sequence was deposited in GenBank under accession number AF548361.

Once the *RAM2* sequence had been obtained, oligonucleotide primers RAMBamComp and RAMHindUra were used to amplify *RAM2* from genomic DNA in a PCR. The PCR fragments were digested with restriction enzymes *Hind*III and *Bam*HI, phenol/chloroform extracted and ethanol precipitated. The PCR fragments were cloned as described above. Plasmid pRAM1.65 contained the promoter, coding and terminator regions of *RAM2*, as determined by restriction enzyme analysis and DNA sequencing.

Southern and Northern blot analyses. Genomic DNA was digested with *Bam*HI, *Bgl*II, *Hind*III, *Xba*I and *Eco*RV and Southern blot analysis was performed as described (Maniatis *et al.*, 1982; Sambrook *et al.*, 1989). A 288 bp sequence of the *RAM2* coding region was available in the *Candida* genome project. This was amplified by PCR and cloned into the PCR-Script Amp SK(+) vector, to generate pRAM-288. Plasmid pRAM-288 was labelled by random priming with [α - 32 P]dATP and the large subunit of DNA polymerase I, and was used for Southern blot analysis (Klenow fragment) (Maniatis *et al.*, 1982; Sambrook *et al.*, 1989).

For Northern blot analysis, overnight cultures were diluted to 4×10^6 cells ml $^{-1}$ and grown to 2×10^7 to 3×10^7 cells ml $^{-1}$ (exponential

growth). Total RNA was prepared and Northern blots were performed as described (Ausubel *et al.*, 1995; Maniatis *et al.*, 1982; Sambrook *et al.*, 1989). Probes used included CDC43-50, RAM1-50, ACT-50 and pRAM1.65 (Table 1). mRNA signals were quantified using the Storm Phosphorimager and IMAGEQUANT software (Molecular Dynamics).

HT test. This was used to determine the essential function of *RAM2* in *C. albicans*. PCR primers 5'RAM80 and HT3'RAM, containing 60 bp of the *RAM2* promoter region and terminator region, respectively, and 20 bp of the polyclonal region of pBME101, were designed (Enloe *et al.*, 2000; Wilson *et al.*, 1999) (Table 1) (plasmid pBME101 contains the *UAU1* cassette important for selections in the HT test). Standard PCRs included $1 \times$ PCR buffer, 0.2 mM each dNTP, 1 μ M each primer and approximately 50 ng linearized pBME101. PCR conditions used were denaturing at 94 °C for 2 min followed by 30 cycles of denaturing at 94 °C for 1 min, annealing at 55 °C for 2 min and elongation at 72 °C for 3 min, with a final extension at 72 °C for 10 min.

Five micrograms of purified PCR products were added to a 40 μ l aliquot of competent BWP17 cells as described previously (De Backer *et al.*, 1999; Thompson *et al.*, 1998). The electroporation conditions used were 1.6 kV, 25 μ F, 200 Ω with a Bio-Rad Gene Pulser. The cells were pelleted and resuspended in 500 μ l of sterile double-distilled H $_2$ O (ddH $_2$ O) and plated onto SD-Arg plates with 250 μ l of culture per

plate; they were then grown at 30 °C for 3–5 days. Transformed colonies were serially transferred three times to ensure a homogeneous transformant.

Heterozygotes (*ram2::UAU1/RAM2*) containing the *UAU1* cassette from pBME101 were PCR-screened for a correct integration at the *RAM2* locus with primers RAM2NA-D3, ARG 1282 and HT 1133 (Table 1). The heterozygotes were plated out onto YEPD plates for 2 days at 30 °C. Lawns of cells were replica-plated onto SD-Arg-Ura plates and grown for 5–6 days at 30 °C. Transformants were screened by PCR with the same primers as described above.

Generation of inducible *RAM2* strain B30. The inducible *PCK1* promoter (Leuker *et al.*, 1997; GenBank accession no. U70473) and a selectable marker, *URA3*, were integrated upstream of the *RAM2* ATG start codon. A plasmid containing *URA3* and the *PCK1* promoter (pETE, constructed by Peter Silver) was linearized and used as the DNA template in PCRs. Oligonucleotides Ppck-crRAM, containing 60 bp of *RAM2* coding sequence including the start codon and 20 bp of homology to the *PCK1* promoter region, and PRam-PL, containing 60 bp of *RAM2* promoter sequence and 20 bp of the polyclonal region upstream of *URA3*, were used as PCR primers (Table 1). PCR products were phenol/chloroform extracted and ethanol precipitated. The PCR fragments were transformed into competent BWP17 as described above. Transformants with the inducible *PCK1* promoter integrated upstream of the *RAM2* ATG start codon were screened and verified by PCR, Southern blot analysis, DNA sequencing and Northern blot analysis.

***RAM2* expression in yeast and hyphal growth states.** After 48 h growth in YAD medium (1.7 g yeast nitrogen base, 5 g ammonium sulfate and 10 g glucose per litre), cells were washed twice in PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ and 0.24 g KH₂PO₄ per litre) at pH 7.4. The culture was diluted to 2 × 10⁶ cells ml⁻¹ and inoculated into either YAD at 25 °C at 180 r.p.m. or pre-warmed M199 medium at 37 °C at 300 r.p.m. (Bahn & Sundstrom, 2001). Total RNA was extracted from yeast and hyphal cells after 4 h growth using TRIzol as described previously (Harry *et al.*, 2002). Total RNA was treated with RQ1 RNase-free DNase at 37 °C for 30 min, followed by phenol/chloroform extraction and ethanol precipitation. RT-PCR was performed using the Access RT-PCR System with 100–400 ng of RNA template according to the manufacturer's instructions. Oligonucleotide primers used for the RT-PCRs were RAM2NA-D3 and RAM2A to amplify *RAM2*, and ACT INTRN and ACTIN R to amplify *ACT1* (Table 1).

Antifungal-susceptibility testing. MIC values were determined according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines for antifungal-susceptibility testing (M27-A) (National Committee for Clinical Laboratory Standards, 1997). YAD supplemented with uridine, histidine and arginine was used as the test medium. MIC values were defined as an 80% reduction in optical density compared with the growth control at 24 and 48 h.

Drugs used for the MIC tests included amphotericin B (stock concn 2.5 mg ml⁻¹ in DMSO), FTase Inhibitor II (stock concn 10 mM in DMSO), GGTI-2147 (stock concn 10 mM in DMSO) and GGTI-286 (stock concn 10 mM in DMSO).

Preparation of cell extract. From an overnight culture grown in YEPD, cells were diluted to 8 × 10⁶ cell ml⁻¹ in 1 l of medium and grown to between 2 × 10⁷ and 3 × 10⁷ cells ml⁻¹. Cells were centrifuged at 6000 g for 5 min at 4 °C. The cell pellet was washed twice with cold ddH₂O and once with freshly prepared lysis buffer (20 mM Tris/HCl, pH 8.0; 5 μM ZnCl₂; 50 mM NaCl; 5 mM DTT). The wet weight of the cell pellet was determined and the pellet was resuspended in 0.5 × the cell weight with lysis buffer supplemented

with protease inhibitors: 1 mM PMSF (stock concn 100 mM in 2-propanol); 10 μg aprotinin ml⁻¹ (stock concn 10 mg ml⁻¹ in water); 10 μg leupeptin ml⁻¹ (stock concn 10 mg ml⁻¹ in water); 10 μg pepstatin A ml⁻¹ (stock concn 5 mg ml⁻¹ in methanol). Cells were transferred to a 50 ml conical tube and an equal volume of glass beads was added to the cell suspension. Cells were vortexed for 30 cycles of 10 s of beating with intervening 10 s of chilling on ice. The bottom of the 50 ml conical tube was pierced with a heated 18 gauge needle and the 50 ml conical tube was placed in 250 ml centrifuge tubes. The cell extracts were collected at 2000 g for 5 min at 4 °C, transferred to an ultracentrifuge tube and centrifuged at 100 000 g for 1 h at 4 °C. Cell extracts were aliquoted into 1.5 ml Eppendorf tubes and stored at -80 °C until use. The concentration of the cell extract was determined by using the Bradford assay (Bio-Rad) according to the manufacturer's instructions with BSA as the standard.

Cell-free enzyme assays. A 2 × reaction buffer was made by adding solution 1 (60 mM KH₂PO₄; 10 mM DTT; 1 mM MgCl₂; 40 μM ZnCl₂) to solution 2 (60 mM K₂HPO₄; 10 mM DTT; 1 mM MgCl₂; 40 μM ZnCl₂) until pH 7.7 was reached. These solutions were aliquoted and stored at -80 °C. Exogenous enzyme substrates, Ras-CVLL (C, cysteine; V, valine; L, leucine) and Ras-WT (CVLS) (C, cysteine; V, valine; L, leucine; S, serine) were used in these assays. The enzyme reactions for the GGTase I were performed with 1 × reaction buffer, 1 μM [³H]GGPP, 10 μM Ras-CVLL, 20 μg cell extract and 0.02% Triton X-100 in a total of 20 μl. The enzyme reactions for the FTase were performed with 1 × reaction buffer, 1 μM [³H]FPP, 10 μM Ras-WT, 20 μg cell extract and 0.02% Triton X-100 in a total of 20 μl. All enzyme reactions were incubated at 30 °C for 90 min. Enzyme reactions were terminated with the addition of 5 × sample buffer (300 mM Tris, pH 6.8; 200 mM DTT; 5% SDS; 0.5% bromophenol blue; 50% glycerol) and heated for 3–5 min at 100 °C. Samples were briefly centrifuged and loaded on to 12% SDS-PAGE gels, which were electrophoresed at 200 V for 45 min.

After electrophoresis, SDS-PAGE gels were soaked in 50% methanol overnight to remove unbound [³H]GGPP or [³H]FPP, then washed three times for 15 min in glacial acetic acid. Gels were soaked in 20% diphenyloxazole (PPO; Amersham Life Sciences) in glacial acetic acid (w/v) for 30 min and washed in water for more than 20 min until white precipitates were gone. The gels were dried at 60 °C for 1.5–2 h and exposed to Kodak BioMAX MS for 4 weeks at -80 °C.

RESULTS

Cloning of the single copy *RAM2*

A partial sequence of the *RAM2* coding region became available early in the *Candida* genome-sequencing project. Based on this sequence, oligonucleotide primers were designed to amplify a 288 bp *RAM2* DNA fragment using PCR. This partial *RAM2* sequence was cloned into a plasmid (pRAM-288) and used as a radiolabelled probe for the Southern blot analysis. The Southern blot analysis identified a single hybridizing band in genomic DNA with five restriction enzymes (*Bam*HI, *Bgl*II, *Hind*III, *Xba*I and *Eco*RV), demonstrating that there is one copy of the *RAM2* gene per *C. albicans* genome (data not shown). The lack of additional bands at low stringency and long exposure demonstrated that additional, related genes were not present in the genome. This is confirmed by analysis of the currently available *Candida* genome sequence (Scherer & Ran, 1996).

Inverted-PCR was used to clone and sequence the entire *RAM2* gene from the laboratory strain SS, using a 3 kb *HindIII* fragment from genomic DNA (see Methods). Plasmid pRAM1.65 was constructed and contained 921 bp of the *RAM2* coding region, 540 bp of the promoter region and 190 bp downstream of the stop codon from strain SS. This construct was later used as a radiolabelled probe for subsequent Southern blots to screen for correct transformants in gene deletion experiments.

Analysis of the *RAM2* sequence

The *RAM2* gene sequence from strain SS has several nucleotide and amino acid differences when compared to the *RAM2* sequences from strain CA124 (Mazur *et al.*, 1999) and strain SC5314 as determined by the *Candida* genome-sequencing project (Scherer & Ran, 1996) (Table 2). In the coding region, there are 8 bp changes between SS and SC5314: two silent base pair differences at positions 92 and 723, and 6 bp changes that result in amino acid changes. There are 5 bp changes between SS and CA124, four of which result in amino acid changes. Of the 6 aa changes between all the strains, two are conservative (positions 38 and 242) and four are non-conservative (positions 41, 168, 176 and 244) (Table 2). The SS and CA124 differences are a subset of the SS and SC5314 differences. In 999 bp of the promoter region upstream of the start codon ATG, there are 7 bp differences between SS and SC5314, 2 bp differences between SS and CA124, and 1 bp difference between SC5314 and CA124. All strains had identical nucleotide sequences 180 bp downstream of the stop codon.

RAM2 is expressed at low levels

To determine if *RAM2* is expressed in yeast and hyphal cells, total RNA was extracted and analysed by Northern blot analysis and RT-PCR. By Northern blot analysis, *RAM2*

mRNA was undetectable in *C. albicans* and *S. cerevisiae* using the homologous gene fragment as a probe, while control *ACT1* mRNA was easily detected (data not shown). RT-PCR did detect low levels of *RAM2* mRNA in the yeast and hyphal forms of *C. albicans* (Fig. 1). Total RNA was DNase-treated and amplified with the primers *RAM2* and *ACT1*. The *ACT1* PCR products indicate the extent of DNA contamination in RNA samples, since the PCR fragment spans the intron within the coding region of the actin transcript. The RT-PCR products amplified with the actin primers indicated that there was no detectable DNA contamination in the DNase-treated RNA samples. As the PCRs were not monitored during linear amplification of the product, it is not possible from this data to compare the levels of *RAM2* mRNA transcript between yeast and hyphal samples.

RAM2 is essential

To determine the function of Ram2p, numerous attempts were made to disrupt both copies of the *RAM2* gene. The

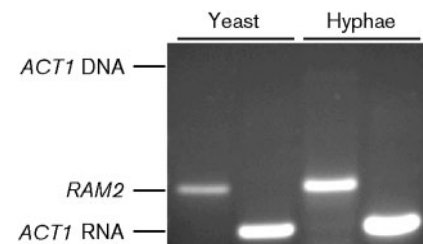


Fig. 1. RT-PCR of *RAM2* from yeast and hyphae. Total RNA was extracted from yeast and hyphae as described in Methods. *RAM2NA-D3* and *RAM2A* were used to amplify *RAM2*, and *ACT INTRN* and *ACTIN R* were used to amplify *ACT1* (Table 1). The *ACT1* PCR products span the intron within the coding region of the actin transcript.

Table 2. *RAM2* nucleotide and protein sequence comparisons

Strain	Nucleotide difference at position*†														
	-847	-811	-603	-539	-386	-38	-34	93	112	121	504	527	723	724	730
SC5314	T	G	T	A	T	C	T	C	A	T	T	A	A	G	A
SS	A	A	C	G	C	T	A	T	T	G	A	G	G	C	C
CA124					T	C	A	T	T	G	T	A	A	G	A
Amino acid difference in the coding region†‡§															
	C 38		NC 41		NC 168		NC 176		C 242		NC 244				
SC5314		I		S		F		D		E				S	
SS		L		A		L		G		Q				R	
CA124		L		A		F		D		E				S	

*Nucleotide position relative to ATG, with A being +1.

†Highlighted areas represent identical nucleotides or amino acids between strains.

‡Amino acid positions.

§C, conservative amino acid change; NC, non-conservative amino acid change.

conventional URA-blaster strategy (Fonzi & Irwin, 1993) and PCR-based disruption cassettes that contain short regions of homology to the gene of interest (Wilson *et al.*, 1999) were used to disrupt *RAM2*. Heterozygote strains (*RAM2/ram2::URA3*) were easily obtained with both gene disruption techniques. However, transformants that had undergone a second round of gene disruption at the *RAM2* locus always retained a wild-type copy. Over 1000 transformants from the two methods were screened using PCR and Southern blot analysis to identify the double deletion of *RAM2* without success (data not shown).

The HT test was developed by Mitchell and co-workers to create gene deletions and test putative essential genes in *C. albicans* (Enloe *et al.*, 2000). For this test, an *UAU1* cassette containing an intact *ARG4* gene flanked by *URA3* deletion derivatives *ura3Δ3'* and *ura3Δ5'* was used. The *URA3* deletion derivatives can recombine to yield an intact *URA3* gene. Hence, *ARG4* and *URA3* were used as selectable markers in this test.

The *UAU1* cassette flanked by DNA sequences homologous to *RAM2* was transformed into BWP17 (*arg4/arg4 ura3/ura3 his1/his1*). PCR primers were designed to screen the correct transformants (Fig. 2a). The insertion of *UAU1* at one allele of the *RAM2* locus (*ram2::UAU1/RAM2*) was confirmed by PCR (Fig. 2b, lane 2) and Southern blot analysis (data not shown). These heterozygotes were able to survive in medium lacking arginine.

On rare occasions, under non-selective conditions, the *UAU1* cassette can become homozygous through gene

conversion or a mitotic recombination event at the gene of interest. Subsequent selection on medium lacking arginine and uridine identifies cells in which one copy of the *UAU1* gene has undergone recombination to yield a functional *URA3* gene and one copy of *UAU1* remains intact. *UAU1* and *URA3* gene insertions in the two alleles of a non-essential gene would allow cells to grow on medium lacking both arginine and uridine. However, if the gene of interest was essential, cells that contain *UAU1* and *URA3* insertions in the two normal alleles will always retain an additional wild-type copy of the gene of interest from trisomy or translocation. Thirty independent heterozygotes of *RAM2* (*ram2::UAU1/RAM2*) were grown in SD-Arg-Ura to select for *URA⁺ ARG⁺* strains. When screened by PCR, all 30 independent *URA⁺ ARG⁺* strains had three alleles of *RAM2*, including *ram2::UAU1*, *ram2::URA3* and *RAM2* (Fig. 2b). Therefore, the HT test and the transformation data strongly suggest that *RAM2* is an essential gene in *C. albicans*.

PTF inhibitors did not affect cell growth

An additional approach used to address the essential role of *RAM2* is to inhibit FTase and GGTase I activities and to examine how this affects the cells. MIC studies were set up with several commercially available GGTase I and FTase inhibitors. Cells were treated with FTase Inhibitor II and two GGTase inhibitors (GGTI-286 and GGTI-2147), ranging from 0.1 nM to 100 μM. There was no change in cell growth in the presence of these inhibitors, even when 100 μM of the drugs were added to the culture (data not shown). Furthermore, when cells were treated with a

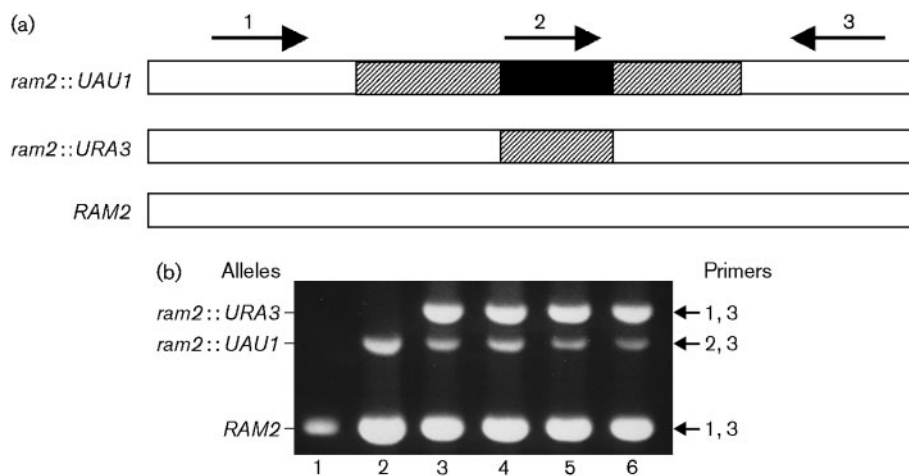


Fig. 2. (a) Oligonucleotide primers for the HT test. PCR primers 1 (*RAM2NA-D3*) and 3 (*HT1133*) were within the *RAM2* gene and outside of the *UAU1* integration site; primer 2 (*ARG 1282*) was within the *ARG4* gene within the *UAU1* construct. Each PCR product size reflects the genotype of a possible transformant. (b) HT test of *RAM2*. Lane 1 contains the parental strain (*RAM2/RAM2*). This allele was detected with primers 1 and 3, generating a 1253 bp PCR fragment. Lane 2 contains the heterozygote strain (*ram2::UAU1/RAM2*). PCR from this strain detected the wild-type *RAM2* allele (1253 bp fragment) and *UAU1* allele (primers 2 and 3 generated a 2023 bp fragment). The PCR product with primers 1 and 3 was too large to be amplified when *UAU1* was present. Lanes 3–6 contain four of the representative 30 independent strains that grew in the SD-Arg-Ura medium. PCR from these strains detected the *ram2::URA3* allele (primers 1 and 3 generated a 2133 bp fragment) as well as *RAM2* and the *ram2::UAU1* allele.

combination of GGTI-2147 and FTase Inhibitor II in a checkerboard study, ranging from 0.1 nM to 100 μ M, cell growth was not affected. Additional inhibitors, including α -hydroxyfarnesylphosphonic acid and FTase Inhibitor III in a similar range of drug concentrations, were also ineffective at inhibiting cell growth (data not shown).

Haploinsufficiency is a condition of diploid organisms in which a single copy of a gene is unable to maintain a wild-type phenotype, while two copies of the gene are necessary and sufficient for the wild-type phenotype (e.g. Giaever *et al.*, 1999). Heterozygote deletion strains of *RAM2* (*RAM2/ram2::URA3*) were tested with the PTF inhibitors. The heterozygote strains, like the wild-type strains, were unaffected by the drugs (data not shown).

The resistance of the fungal cells to PTF inhibitors may be due to efflux of the drug from the cell by efflux pumps such as *CDR1*, *CDR2* and *MDR1*, pumps known to be involved in azole drug resistance in *C. albicans* (White *et al.*, 1998). A strain containing a deletion of all three pumps (*CDR1*, *CDR2* and *MDR1*) was tested with the PTF inhibitors. The strain grew similar to wild-type strains (data not shown).

Enzyme assays indicated that FTase and GGase I were inhibited *in vitro*

The lack of cell-growth inhibition by the PTF drugs may be due to the inability of the PTF drugs to inhibit *Candida* enzymes or to penetrate the cell wall and/or plasma membrane of *C. albicans*. The effect of PTF inhibitors on *C. albicans* FTase and GGase I activities was therefore examined with cell-free enzyme assays. Cell extracts were incubated with [³H]FPP and exogenous substrate Ras-WT to assay for FTase activity or [³H]GGPP and exogenous substrate Ras-CVLL to assay for GGase I activity. Ras-WT and Ras-CVLL are commercially available proteins that contain signal sequences that are specific for FTase and GGase I, respectively. The FTase adds [³H]FPP onto the Ras-WT substrate, and the GGase I adds [³H]GGPP onto the Ras-CVLL substrate.

FTase and GGase I activities were observed in *C. albicans* cell extracts in the absence of drug (Fig. 3, lanes 2 and 5) and in the drug solvent DMSO (Fig. 3, lanes 3 and 6). The prenylated exogenous substrates are indicated by the arrow in Fig. 3; the prenylated endogenous substrates are the upper and lower bands observed in some lanes. FTase Inhibitor II (50 μ M) did not inhibit FTase enzyme activity (Fig. 3, lane 4); however, GGTI-2147 (50 μ M) abolished GGase I enzyme activity (Fig. 3, lane 7). In the presence of both 50 μ M FTase Inhibitor II and 50 μ M GGTI-2147, the FTase and GGase I enzyme activities were abolished (Fig. 3, lane 9).

Regulation of the α - and β -subunits of FTase and GGase I

Since *RAM2* encodes the common α -subunit of both the FTase and GGase I, it was of interest to examine the

regulation of these different subunits. To study how *RAM2* overexpression might affect the regulation of the other two β -subunits at the transcriptional level, an inducible promoter from *PCK1* was ligated upstream of the *RAM2* ATG start codon. The *PCK1* promoter is repressed by glucose (YEPD) and induced by gluconeogenic carbon sources, such as Casamino acids (YEPC) (Leuker *et al.*, 1997).

PCR fragments containing *URA3*, 1435 bp of the *PCK1* promoter region and flanking *RAM2* sequences were transformed into BWP17 to generate strain B30, which contains the inducible *PCK1* promoter ligated upstream of the *RAM2* start codon. Strains BWP17 and B30 were grown in YEPD (repressing medium) and YEPC (inducing medium). Total RNA was prepared at the exponential growth phase. Northern blots were first hybridized with either the *CDC43* probe or the *RAM1* probe, followed by the *RAM2* probe and then the *ACT1* probe. The *RAM2*, *RAM1* and *CDC43* mRNA signals for each Northern blot were normalized to the *ACT1* signal.

Northern blot analyses indicated that *CDC43* and *RAM1* were not upregulated when *RAM2* was overexpressed. In repressing YEPD medium, there were no detectable levels of *RAM2* in BWP17 and B30, but low levels of *CDC43* and *RAM1* were detectable (Fig. 4). When B30 was grown in YEPC, the level of *RAM2* RNA transcripts increased at least 11- to 13-fold above background, whereas both the *CDC43* and *RAM1* RNA transcript levels remained approximately the same. Therefore, an increase in *RAM2* expression does not lead to an upregulation of *RAM1* or *CDC43* at the transcriptional level.

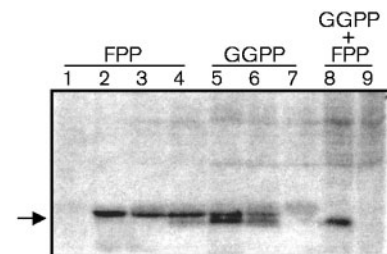


Fig. 3. Cell-free enzyme assay. Lanes 1–4 contained exogenous Ras-WT substrate and [³H]FPP (FPP) to assay for FTase, and lanes 5–7 contained the Ras-CVLL and [³H]GGPP (GGPP) to assay for GGase I. Lanes 8 and 9 contained Ras-WT, Ras-CVLL, [³H]FPP and [³H]GGPP to assay both FTase and GGase I. Lanes: 1, control without cell extract; 2, extract without drug or DMSO (drug solvent); 3, extract with DMSO; 4, extract with FTase Inhibitor II (50 μ M); 5, extract without drug or DMSO; 6, extract with DMSO; 7, extract with GGTI-2147 (50 μ M); 8, extract without drug or DMSO; 9, extract with FTase Inhibitor II (50 μ M) + GGTI-2147 (50 μ M). FTase was not inhibited by FTase Inhibitor II (lane 4), while FTase and GGase I enzyme activities were abolished in the presence of FTase Inhibitor II and GGTI-2147 (lane 9). The arrow indicates the position of prenylated Ras-WT and Ras-CVLL.

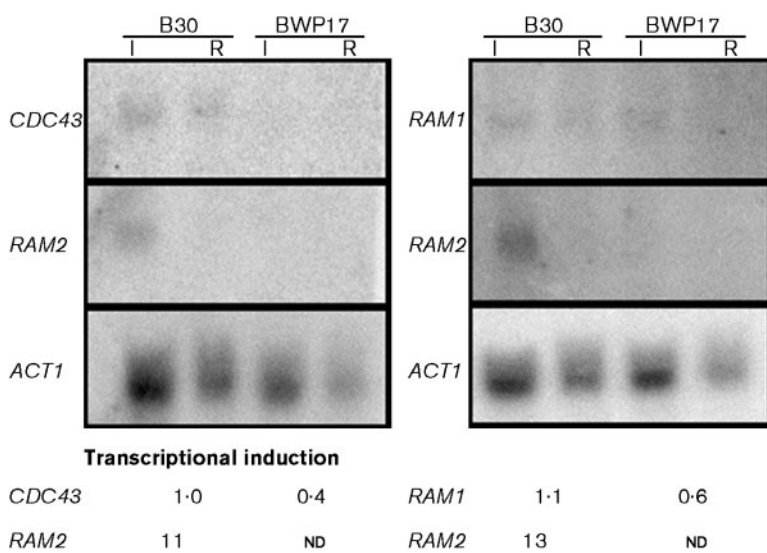


Fig. 4. *CDC43* and *RAM1* are not co-regulated with *RAM2* transcriptionally. Strain BWP17 and the inducible strain B30 were grown in either inducing medium (I) or repressing medium (R) to mid-exponential phase at 30 °C and 180 r.p.m. as described in Methods. Two sets of Northern blots were prepared. The left panel was hybridized with *CDC43*-50, followed by *pRAM1.65* and *ACT*-50. The right panel was hybridized with *RAM1*-50, followed by *pRAM1.65* and *ACT*-50. Each mRNA signal was quantified using the Storm Phosphoimager and IMAGEQUANT software. The *CDC43*, *RAM1* and *RAM2* mRNA signals were normalized to *ACT1* mRNA signals. The normalized mRNA levels in the inducing medium were divided by the normalized mRNA levels in the repressing medium, to compare the level of transcriptional induction. ND, Not detectable.

DISCUSSION

In this study, the *RAM2* gene, which encodes the common α -subunit of FTase and GGTase I, was examined in *C. albicans*. The results of the HT test strongly suggest that *RAM2* is essential for the viability of *C. albicans*, similar to its orthologue in *S. cerevisiae* (He *et al.*, 1991). Low levels of *RAM2* RNA transcripts were detected by RT-PCR in the yeast and hyphal growth states. The MIC studies of the PTF drugs against FTase and GGTase I showed that the drugs did not inhibit cell growth. However, cell-free enzyme assays indicated that at least one PTF drug inactivated FTase and GGTase I enzyme activities in *C. albicans*, suggesting that this drug may not be imported into the cells.

Previous sequence analysis of *C. albicans CDC43* identified allelic and strain variations, including differences in the repetitive regions of the protein, deletions and amino acid changes among the three strains examined (Mazur *et al.*, 1999). Also, *C. albicans Ram2p* contained a D155G change in strains MY1055 and CA124 (Mazur *et al.*, 1999). Our sequence analysis of the *Ram2p* in strains SC5314, SS and CA124 indicated that there are more strain differences than previously described, suggesting that the coding sequence of *RAM2* can also vary considerably (Table 2). This may have important implications for the development of drugs against *Candida* PTFs, since ideally the drugs would target the conserved regions of the PTFs.

In this study, it was hypothesized that a disruption of FTase and GGTase I activities would be detrimental to the *Candida* cells. Consistent with this hypothesis, deletion of both copies of *RAM2* using the standard URA-blaster technique and the PCR-based short-homology technique were unsuccessful. Consistent with these observations, the results from the HT test showed that all 30 independent

URA⁺ ARG⁺ mutants retained a wild-type copy of *RAM2*, indicating that *RAM2* is most likely essential for the viability of *C. albicans* (Fig. 2b). In Results, we also described the use of the inducible promoter *PCK1*. Inducible promoters can be used to study essential genes by turning expression of the gene off under repressing conditions. However, the *PCK1* promoter does express under repressing conditions at levels that would be sufficient to complement a low-level expressed gene such as *RAM2* (Ernst & Bockmuhl, 2002).

It is a formal possibility that deletion of the *RAM2* gene with the *UAU1* cassette might disrupt the essential function of adjacent genes on the chromosome and that *RAM2* might not be essential to *C. albicans*. While this possibility can not be eliminated by these results, several points can be made. First, the *UAU1* cassette only replaced the coding region of *RAM2*, making it unlikely that it altered expression of adjacent genes. Second, the upstream adjacent gene *IPF19970* has an unknown function but has some homology to a non-essential gene in *S. cerevisiae*. The downstream adjacent gene *IPF14634* is an APP-1 binding protein (by homology), again a non-essential gene in *S. cerevisiae*. These two genes adjacent to *RAM2* are encoded on the opposite strand, so the downstream gene is unlikely to be regulated by a change in *RAM2*. The gene upstream of *IPF19970* is *YTM1*, which is an essential gene in *S. cerevisiae*. The gene downstream of *IPF14634* is *EFT3*, which is a non-essential gene in *S. cerevisiae*. Therefore, adjacent genes are unlikely to explain the *UAU1* result unless the construct is affecting the *YTM1* gene function.

As *RAM2* appears to be an essential gene, it is not surprising that *RAM2* RNA transcripts were detected in the yeast and hyphal forms of *C. albicans* by RT-PCR (Fig. 1). Since the

RT-PCR performed was not quantitative and continued for 35 cycles, the relative levels of *RAM2* transcripts in yeast and hyphae growth states can not be compared in Fig. 1.

The role of *RAM2* is further examined by inhibiting both the FTase and GGTase I activities using pharmacological agents. Inhibition of both FTase and GGTase I activities would be expected to severely impair important cellular functions. PTF inhibitors of FTase and GGTase I had no effect on cell growth. Several possibilities exist: (i) the drugs may not penetrate the *Candida* cell membrane and/or cell wall; (ii) the drugs may be imported into the cells but do not inhibit *Candida* FTase and GGTase I; (iii) the drugs may be imported into the cells and may inhibit *Candida* FTase and GGTase I, but do not inhibit cell growth, as a result of drug modification, degradation, sequestration and/or efflux; (iv) the combined enzyme activities of FTase and GGTase I may not be essential to *Candida*. A heterozygote deletion was used to demonstrate that strains containing a single copy of *RAM2* were similarly unaffected by the drugs.

In vitro enzyme assays were conducted with *Candida* cell extracts to determine if the drugs did inhibit PTF activity. The results demonstrated that FTase enzyme activity was not inhibited by 50 μ M of FTase Inhibitor II, but GGTase I enzyme activity was inhibited by 50 μ M of GGTI-2147. FTase and GGTase I enzyme activities were assayed together in the presence of both drugs at 50 μ M. The two drugs together were able to inhibit FTase and GGTase I activities at the concentrations tested (Fig. 3, lane 9). The most likely explanation is that 50 μ M of GGTI-2147 is active against both enzymes, since FTase Inhibitor II is ineffective against *Candida* FTase at 50 μ M. However, GGTI-2147 did not affect cell growth at 100 μ M, in the presence or absence of FTase Inhibitor II. The IC_{50} of GGTI-2147 for mammalian enzymes is 0.5 μ M for GGTase I and >30 μ M for FTase (Vasudevan *et al.*, 1999). Therefore, at 50 μ M, GGTI-2147 may inhibit both *Candida* FTase and GGTase I *in vitro*, but does not affect cell growth. A subsequent experiment indicated that *C. albicans* FTase activity was significantly reduced in the presence of 100 μ M of the FTase Inhibitor II (data not shown), indicating that a higher concentration of this drug alone is effective against *C. albicans* FTases. These results, together with the results from the HT test, indicated that the lack of inhibition on cell growth observed in the MIC studies might be due to the inability of GGTI-2147 to penetrate *C. albicans*. This suggested that the development of PTF inhibitors against *Candida* needs to focus on drug import. However, the possibility exists that GGTI-2147 might have been imported into the cell and is active against the enzyme, but is further modified, degraded, sequestered or effluxed from the cell. If efflux is involved, it is unlikely to be associated with the three efflux pumps associated with azole drug resistance (*CDR1*, *CDR2* and *MDR1*), as a strain deleted of these three pumps was not altered in growth in the presence of these drugs. A more direct assay to measure prenylation activities in the living cells is needed to determine if the GGTI-2147 was imported

into the cells and to determine its effect on PTF activities *in vivo*.

To gain a better understanding of the regulation of the α - and β -subunits of FTase and GGTase I, the transcriptional regulation of the β -subunits was analysed in strain B30 containing the *RAM2* gene, which encodes the α -subunit, fused to the inducible promoter. Surprisingly, little research has been done on the regulation of PTF subunits. Previous studies have demonstrated that overexpression of the Rab Escort Protein (REP), depletion of the GGPP pool and binding of the α -subunit to a protein tyrosine phosphatase may play a role in regulating GGTase II activity in *S. cerevisiae* (Miaczynska *et al.*, 2001; Si *et al.*, 2001). The regulation of farnesylation has mostly been studied in mammalian cell systems, because of the intense interest in FTase inhibitors as chemotherapeutic agents. Insulin has been found to promote the activation of FTase and GGTase I, and the phosphorylation of the common α -subunit of these two enzymes via the Shc branch of intracellular signalling in CHO (Chinese hamster ovary) cells (Goalstone *et al.*, 2001).

The results from the Northern blot analysis indicated that *RAM2* is undetectable and that *CDC43* and *RAM1* are detectable, although not in abundant levels in a standard strain (Fig. 4). Additionally, when *RAM2* was overexpressed at least 11- to 13-fold in strain B30, which has one copy of *RAM2* under the control of a regulable promoter, the *RAM1* and *CDC43* RNA levels were not upregulated, suggesting that these genes may be regulated independently of each other. Overexpression of *RAM2* in strain B30 did not produce a difference in cellular morphology, growth rate or yeast-to-hyphal transition in repressing or inducing medium (data not shown).

This research has demonstrated that *RAM2* is most likely an essential gene in *C. albicans* and has contributed to the fundamental understanding of protein prenylation, which is a crucial cellular process that may be explored as a new antifungal drug target in *C. albicans*.

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