Purification of geranylgeranyltransferase I from *Candida albicans* and cloning of the *CaRAM2* and *CaCDC43* genes encoding its subunits

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All previously characterized protein geranylgeranyltransferases I (GGTase I) are heterodimeric zinc metalloenzymes which catalyse geranylgeranylation of a cysteine residue in proteins containing a C-terminal CaaL motif (C, Cys; a, aliphatic amino acid; L, Leu). The α and β subunits of GGTase I of *Saccharomyces cerevisiae* are encoded by *RAM2* and *CDC43*, respectively, and are essential for yeast viability. The authors are therefore investigating the role of geranylgeranylation in the related pathogenic yeast, *Candida albicans*, which is the most prevalent human fungal pathogen. GGTase I was purified to near homogeneity and also found to be a heterodimeric magnesium-dependent, zinc metalloenzyme displaying selectivity for CaaL-containing protein substrates. GGTase I peptide sequences were obtained from the purified protein and used to clone the genes encoding both subunits. *CaRAM2* and *CaCDC43* encode proteins that are 42 and 34% identical to their corresponding *S. cerevisiae* homologues, respectively, and 30% identical to their human homologues. Despite the limited overall homology, key zinc- and substrate-binding residues of the β subunit (Cdc43p) are conserved. A unique feature of CaCdc43p is a tract of polyasparagine whose length varies from 6 to 17 residues among *C. albicans* strains and between alleles. Coexpression of both *CaCDC43* and *CaRAM2* under their native promoters complemented the *ts* defect of a *S. cerevisiae cdc43* mutant but expression of the β -subunit alone did not correct the growth defect, suggesting that hybrid GGTase I heterodimers are nonfunctional.

Keywords: *Candida albicans*, protein prenylation, protein geranylgeranyltransferase I, heterologous expression

INTRODUCTION

Prenylation is a post-translational modification that facilitates membrane localization of a protein by increasing its hydrophobicity, and is often essential for

function (Schafer & Rine, 1992; Omer & Gibbs, 1994). One class of protein prenyltransferases consists of heterodimeric zinc-dependent enzymes catalysing prenyl group transfer from either farnesyl diphosphate (farnesyltransferase; FTase) or geranylgeranyl diphosphate (geranylgeranyltransferase I; GGTase I). The prenyl group is linked to a cysteine residue in the C-terminal amino acid sequence referred to as a CaaX motif [C, cysteine; a (usually), aliphatic amino acid; X, any amino acid]. The specificity of prenyl group addition is largely determined by the X-residue of the CaaX motif (Zhang & Casey, 1996a). Following prenylation, the

Abbreviations: FPP, farnesyl diphosphate; FTase, farnesyltransferase; 5-FOA, 5-fluoroorotic acid; GGPP, geranylgeranyl diphosphate; GGTase, geranylgeranyltransferase.

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three C-terminal amino acids are removed by proteolysis and the free carboxyl group of the prenyl-cysteine is carboxy-methylated (Clarke, 1992). A second and distinct class of protein prenyltransferases found in yeast and mammalian cells is GGTase II, which specifically catalyses geranylgeranylation at both cysteines of C-terminal -CC or -CXC sequences found in yeast Rab proteins (Farnsworth *et al.*, 1994; Zhang & Casey, 1996a).

GGTase I has a preference for substrates in which X of the CaaX motif is leucine, whereas FTase farnesylates proteins that terminate in alanine, cysteine, glutamine, methionine or serine (Moores *et al.*, 1991; Caplin *et al.*, 1994). The α and β subunits of GGTase I from *Saccharomyces cerevisiae* are encoded by *RAM2* and *CDC43*, respectively, and each is essential for viability (Adams *et al.*, 1990; Finegold *et al.*, 1991; He *et al.*, 1991; Ohya *et al.*, 1991; Mayer *et al.*, 1992). FTase shares a common α subunit with GGTase I in both yeast and mammals. *RAM1* encodes the β subunit of *S. cerevisiae* FTase, and null mutants of this gene exhibit a temperature-sensitive lethality (He *et al.*, 1991; Kohl *et al.*, 1991).

Geranylgeranylated proteins are typically small GTP-binding proteins involved in cytokinesis, cell polarity and morphogenesis (Schafer & Rine, 1992). The two most critical substrates of *S. cerevisiae* GGTase I appear to be the essential proteins Cdc42p and Rho1p, since the simultaneous overexpression of both suppresses a GGTase I defect (Ohya *et al.*, 1993; Trueblood *et al.*, 1993). Compensation presumably occurs by FTase-mediated prenylation of these substrates, since FTase, not normally essential, becomes required for cell viability in this situation. Rho1p has at least three important roles in morphogenesis in *S. cerevisiae*: (1) it is required as an activator of protein kinase C, which plays a fundamental and essential role in maintaining cell wall integrity (Nonaka *et al.*, 1995; Kamada *et al.*, 1996); (2) it has been shown to activate Bin1p, a protein involved in reorganization of the actin cytoskeleton during polarized morphogenesis (Kohno *et al.*, 1996; Imamura *et al.*, 1997); and (3) it has been identified as the GTP-binding regulatory subunit of 1,3- β -D-glucan synthase, an essential enzyme involved in fungal cell wall biosynthesis (Drgonová *et al.*, 1996; Mazur & Baginsky, 1996; Qadota *et al.*, 1996). In this last role, Rho1p is found associated with Fks1p, an essential subunit of *S. cerevisiae* 1,3- β -D-glucan synthase (Douglas *et al.*, 1994).

Candida albicans, a dimorphic yeast related to *S. cerevisiae*, is a major opportunistic human fungal pathogen, causing life-threatening infections in many immunocompromised patients (Cox & Perfect, 1993). We are interested in exploiting cell wall synthesis and related processes, such as protein prenylation, for antifungal targets. The recent confirmation of CaRho1p as the regulatory subunit of glucan synthase in *C. albicans* (Kondoh *et al.*, 1997) suggests that the essential regulatory role of the RHO-family GTPases is conserved

in *Candida* species. Rho1p (Kondoh *et al.*, 1997) and Cdc42p (Mirbod *et al.*, 1997) of *C. albicans* contain CaaL motifs and are probably GGTase I substrates. Hence GGTase I may play an important role in morphogenesis and cell wall biosynthesis in *C. albicans*. Here we report the purification of GGTase I from *C. albicans* and the subsequent cloning and sequence analysis of its subunits. We show that despite the limited overall homology of the subunits to those of other organisms, key structural and substrate-recognition elements of the β subunit (Cdc43p) are conserved. Function of the cloned *C. albicans* genes was confirmed by complementation of a *S. cerevisiae* conditional GGTase I mutant.

METHODS

General. Chromatography columns and media were from Pharmacia. Protein concentrations were determined with the BCA protein assay reagent (Pierce) or Protein-Gold reagent (Integrated Separation Systems) with BSA as the standard. SDS-PAGE gels were from Novex.

Yeast strains, growth media and microbiological methods. CJ198-2B (*MAT α cdc43-2 ura3 trp1 his4 gal2*) is a *ts cdc43 S. cerevisiae* mutant kindly provided by D. Johnson (Adams *et al.*, 1990). RS51-3A (*MAT α ram2-1 ura3 his3 ade8 trp1 can1 RAS2^{val19}*) is a *ts ram2* mutant obtained from S. Powers (Goodman *et al.*, 1990). *S. cerevisiae* diploid W303 (*MAT α /MAT α ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1*) was constructed by mating haploid W303-1A and W303-1B (Thomas & Rothstein, 1989). *S. cerevisiae* S288C (*MAT α*) was obtained from the Yeast Genetics Stock Center. *C. albicans* CAI4 (*Δ ura3::limm434/ Δ ura3::limm434*) was a gift of W. Fonzi (Fonzi & Irwin, 1993) and MY1055 is a clinical isolate (Abruzzo *et al.*, 1995). Mating and sporulation of yeast strains, and tetrad dissections, were performed as described and all media were standard for yeast genetics (Sherman *et al.*, 1986).

Protein prenyltransferase assays. Protein prenyltransferase activities were routinely determined by an acid quench-filtration assay with prenyl acceptor substrates based on *S. cerevisiae* Ras1p (Moores *et al.*, 1991). The protein substrates, referred to as Ras-CaaX, were expressed in *Escherichia coli* from the previously described plasmids [⁶⁸Leu]RAS1(term.)-SLK CaaX (CaaX = CAIL, CVLS, CVLM) obtained from C. Omer (Moores *et al.*, 1991). Additional Ras-CaaX plasmids (CaaX = CVIL, CTIL, CVVL, CVIA) were constructed by the strategy previously described (Moores *et al.*, 1991). The integrity of Ras-CaaX protein samples was verified by mass-spectroscopic analysis. Typical GGTase I reactions were performed under either of two sets of conditions: 50 mM Bistris propane pH 9.0, 10 mM MgCl₂, 0.1 mM ZnCl₂, 0.05% (w/v) dodecyl maltoside, 2–4 μ M [³H]geranylgeranyl diphosphate (³H-GGPP), 20 μ M Ras-CAIL or Ras-CVIL; or 50 mM HEPES pH 7.5, 1–5 mM DTT, 10 mM MgCl₂, 0.05 mM ZnCl₂, 0.05% dodecyl maltoside, 2–4 μ M ³H-GGPP, 20 μ M Ras-CAIL or Ras-CVIL. Detergent was required to maintain GGPP solubility and in some cases 0.02% (w/v) Triton X-100 was substituted for the 0.05% dodecyl maltoside with little change in enzyme activity. Typical conditions for measurement of FTase activity were 50 mM HEPES pH 7.5, 1 mM MgCl₂, 0.1 mM ZnCl₂, 0.05% dodecyl maltoside, 2–4 μ M [³H]farnesyl diphosphate (³H-FPP), 20 μ M Ras-CVLS or Ras-

CVLM. All reactions were performed in duplicate and were initiated by addition of prenyltransferase activity to a final volume of 25 or 50 μ l in polypropylene 96-well plates or microcentrifuge tubes, respectively, and incubated at 30 °C for 30–90 min. Reaction samples (25 μ l) were quenched by addition of 200 μ l 10% (v/v) concentrated HCl/ethanol, incubated at 37 °C for 30 min, then vacuum filtered through Whatman GF/B filters and rinsed with ethanol. Dried filters were subjected to liquid scintillation counting (Beckman Ready Safe). ^3H -GGPP [12–20 Ci ($4.4\text{--}7.4 \times 10^{11}$ Bq) mmol^{-1}] and ^3H -FPP [15–20 Ci ($5.6\text{--}7.4 \times 10^{11}$ Bq) mmol^{-1}] were purchased from Amersham and typically used in assays at 1000–2000 d.p.m. pmol^{-1} ; non-radioactive GGPP and FPP were purchased from American Radiolabelled Chemicals. For the determination of Michaelis constants for Ras-CVIL and GGPP, purified GGTase I was assayed in 50 mM HEPES pH 7.5, 10 mM MgCl_2 , 0.1 mM ZnCl_2 and 0.02% Triton X-100 containing the varied substrate and 2 μM ^3H -GGPP or 50 μM Ras-CVIL, respectively. K_m values were determined by fitting saturation curves to $V = V_{\text{max}} \cdot c / (K_m + c)$.

Purification of *C. albicans* GGTase I. *C. albicans* MY1055 cultures were grown overnight in YPD at 30 °C, harvested by centrifugation at an OD_{600} of 10–15 (approx. 20 g cell paste l^{-1}), and stored at -80 °C. All purification procedures were conducted at 4 °C. The cell paste (36 g) was thawed in an equal volume of lysis buffer containing 50 mM Bistris propane/HCl, pH 7, 1 mM β -mercaptoethanol, 1 $\times$ Complete protease inhibitor cocktail (Boehringer Mannheim) and 1 mM Pefabloc protease inhibitor (Boehringer Mannheim). The cell slurry was homogenized with an equal volume of 0.5 mm glass beads for 30 s and cooled for 2.5 min in an ice-cooled Bead-Beater (Biospec); this cycle was repeated 8–10 times. The crude homogenate was centrifuged at 31000 g for 15 min at 4 °C and the supernatant (89 ml) subjected to buffer exchange by gel filtration on Sephadex G-25 (2.6×70 cm) equilibrated in buffer A (20 mM Bistris propane/HCl, pH 7, 0.02 mM ZnCl_2 , 1 mM MgCl_2). The eluate containing the protein peak was clarified by ultracentrifugation at 100000 g for 1 h at 4 °C. After centrifugation, the supernatant was retained and loaded onto a Q-Sepharose HP column (2.6×24 cm) equilibrated in buffer A. The column was washed with buffer A and eluted with a 10-column volume linear gradient of NaCl (0–0.5 M) in buffer A at a flow rate of 3 ml min^{-1} and fractions (15 ml) were collected. This step completely resolved GGTase I activity (eluted at 0.3 M NaCl) from FTase activity (eluted at 0.35 M NaCl). Fractions containing the GGTase I activity were pooled (75 ml), adjusted to 0.8 M ammonium sulfate by the addition of solid ammonium sulfate (8.1 g), and the sample applied to a Phenyl Sepharose HP column (1.6×5 cm) equilibrated in buffer A plus 0.8 M ammonium sulfate. The column was washed in buffer A plus 0.8 M ammonium sulfate and eluted with a 10-column volume descending linear gradient of ammonium sulfate (0.8–0 M) in buffer A at a flow rate of 2.5 ml min^{-1} . Fractions (2.5 ml) were collected and those containing GGTase I activity were pooled (eluted at 0.4–0.2 M ammonium sulfate; 27.5 ml), dialysed into buffer A, concentrated (0.28 ml) with concentrating solution (Pierce) and/or by ultrafiltration (30 kDa cutoff; Amicon), and subjected to gel filtration on a Superdex-200 HR 10/30 FPLC column equilibrated in buffer A. The column was eluted at a flow rate of 0.4 ml min^{-1} and fractions (0.2 ml) were collected. The GGTase I activity was pooled and applied to a Mono-Q HR 5/5 FPLC column equilibrated in buffer A and eluted with a two-step NaCl gradient (0–0.35 M in 5 ml; 0.35–0.7 M in 20 ml) at a flow rate of 0.5 ml min^{-1} . Fractions (0.25 ml) were collected. The GGTase I activity eluted as an apparent single

peak at approximately 0.38 M NaCl. The pooled activity was dialysed into buffer A and stored at -80 °C. The FTase activity could be further purified by Phenyl-Sepharose chromatography under the same conditions as described above for GGTase I. The FTase activity eluted at 0.2–0 M ammonium sulfate and was pooled, dialysed into buffer A, and stored at -20 °C (significant loss of activity occurred when the preparation was stored at -80 °C).

Protein chemistry. Approximately 5 μg (~ 60 pmol) purified GGTase I was subjected to electrophoresis on a 10% Tris/glycine SDS-PAGE gel and blotted to PVDF (Bio-Rad). The blot was stained with Ponceau S (Sigma) and the two main bands detected (approx. 41 and 45 kDa) were excised. Trypsin digest/HPLC and peptide sequencing/mass spectroscopic analyses were performed by the Harvard University Microchemistry facility. Two sequences were obtained from the 41 kDa subunit [41-1, NF(W or T)DYQ; and 41-2, LHITELGINELASHYT] and five sequences from the 45 kDa subunit [45-1, DIGGFNGR; 45-2, DYPENLYPK; 45-3, TQNQLFGGFR; 45-4, FALQEINPILTITK; and 45-5, GGFVPTLYYNEENx₂DY].

Nucleic acid isolation, hybridization, and sequence analysis. Plasmid DNA was isolated using the QIAprep spin miniprep or QIAGEN-tip 500 or 100 kits (QIAGEN). Genomic DNA of *C. albicans* was isolated by the glass-bead lysis method of Hoffman & Winston (1987). All DNA blots were performed with Zeta-ProbeGT derivatized nylon membranes (Bio-Rad) and were hybridized under stringent conditions recommended by the manufacturer. The probes were radiolabelled with [α - ^{32}P]dCTP using a random-primed DNA labelling kit (Stratagene). The DNA sequence of both strands of designated clones was determined on a model 377A automated DNA sequencer with a Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Sequences were assembled with Gene Codes Corporation Sequencher 3.0 and analysed primarily with the Genetics Computer Group software package. Percentage identity between protein sequences was determined with the GAP program. Multiple sequence alignments were performed by the CLUSTAL program (Thompson *et al.*, 1994).

Cloning of the GGTase I subunit genes. Fragments of *CaCDC43* and *CaRAM2* were amplified from genomic *C. albicans* DNA by PCR with degenerate oligonucleotide primers encoding the least degenerate regions of the determined GGTase I peptide sequences (Compton, 1990). In unavoidable cases where sequencing indicated two possible amino acids or a primer encoded an amino acid potentially encoded by five or more codons (L, S, R), primer degeneracy was minimized by preparing two separate primer pools. PCR products were purified and directly subcloned into pCR2.1 (Invitrogen) for DNA sequencing. PCRs with the primers (*Eco*RI linkers are underlined) 5' GCG CGC GAA TTC (T/C)TG (G/A)TA (G/A)TC CCA (G/A)AA (G/A)TT 3' (antisense) and 5' GCG CGC GAA TTC GA(G/A) CT(A/C/T) GG(A/C/T/G) AT(A/C/T) AA(C/T) GA(G/A) 3' (sense), encoding regions of peptides 41-1 (NFWDYQ) and 41-2 (ELGINE), respectively, generated a predominant 729 bp PCR product (pCaRAM2-9) from *C. albicans* CAI4 DNA. The PCR reactions (50 μ l volume) contained 100 ng genomic DNA, 1.0 μM each primer, 3 mM Mg^{2+} , 200 μM each dNTP and 1.25 units *Taq* polymerase (Fisher Biotech). The thermal-cycler profile (40 cycles) was 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, followed by a final incubation at 72 °C for 10 min. The DNA sequence of this product suggested that it encoded a peptide of *C. albicans* Ram2p by homology to *S. cerevisiae* Ram2p. Likewise, sense and antisense degenerate

oligonucleotides 5' GCG CGC GAA TTC TA(C/T) TA(C/T) AA(C/T) GA(A/G) AA(A/G) AA(C/T) 3' and 5' GCG CGC GAA TTC (A/C/G/T)CC (G/A)AA (T/C)AA (T/C)TG (G/A)TT (T/C)TG 3' partially encoding peptide 45-5 (YYNEEN) and encoding 45-3 (QNQLFG), respectively, were employed in PCRs with *C. albicans* MY1055 genomic DNA as template. The PCR reactions were performed as described above with a thermocycler profile of (35 cycles) 94 °C for 1 min, 40 °C for 1 min, 72 °C for 1 min, followed by a final incubation at 72 °C for 10 min. A 573 bp PCR product (pCR2.1-600) was obtained whose DNA sequence revealed an open reading frame which encoded peptides 45-1 and 45-2 in addition to exhibiting homology to *S. cerevisiae* Cdc43p.

To isolate genomic clones of each subunit, probes derived from the PCR clones of each gene were used to screen a *C. albicans* genomic library, CLS2, that had been constructed from strain CA124 in the *S. cerevisiae* vector YEp24 and was obtained from J. Rosamond (Sherlock *et al.*, 1994). The *CaRAM2* and *CaCDC43* probes were a radiolabelled 0.6 kb *NheI*-*XbaI* fragment from pCaRAM2-9 and a 0.6 kb *EcoRI* fragment from pCR2.1-600, respectively. Four independent *CaRAM2* and three *CaCDC43* clones that hybridized to their respective probes were isolated. The clones for each subunit had insert sizes ranging from approximately 5 to 7 kb and contained common overlapping restriction fragments. Restriction and partial sequence analysis indicated that the clones contained full-length genes based on comparison of predicted peptide sequences to their *S. cerevisiae* counterparts. One *CaRAM2* clone, pCaRAM16, and one *CaCDC43* clone, pCaCDC7, contained approximately 3.0 kb *HindIII* fragments that were collinear with genomic DNA and were chosen for completion of sequence determination and further analysis.

Primers based on sequence of the genomic clones were used to amplify the genes from genomic DNA of *C. albicans* strains CA14 and MY1055 by PCR. A *CaRAM2* PCR product, encompassing the entire coding region as well as 1279 bp upstream and 404 bp downstream from the ORF, was amplified from CA14 with the primers 5' GCGCGCTGCAG-CATAAGGGATCAACGCCG 3' and 5' CCGGCGGTACC-AGGAGAAAATTGATTCCCCG 3' (underlined sequences are *PstI* and *KpnI* restriction sites, respectively). A *CaCDC43* PCR product, containing the entire coding region as well as 647 bp 5' and 733 bp 3' of the ORF, was amplified from CA14 with primers 5' GCGCTCTAGACGTATAAACCCCTTGTC-CCG 3' and 5' CCGCGGTACCTTGAAGGTAATGTAC-CACGCC 3' (underlined sequences are *XbaI* and *KpnI* sites, respectively). The PCR reactions (50 µl volume) contained 100 ng genomic DNA, 0.2 µM each primer, 2 mM Mg²⁺, 200 µM each dNTP, 1 unit Elongase and 1× buffers A and B (GibcoBRL). Cycle profile (30 cycles) was 94 °C for 1 min, 55 °C for 1 min, 72 °C for 6 min, followed by a final incubation at 72 °C for 20 min. The *CaRAM2* and *CaCDC43* PCR products were cloned into YEplac181 (Leu⁺; Gietz & Sugino, 1988) and pUC19 (Yanisch-Perron *et al.*, 1985), respectively. Five clones of each gene were sequenced and the data indicated that four clones of one allele and one clone of the other allele were isolated. Plasmids pCaRAM2-22 and pCaCDC43-43 contain the most frequently isolated allele of each gene while pCaRAM2-25 and pCaCDC43-40 harbour the other allele. Similarly, a *CaRAM2* PCR product encompassing the complete ORF and 131 bp of 5' and 155 bp of 3' non-coding sequence was amplified from MY1055 DNA with the primers 5' GCGGGATCCTCATGAACTTTTCAAACAAACCA 3' and 5' GCGGGATCCCTTAATTGATTGCAATCATT-CCT 3' (underlined sequences are *BamHI* sites). A *CaCDC43* PCR product containing the ORF and 159 bp of 5' and 173 bp

of 3' non-coding sequence was amplified from MY1055 DNA with the primers 5' GCGGGATCCTCACCGCACACCAA-AAAGTA 3' and 5' GCGGGATCCTGTGGGAGAACAC-TCCGATT 3'. The PCR reactions (50 µl) contained 1× reaction buffer, 100 ng genomic DNA, 0.5 µM primers, 200 µM each dNTP and 2 units *Pfu* (Stratagene) or *Vent* (NEB) polymerase. The cycle profile (30 cycles) was 94 °C for 1 min, 50 °C for 1 min, 72 °C for 3–4 min. T-overhangs were added with *Taq* polymerase and the PCR products cloned into pCR2.1 (Invitrogen). Six clones were sequenced for each product. Three clones were obtained from each *CaRAM2* allele as exemplified by pCR2.1-RAM2-21 and pCR2.1-RAM2-27; four and two clones were obtained for the two *CaCDC43* alleles and are represented by pCR2.1-CDC43-24 and pCR2.1-CDC43-14, respectively.

Construction of *CaCDC43* and *CaRAM2* subclones in *S. cerevisiae* vectors. The 3.0 kb *CaRAM2* *HindIII* fragment from genomic clone pCaRAM16 was subcloned into YEplac-112 (Trp⁺; Gietz & Sugino, 1988) to construct pCaRAM2-1. Plasmid pCaRAM2T was constructed by ligating the *CaRAM2* insert from PCR clone pCaRAM2-22 into *KpnI*/*PstI*-digested YEplac112. The *CaCDC43* insert of PCR clone pCaCDC43-40 was subcloned into *EcoRI*/*Sall*-digested pRS426 (Ura⁺; Christianson *et al.*, 1992) to construct p40CDCU and the same fragment from both pCaCDC43-43 and pCaCDC43-40 was subcloned into pRS423 (His⁺; Christianson *et al.*, 1992) to construct p43CDC and p40CDC, respectively.

Complementation of *S. cerevisiae* GGTase I mutants with *C. albicans* subunits. The *S. cerevisiae* *ts* mutants RS51-3A (*ram2*) and CJ198-2B (*cdc43*) were first transformed to Trp⁺ with either *CaRAM2* plasmids pCaRAM2-1 and pCaRAM2T or empty vector YEplac112. Transformation was as described by Elble (1992). The transformants were subsequently transformed to Ura⁺ with either *CaCDC43* plasmids pCaCDC7 and p40CDCU or their respective vector controls YEplac112 and pRS426. Transformants selected at 30 °C were tested for complementation at 37 °C.

A *S. cerevisiae* *ram2* disruptant was also tested for complementation and was constructed as follows. First, the *S. cerevisiae* *RAM2* gene was amplified by PCR from genomic DNA of strain S288C with primers 5' GCGCCGCTCGAG-TTGTGATTAGTCGGTTAGAGG 3' and 5' CGGTGG-CTCGAGGGGTTTGGACGCATCAATGAT 3' (underlined sequences are *XhoI* sites). The PCR reaction (50 µl volume) contained 100 ng genomic DNA, 1.0 µM each primer, 200 µM each dNTP, 1 unit Elongase and 1× buffers A and B (GibcoBRL). The cycle profile (35 cycles) was 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1.5 min, followed by one cycle at 72 °C for 10 min. The 1566 bp PCR product was cloned into the *XhoI* site of pRS423 (His⁺) to construct pRAM2-31. The *RAM2* *XhoI* fragment from pRAM2-31 was subsequently cloned into pGEM5Zf(+) to construct pFTA1 and into YEplac195 (Ura⁺; Gietz & Sugino, 1988) to construct pFTA4. An insertion-deletion of *RAM2* was made by replacing the 0.5 kb *BglII*-*EcoRI* fragment of pFTA1 with a 0.8 kb *BglII*-*EcoRI* *TRP1* fragment from pJJ246 (Jones & Prakash, 1990) to construct pFTA1d. Trp⁺ transformants of diploid W303 were obtained with a *HincII*-*SphI* fragment of pFTA1d containing the *RAM2* disruption and Southern blot analysis confirmed that a gene replacement had taken place on one allele in several transformants. Since we expected a haploid *ram2* disruptant to be lethal as reported in the literature (He *et al.*, 1991), one diploid heterozygous for the disruption was transformed with either the *URA3* *RAM2* plasmid pFTA4 or

control vector YEplac195. Subsequently, the transformants were sporulated and tetrads dissected. Three tetrads in which all four spores were viable were obtained from the dissection of a Ura⁺ RAM2 transformant and a transformant containing vector YEplac195 yielded tetrads which contained only two viable spores, consistent with the disruption of an essential gene. Three four-spore tetrads from a Ura⁺ RAM2 transformant were propagated on 5-fluoroorotic acid (5-FOA)-containing medium (Boeke *et al.*, 1984) to select against the RAM2 Ura⁺ plasmid, and on SD medium supplemented with uracil. All four segregants from the three tetrads grew on the control medium while only two of the four spores grew on the 5-FOA-containing medium; the spores which did not grow on 5-FOA medium were nonviable upon loss of the RAM2 plasmid and therefore contained the *ram2* disruption. The Ura⁺ RAM2 transformant of one of the latter spores, designated W303dR2, was chosen for complementation studies.

Leu⁺ His⁺ transformants of *ram2* disruptant W303dR2 were obtained with pCaRAM2-22 and either p43CDC or p40CDC and appropriate vector controls. The transformants were streaked onto minimal medium at 30 °C and subsequently grown overnight in YPAD broth. The strains were plated on 5-FOA-containing medium and incubated at 30 °C.

RESULTS

Purification of *C. albicans* GGTase I

GGTase I and FTase activities were detected in cell-free extracts of *C. albicans* in protein prenyltransferase assays containing ³H-GGPP and a typical GGTase I prenyl acceptor, Ras-CAIL, or ³H-FPP and a typical FTase prenyl acceptor, Ras-CVLS, respectively (data not shown). Interestingly, both the GGTase I and FTase activities were elevated approximately 10- to 15-fold following clarification of the supernatant by ultracentrifugation (Table 1), perhaps resulting from removal of inhibiting lipids or some other membrane associated factor. Fractionation by anion exchange afforded the resolution of a single peak of GGTase I activity and a single major peak of FTase activity (Fig. 1a). GGTase I was purified to near homogeneity by additional con-

ventional chromatographic steps as described in Methods. Purification results from a typical preparation of GGTase I are presented in Table 1. The purification enriched two polypeptides migrating at approximately 45 kDa and 41 kDa by SDS-PAGE (Fig. 1b) and the protein size, estimated as approximately 65 kDa by gel filtration, suggests that CaGGTase I exists as an α/β heterodimer. Internal peptide sequences were obtained for both subunits (see Methods) and sequence comparisons to the *S. cerevisiae* GGTase I subunits, Ram2p and Cdc43p, revealed homology with peptide sequences from the 41 kDa and 45 kDa subunits, respectively, suggesting that the *C. albicans* GGTase I homologue had been purified.

Characterization of *C. albicans* GGTase I

Detergent was absolutely required to maintain the solubility of the GGPP substrate in the enzyme assay and dodecyl maltoside or Triton X-100 (0.02–0.05 %) were found to be satisfactory for maintaining substrate solubility and enzyme activity. Initial assays were performed with Ras-CAIL in the absence of DTT, and under these conditions a pH optimum of 9 was observed. The addition of DTT (1–10 mM) was found to be inhibitory at alkaline pH, and was routinely omitted until it was subsequently discovered that DTT shifted the pH optimum to 7.5–8 (data not shown). The effect of DTT resulted from stimulation of enzyme activity at the lower pH and inhibition at higher pH. For example, with Ras-CVIL at pH 7.5 in the presence and absence of 5 mM DTT, the relative activities observed were 100 % and 45 %, respectively, while at pH 9 the activities observed were 38 % and 78 %, respectively. The DTT dependence of the pH optimum was observed with both Ras-CAIL and Ras-CVIL. Metal requirements for *C. albicans* GGTase I were also examined (Fig. 2) since all previously characterized prenyltransferases are magnesium-dependent, zinc metalloenzymes, with the apparent exception of mammalian GGTase I which has been reported to require only zinc (Zhang & Casey,

Table 1. Purification of *C. albicans* GGTase I

Sample	Protein (mg)	Specific activity (units mg ⁻¹)*	Total activity (units)*	Purification (-fold)	Recovery (%)
Homogenate	1647	0.44	725	—	—
100 000 g supernatant	825	7.4	6110	1	100
Q-Sepharose	39	145	5660	20	93
Phenyl-Sepharose	6.1	714	4360	96	71
S-200 10/30 HR	0.062†	48 000	2960	6500	48
Mono-Q 5/5 HR	0.01†	183 000	1800	24700	29

* 1 unit is defined as 1 pmol [³H]GGPP transferred to Ras-CVIL min⁻¹ (assay conditions: 50 mM Bistris propane, pH 9, 10 mM MgCl₂, 0.1 mM ZnCl₂, 20 μ M Ras-CVIL, 2–4 μ M ³H-GGPP, 0.05 % dodecyl maltoside, 30 °C).

† Protein determined with Protein-Gold assay.

Fig. 1. (a). Separation of GGTase I and FTase activities by anion exchange on Q-Sepharose HP. Chromatographic conditions are described in Methods and the sample load and recovery are reported in Table 1. Portions (10 μ l) of the indicated fractions were assayed for GGTase I (●) and FTase (○) activities as described in Table 1 and Methods, respectively. The entire column profile had been assayed in prior preparations and no significant prenyltransferase activity was detected other than what is indicated. (b) Subunit composition of the purified *C. albicans* GGTase I. A sample (2.5 μ l; 25 ng) of the Mono-Q-purified GGTase I (lane 2) was subjected to SDS-PAGE and the protein bands visualized by silver staining. Lane 1 contains molecular mass standards (Mark12, Novex).

Fig. 2. Zinc and magnesium dependence of *C. albicans* GGTase I. Purified GGTase I was assayed for activity in the presence of the indicated final concentrations of ZnCl₂ and MgCl₂. Assays were performed in 50 mM HEPES, pH 7.5, 5 mM DTT, 0.02% Triton X-100, containing 25 μ M Ras-CVIL and 0.25 μ M ³H-GGPP. Data in the lane marked with asterisks (*) were obtained from an assay which also contained 2.5 mM EDTA. The data are means of duplicate determinations; error bars indicate the difference between replicates.

1996b). Purified GGTase I was nearly inactive in the absence of added zinc and magnesium salts, and the residual activity was further reduced by the addition of 2.5 mM EDTA. In contrast to mammalian GGTase I, the addition of 100 μ M ZnCl₂ alone restored only 15% of full enzyme activity. Full activity required the further addition of 10–20 mM MgCl₂, which stimulated the zinc-containing enzyme an additional sevenfold. The addition of 10 mM MgCl₂ alone restored 65% of full activity. Full activity was achieved by the further addition of 10 μ M ZnCl₂, suggesting that the purified

enzyme maintained a significant amount of bound zinc. Michaelis constants (K_m) were determined for Ras-CVIL and GGPP as 23 μ M and 0.024 μ M (means of two determinations) respectively, by fitting plots of initial velocity (pH 7.5) versus substrate concentration to the Michaelis–Menten equation by non-linear regression analysis (data not shown). Approximate K_m values of 110 μ M and 90 μ M were obtained for Ras-CAIL and Ras-CTIL, respectively.

Substrate preferences of *C. albicans* GGTase I and FTase

The prenyl acceptor substrate specificity of FTase and GGTase I is a critical issue regarding the functional requirement for GGTase I in *C. albicans*. FTase cross-specificity for typical GGTase I substrates could obviate the presumed requirement for the latter. Therefore, the substrate specificity of the partially purified GGTase I and FTase activities was examined with a series of Ras-CaaX homologues as the prenyl acceptor and either GGPP or FPP as the prenyl donor (Fig. 3). The CaaX sequences used included CVIL (*Candida* CRL1; RHO homologue), CVVL (*Candida* RHO1), CTIL (*Candida* CDC42), CAIL, CVLM, CVLS and CVIA (*S. cerevisiae* a mating factor). GGTase I showed a preference for typical GGTase I CaaX prenyl acceptor substrates where X is a leucine residue and showed almost complete selectivity for GGPP as the prenyl donor (Fig. 3a). Within the Ras-CaaL series the ranking of GGTase I activity was CVIL > CTIL > CAIL ~ CVVL. Modest activity was observed with CVLM and no activity was observed with CVLS or CVIA, typical acceptor substrates for FTase. FTase exhibited a strong preference for Ras-CaaX ending with methionine (CVLM) and showed much less activity with other typical FTase

Fig. 3. Prenyl acceptor substrate specificity of *C. albicans* GGTase I (a) and FTase (b). GGTase I and FTase activities were separated by the first two columns (Q-Sepharose and Phenyl-Sepharose) as described in Methods and assayed with the indicated Ras-CaaX substrate (20 μ M) and either GGPP ($\square$) or FPP ($\blacksquare$) at 4 μ M. All assays were performed at pH 7.5 as described in Methods, and included 1 mM DTT. The data are means of duplicate determinations; error bars indicate the difference between replicates.

acceptors examined (CVLS and CVIA) (Fig. 3b). FTase was also observed to catalyse some farnesylation of all CaaL substrates examined. The ranking for FTase farnesylation of the CaaL series was CVIL > CVVL ~ CTIL > CAIL and occurred with an efficiency of 19, 6, 6 and 2%, respectively, relative to farnesylation of Ras-CVLM. The enzyme samples used in this experiment were both partially purified by anion exchange and hydrophobic interaction chromatography (see Methods) and purified to a similar extent (approx. 100-

fold). A comparison of the specific activity data in Fig. 3 indicates that FTase-catalysed farnesylation of CaaL substrates occurs at a level similar to that of GGTase I-catalysed geranylgeranylation.

Cloning of CaRAM2 and CaCDC43 from *C. albicans*

Purification and sequencing of the protein subunits allowed for isolation of gene fragments by PCR as described in Methods. A 729 bp CaRAM2 PCR product contained an open reading frame exhibiting approximately 37% sequence identity to the *S. cerevisiae* RAM2 (α -subunit) protein sequence and a 573 bp CaCDC43 PCR product was found to contain an ORF exhibiting approximately 29% identity to the *S. cerevisiae* CDC43 protein sequence (β -subunit).

The PCR clones of each gene were then used to isolate genomic clones and the DNA and predicted peptide sequences of each subunit were determined. Open reading frames of 918 and 1194 bp encoding putative peptides of 306 and 398 amino acids were identified for CaRAM2 and CaCDC43, respectively. The predicted molecular masses for CaRam2p and CaCdc43p were 36.9 and 47.8 kDa, respectively, which agreed reasonably well with the determinations for the purified subunits. All of the peptide sequences determined from the purified *C. albicans* GGTase I subunits were found in CaCdc43p and CaRam2p, confirming that the genes isolated corresponded to the purified proteins. CaRam2p and CaCdc43p are 42 and 34% identical to their corresponding *S. cerevisiae* homologues, respectively, and 61 and 55% similar, as summarized in Table 2. Both *C. albicans* subunits are approximately 30% identical to their mammalian, plant and *Schizosaccharomyces pombe* homologues.

One CUG codon, which is decoded *in vivo* as serine in *C. albicans* instead of the conventional leucine (Tuite & Santos, 1996), was identified near the C-terminus of Cdc43p encoding amino acid 388. A direct repeat of the trinucleotide ACT/C/G (the third nucleotide is most frequently T) was found 72 nucleotides upstream of the coding sequence of CaCDC43 and was repeated 22

Table 2. Comparisons of amino acid sequences of *C. albicans* GGTase I subunits with GGTase I of other organisms

Organism	CaRam2p		CaCdc43p	
	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)
<i>S. cerevisiae</i>	42	61	34	55
<i>Sch. pombe</i>	36	46	30	53
Human	30	58	30	53
Rat	30	57	29	54
Bovine	30	57	NA	NA
Tomato	29	55	NA	NA

NA, Not available.

[illegible]

Fig. 4. For legend see facing page.

times. The significance of this repeat in *CaCDC43* is unknown, but it may play a regulatory role. A BLAST search revealed similar sequences in 5' and 3' noncoding regions of other genes as well as in centromeric DNA of *S. cerevisiae* and *Sch. pombe*. The 3' untranslated region of *CaCDC43* contains the *S. cerevisiae* consensus sequence for transcription termination TAG...TAGT...TTT described by Zaret & Sherman (1982) beginning at nucleotide 1671. Three potential TATA boxes were noted in the 5' noncoding region of *CaRAM2*.

A multiple sequence alignment of the α -subunit (Ram2p) from yeast, human and a plant (Fig. 4a) revealed that CaRam2p contains several small regions of homology including five sequence repeats previously noted for prenyltransferase α -subunits. The first three of these regions are a close match to the consensus sequence motif NYxxWxYR (x is an undefined amino acid; Boguski *et al.*, 1992) while the latter two repeats differ by two amino acids. From the rat FTase crystal structure this motif is known to occur in α -subunit 'helical hairpins' which form part of the β -subunit interface (Park & Beese, 1997).

Fig. 4(b) presents a multiple sequence alignment for the β -subunit (Cdc43p). A unique feature of CaCdc43p is an insertion of a tract of 17 asparagines at amino acid 178. The putative polyasparagine tract of CaCdc43p is encoded by an AT-rich region resembling noncoding sequence, but is most probably translated. No good matches to intron consensus sequences were found and introns occur infrequently in *C. albicans* genes. Three residues which are completely conserved in all β -subunits of GGTase I, Asp 302, Cys 304 and His 357, are putatively assigned as the CaCdc43p β -subunit zinc ligands based on comparison to the FTase crystal structure (Park *et al.*, 1997). Several residues implicated in substrate (prenyl donor and acceptor) binding by mutagenesis studies of FTase (Kral *et al.*, 1997) or GGTase I (Ohya *et al.*, 1996) and the FTase crystal structure (Park & Beese, 1997) are conserved in all β -subunits of GGTase I including CaCdc43p and are indicated in Fig. 4(b).

Allelic and strain variations are found in both subunits of *C. albicans* GGTase I

PCR clones containing the coding region of each subunit and various amounts of flanking sequence were isolated from strain CA14, the strain most routinely used for *Candida* genetics, and from MY1055, a pathogenic strain that we use in mouse models for virulence. The

(a)

CA124	Y	D	D	H	N	N	N	N	N	N	N	N	N	N	N	N	N	N	R	E	D
MY1055-14	Y	D	D	.	N	N	N	N	N	N	N	.	.	.	.	.	.	.	R	E	D
MY1055-24	Y	D	D	.	N	N	N	N	N	N	N	.	.	.	.	.	.	.	R	E	D
CA14-40	Y	D	D	.	N	N	N	N	N	N	N	.	.	.	.	.	.	.	R	E	D
CA14-43	Y	D	D	.	N	N	N	N	N	N	N	N	N	N	.	.	.	.	R	E	D

(b)

CA124	N	L	Y	P	K	.	.	.	.	D	G	N	D	N	G	D	N	Y	E	Y		
MY1055-14	N	L	Y	P	K	.	.	.	.	D	G	N	G	N	G	N	G	D	N	Y	E	Y
MY1055-24	N	L	Y	P	K	D	G	N	G	D	G	N	G	N	G	N	G	D	N	Y	E	Y
CA14-40	N	L	Y	P	K	.	.	.	.	D	G	.	.	N	G	D	N	Y	E	Y	Y	
CA14-43	N	L	Y	P	K	D	G	N	G	D	G	N	G	N	G	D	N	Y	E	Y	Y	

Fig. 5. (a, b) Two divergent regions among CaCdc43ps. CA124, MY1055 and CA14 are different *C. albicans* strains. MY1055-14 and -24 and CA14-40 and -43 represent different alleles.

predicted amino acid sequences of CaCdc43ps from CAI4 and MY1055 differed substantially from Cdc43p of CA124 in two regions. The most notable difference is the large variation in the number of asparagines and the absence of a histidine residue directly preceding the asparagine tract in the predicted translation products of CAI4 and MY1055 (Fig. 5a). The strains differ at another repetitive region of the protein: approximately 80 amino acids downstream of the asparagine tract, one allele of both CAI4 and MY1055 encodes an insertion of 'DGNG' (Fig. 5b). CAI4-40 contains a deletion of 'ND' and the other alleles have a D(277)G change. Four additional changes compared to Cdc43p of CA124 were noted throughout the protein. One was an E(395)K change in three out of four alleles analysed whereas the other changes were only found in one of the alleles. Fewer differences were found in the predicted Ram2 polypeptides. One allele from CAI4 encoded no changes compared to CA124 while the other allele had one silent base change. Likewise, one allele of MY1055 was identical to CA124 whereas the other contained four base changes and encoded a single D(155)G change.

Complementation of a *S. cerevisiae* *ts cdc43* mutant requires both CaCDC43 and CaRAM2

Complementation of the *ts* defect of *S. cerevisiae cdc43* and *ram2* mutants, CJ198-2B and RS51-3A, respectively, was attempted by coexpression of both subunits of *C. albicans* GGTase I. The *C. albicans* GGTase I subunit genes from strains CA124 or CA14 were expressed under their respective *C. albicans* promoters on high-copy *S. cerevisiae* plasmids. The *ts* defect of the *cdc43* mutant was complemented in transformants containing both subunits as shown in Fig. 6; 13 and 9 transformants

Fig. 4. Amino acid sequence alignments of protein geranylgeranyl transferase α (a) and β (b) subunits. Alignments were determined by the CLUSTAL program. Amino acid identities are indicated by boxes. Dashes denote gaps formed by the alignment algorithm. (a) The five regions matching the consensus 'N^yxxW^xYR' are marked by bars above the sequence. The GenBank accession numbers for the sequences of the GGTase I α subunits are as follows: *C. albicans* CaRAM2, AF110691; *S. cerevisiae* RAM2, M88584; tomato LeFTA, U83707; and human α , L00634. (b) Residues implicated in zinc, prenyl acceptor and prenyl donor binding are indicated by the superscripts z, a, d, respectively. The GenBank accession numbers for the aligned sequences of the β subunits of GGTase I are as follows: *C. albicans* CaCDC43, AF110690, *S. cerevisiae* CDC43, M31114; human β , L25441; *S. pombe* Cwg2+, Z12155.

Fig. 6. Complementation of the *ts* defect of *S. cerevisiae cdc43* mutant CJ198-2B by *CaCDC43* and *CaRAM2*. CJ198-2B was transformed with: A, vectors pRS426 and Yeplac112; B, p40CDCU (*CaCDC43*) and Yeplac112; and C, p40CDCU (*CaCDC43*) and pCaRAM2T (*CaRAM2*).

containing both *CaCDC43* and *CaRAM2* from CA124 and CA14, respectively, grew at the nonpermissive temperature. Transformants containing *CaCDC43* alone were not viable at the restrictive temperature. No complementation of the *ts ram2* mutant was obtained with both *CaCDC43* and *CaRAM2* (tested from CA124 only) for nine transformants tested at the nonpermissive temperature.

The *S. cerevisiae ts ram2* mutant is defective in FTase as well as GGTase I at the nonpermissive temperature since the α subunit encoded by *RAM2* is common to both GGTase I and FTase. The lack of complementation by both subunits of *C. albicans* GGTase I is most probably due to nonfunctional FTase since we were able to complement the *ts* defect of the *cdc43* mutant. FTase of *S. cerevisiae* is essential at 37 °C but not at 30 °C as *S. cerevisiae* GGTase I compensates for FTase at 30 °C (Ohya *et al.*, 1993; Trueblood *et al.*, 1993). We tested to see if the lethality of a *S. cerevisiae ram2* disruptant could be corrected by coexpression of the *C. albicans* GGTase I subunits at 30 °C. We expected that the GGTase I defect of the *ram2* disruptant strain, W303dR2, would be corrected by the *C. albicans* enzyme and we wanted to know if *C. albicans* GGTase I would compensate for FTase as well. We used the 'plasmid shuffle technique' to determine if the *C. albicans* GGTase I subunits expressed under their respective promoters in the *ram2* disruptant strain W303dR2 would allow loss of the *Ura*⁺ *RAM2* plasmid. To select against the *Ura*⁺ plasmid containing the *S. cerevisiae* *RAM2* gene, transformants containing the *C. albicans* genes were subsequently propagated at 30 °C on medium containing 5-FOA. No 5-FOA-resistant colonies were obtained, indicating that it was not possible to maintain viability upon loss of the *S. cerevisiae URA3 RAM2* plasmid.

DISCUSSION

We have purified the *C. albicans* GGTase I protein and isolated and sequenced the genes (*CaRAM2* and *CaCDC43*) encoding the heterodimeric enzyme. The functionality of the cloned *C. albicans* GGTase I

subunits was confirmed by coexpression under their native promoters in a *S. cerevisiae ts cdc43* mutant which enabled growth at the nonpermissive temperature. Complementation was not obtained by expression of *CDC43* alone, suggesting that heterodimers between the *S. cerevisiae* α and *C. albicans* β subunits of GGTase I are either not functional or not formed. This result explains our earlier failure to clone *CaCDC43* by complementation of the *ts cdc43* mutant (data not shown). Similarly, Yalovsky *et al.* (1997) have shown that coexpression of both the α and β subunits of tomato FTase was required to complement the *ts* defect of a *S. cerevisiae ram1* mutant. It is also noteworthy that the *C. albicans* subunits functioned in *S. cerevisiae* despite the presence of a CUG codon in *CaCDC43* which is usually translated as serine in *C. albicans* instead of leucine. However, we were unable to detect any increase in GGTase I activity in cell-free extracts prepared from the *cdc43* mutant complemented with *CaCDC43* and *CaRAM2* (data not shown). The extracts were made from cells grown at 30 °C and assayed at 30 °C, where we detected a low level of *S. cerevisiae* GGTase I activity (3–5% of wild-type) from the *cdc43* mutant control, as previously reported (Ohya *et al.*, 1996). While poor expression of the *C. albicans* genes or poor *in vitro* enzyme activity with the Ras-CVIL or Ras-CAIL substrates (high *K_m* values) could explain our inability to detect *CaGGTase I* activity, presumably the critical ScRho1 and ScCdc42 proteins are being adequately geranylgeranylated in the whole cell.

Correction of the growth defects of the *S. cerevisiae ram2* mutants requires restoration of both GGTase I and FTase activity, as the α subunit encoded by *RAM2* is common to both GGTase I and FTase. FTase is essential for survival of *S. cerevisiae* at 37 °C but is not required at 30 °C. Since the *C. albicans* subunits complemented the *ts* GGTase I mutant, the inability to complement the *ts ram2* mutant suggests that the FTase defect has not been corrected and that heterodimers between *C. albicans* Ram2p and *S. cerevisiae* Ram1p are probably not functional or do not form. The lack of complementation of the *ram2* disruptant at 30 °C is most probably due to defective FTase as well. All of

these experiments suggest that *C. albicans* GGTase I expressed from its native promoters in *S. cerevisiae* can compensate for defective GGTase I, but cannot additionally substitute for *S. cerevisiae* FTase. It is possible that greater expression of the *C. albicans* GGTase I subunits, i.e. under *S. cerevisiae* promoters, would rescue the *ram2* null mutant. Expression in *S. cerevisiae* might also be improved by mutating the CUG codon encoding amino acid 388 of Cdc43p to encode serine. Alternatively, *C. albicans* GGTase I may exhibit tighter prenyl acceptor (CaaX) specificity than *S. cerevisiae* GGTase I and may be incapable of sufficiently prenylating the requisite Ras proteins (Ras1=CIIC; Ras2=CIIS). This possibility is consistent with the observed inability of *C. albicans* GGTase I to geranylgeranylate RasCVLS.

A striking feature of CaCdc43p is an insertion of a tract of asparagines at amino acid 178 that is not found in other prenyltransferases and whose length varies among strains and between alleles. Several proteins from *Dictyostelium discoideum* contain translated poly-asparagine tracts and similar tracts have been identified in at least two *S. cerevisiae* proteins, Swl1p and Abf1p (Diffley & Stillman, 1989; Schmalix & Bandlow, 1994). The function of the polyasparagine tracts is unknown and the significance, if any, of this region in CaCdc43p remains to be determined. We do know that CaCdc43p containing either 6 or 17 asparagines is functional, as complementation of the *ts cdc43* mutant was obtained with either of these homologues. Restriction site polymorphisms between alleles of *C. albicans* have been identified previously (Kelly *et al.*, 1987), but to our knowledge, this is the first time that differences between the coding sequence of two alleles has been documented. It will be interesting to see what other types of changes occur between ORFs of different alleles and how frequently this occurs when more sequence of the *C. albicans* genome becomes available.

Studies on the substrate specificity of *S. cerevisiae* GGTase I and FTase have shown that prenyl acceptor specificity is largely defined by the C-terminal X-residue of the CaaX motif and, moreover, is largely independent of the remaining N-terminal protein sequence (Moore *et al.*, 1991). *S. cerevisiae* GGTase I has a preference for CaaX substrates ending in leucine and to a lesser extent, methionine, while *S. cerevisiae* FTase preferentially farnesylates CaaX sequences ending in methionine, serine, cysteine and alanine. Enzyme specificity studies with recombinant yeast FTase from *S. cerevisiae* have in fact shown Ras-CaaL substrates to be modestly farnesylated (3–8%) relative to Ras-CAIM (Caplin *et al.*, 1994). However, the lethality of the GGTase I null mutation presumably results from the inability of *S. cerevisiae* FTase to sufficiently prenylate wild-type levels of Cdc42p (CTIL) and Rho1p (CVLL) in the mutant yeast cells. The *C. albicans* GGTase I exhibits the same preference for the geranylgeranylation of Ras-CaaL substrates as observed with both *S. cerevisiae* and mammalian GGTase I enzymes. However, the K_m values determined for Ras-CaaL substrates, particularly CTIL

and CAIL, were unusually high compared to values observed for *S. cerevisiae* and mammalian GGTase I, which are typically 5 μ M or less. The reduced GGTase I activity with RasCTIL and RasCAIL probably reflects the higher K_m values observed with these substrates. The *C. albicans* FTase has a strong preference for farnesylation of Ras-CVLM as does *S. cerevisiae* FTase, but in contrast, has quite modest activity with Ras-CVLS or Ras-CVIA (yeast α -factor mating pheromone). Interestingly, the *C. albicans* FTase also possesses the ability to farnesylate all Ras-CaaL substrates examined at levels ranging from 2% (CAIL) to 20% (CVIL) relative to that observed with CVLM. Moreover, the specific activities determined for CaaL farnesylation are comparable to those observed for GGTase I catalysed geranylgeranylation. It is unclear whether the cross-specificity of *C. albicans* FTase for CaaL substrates observed *in vitro* will affect the functional requirements for GGTase I in *C. albicans* cells.

The cloning of the *C. albicans* GGTase I subunit genes is an important step towards understanding the role of protein geranylgeranylation in *C. albicans* morphogenesis. The sequence data presented suggest that it may be possible to identify inhibitors with selectivity for the *C. albicans* versus the human enzyme. Further experiments to validate GGTase I as a broad-spectrum fungicidal target for antimycotic therapy are under way.

NOTE ADDED IN PROOF

A report on the complete cloning and characterization of GGTase I from the fission yeast *Schizosaccharomyces pombe* was published recently (Arellano *et al.*, 1998).

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REFERENCES

- Abruzzo, G. K., Flattery, A. M., Gill, C. J., Kong, L., Smith, J. G., Krupa, D., Pikounis, V. B., Kropp, H. & Bartizal, K. (1995). Evaluation of water-soluble pneumocandins L-733,560, L-705,589, and L-731,373 in mouse models of disseminated aspergillosis, candidiasis, and cryptococcosis. *Antimicrob Agents Chemother* 39, 1077–1081.
- Adams, E. M., Johnson, D. I., Longnecker, R. M., Sloat, B. F. & Pringle, J. R. (1990). CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast *Saccharomyces cerevisiae*. *J Cell Biol* 111, 131–142.
- Arellano, M., Coll, P. M., Yang, W., Duran, A., Tamanoi, F. & Perez, P. (1998). Characterization of the geranylgeranyl trans-

- ferase type I from *Schizosaccharomyces pombe*. *Mol Microbiol* **29**, 1357–1367.
- Boeke, J. D., LaCroute, F. & Fink, G. R. (1984). A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol Gen Genet* **197**, 345–346.
- Boguski, M. S., Murray, A. W. & Powers, S. (1992). Novel repetitive sequence motifs in the α and β subunits of prenyl-protein transferases and homology of the α subunit to the MAD2 gene product of yeast. *New Biol* **4**, 408–411.
- Caplin, B. E., Hettich, L. A. & Marshall, M. S. (1994). Substrate characterization of the *Saccharomyces cerevisiae* protein farnesyl-transferase and type-I protein geranylgeranyltransferase. *Biochim Biophys Acta* **1205**, 39–48.
- Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H. & Hieter, P. (1992). Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**, 119–122.
- Clarke, S. (1992). Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. *Annu Rev Biochem* **61**, 355–386.
- Compton, T. (1990). Degenerate primers for DNA amplification. In *PCR Protocols: a Guide to Methods and Applications*, pp. 39–45. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. New York: Academic Press.
- Cox, G. M. & Perfect, J. R. (1993). Fungal infections. *Curr Opin Infect Dis* **6**, 422–426.
- Diffley, J. F. X. & Stillman, B. (1989). Similarity between the transcriptional silencer binding proteins ABF1 and RAP1. *Science* **246**, 1034–1038.
- Douglas, C. M., Foor, F., Marrinan, J. A. & 11 other authors (1994). The *Saccharomyces cerevisiae* FKS1 (*ETG1*) gene encodes an integral membrane protein which is a subunit of 1,3- β -D-glucan synthase. *Proc Natl Acad Sci USA* **91**, 12907–12911.
- Drgonová, J., Drgon, T., Tanaka, K., Kollár, R., Chen, G.-C., Ford, R. A., Chan, C. S. M., Takai, Y. & Cabib, E. (1996). Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. *Science* **272**, 277–279.
- Elble, R. (1992). A simple and efficient procedure for transformation of yeasts. *Biotechniques* **13**, 18–20.
- Farnsworth, C. C., Seabra, M. C., Ericsson, L. H., Gelb, M. H. & Glomset, J. A. (1994). Rab geranylgeranyl transferase catalyzes the geranylgeranylation of adjacent cysteines in the small GTPases Rab1A, Rab3A, and Rab5A. *Proc Natl Acad Sci USA* **91**, 11963–11967.
- Finegold, A. A., Johnson, D. I., Farnsworth, C. C., Gelb, M. H., Judd, S. R., Glomset, J. A. & Tamanoi, F. (1991). Geranylgeranylprotein transferase of *Saccharomyces cerevisiae* is specific for Cys-Xaa-Xaa-Leu motif proteins and requires the CDC43 gene product, but not the DPR1 gene product. *Proc Natl Acad Sci USA* **88**, 4448–4452.
- Fonzi, W. A. & Irwin, M. Y. (1993). Isogenic strain construction and gene mapping in *Candida albicans*. *Gene* **134**, 717–728.
- Gietz, R. D. & Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**, 527–534.
- Goodman, L. E., Judd, S. R., Farnsworth, C. C., Powers, S., Gelb, M. H., Glomset, J. A. & Tamanoi, F. (1990). Mutants of *Saccharomyces cerevisiae* defective in the farnesylation of Ras proteins. *Proc Natl Acad Sci USA* **87**, 9665–9669.
- He, B., Chen, P., Chen, S., Vancura, K. L., Michaelis, S. & Powers, S. (1991). RAM2, an essential gene of yeast, and RAM1 encode the two polypeptide components of the farnesyltransferase that prenylates a-factor and Ras proteins. *Proc Natl Acad Sci* **88**, 11373–11377.
- Hoffman, C. S. & Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmid for transformation of *Escherichia coli*. *Gene* **57**, 267–272.
- Imamura, H., Tanaka, K., Hihara, T., Umikawa, M., Kamei, T., Takahashi, K. & Sasaki, T. (1997). Bni1p and Bnr1p: downstream targets of the Rho family small G-proteins which interact with profilin and regulate actin cytoskeleton in *Saccharomyces cerevisiae*. *EMBO J* **16**, 2745–2755.
- Jones, J. S. & Prakash, L. (1990). Yeast *Saccharomyces cerevisiae* selectable markers in pUC18 polylinkers. *Yeast* **6**, 363–366.
- Kamada, Y., Qadota, H., Python, C. P., Anraku, Y., Ohya, Y. & Levin, D. E. (1996). Activation of yeast protein kinase C by Rho1 GTPase. *J Biol Chem* **271**, 9193–9196.
- Kelly, R., Miller, S. M., Kurtz, M. B. & Kirsch, D. R. (1987). Directed mutagenesis in *Candida albicans*: one-step gene disruption to isolate *ura3* mutants. *Mol Cell Biol* **7**, 199–208.
- Kohl, N. E., Diehl, R. E., Schaber & 9 other authors (1991). Structural homology among mammalian and *Saccharomyces cerevisiae* isoprenyl-protein transferases. *J Biol Chem* **266**, 18884–18888.
- Kohno, H., Tanaka, K., Mino, A., Umikawa, M., Imamura, H., Fujiwara, T., Fujita, Y., Hotta, K. & Qadota, H. (1996). Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein. *EMBO J* **15**, 6060–6068.
- Kondoh, O., Tachibana, Y., Ohya, Y., Arisawa, M. & Watanabe, T. (1997). Cloning of the *RHO1* gene from *Candida albicans* and its regulation of β -1,3-glucan synthesis. *J Bacteriol* **179**, 7734–7741.
- Kral, A. M., Diehl, R. E., deSolms, S. J., Williams, T. M., Kohl, N. E. & Omer, C. A. (1997). Mutational analysis of conserved residues of the β -subunit of human farnesyl:protein transferase. *J Biol Chem* **272**, 27319–27323.
- Mayer, M. L., Caplin, B. E. & Marshall, M. S. (1992). CDC43 and RAM2 encode the polypeptide subunits of a yeast type I protein geranylgeranyltransferase. *J Biol Chem* **267**, 20589–20593.
- Mazur, P. & Baginsky, W. (1996). In vitro activity of 1,3- β -D-glucan synthase requires the GTP-binding protein Rho1. *J Biol Chem* **271**, 14604–14609.
- Mirbod, F., Nakashima, S., Kitajima, Y., Cannon, R. D. & Nozawa, Y. (1997). Molecular cloning of a Rho family, CDC42Ca gene from *Candida albicans* and its mRNA expression changes during morphogenesis. *J Med Vet Mycol* **35**, 173–179.
- Moore, S. L., Schaber, M. D., Mosser, S. D., Rands, E., O'Hara, M. B., Garsky, V. M., Marshall, M. S., Pompliano, D. L. & Gibbs, J. B. (1991). Sequence dependence of protein isoprenylation. *J Biol Chem* **266**, 14603–14610.
- Nonaka, H., Tanaka, K., Hirano, H., Fujiwara, T., Kohno, H., Umikawa, M., Mino, A. & Takai, Y. (1995). A downstream target of RHO1 small GTP-binding protein is PKC1, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in *Saccharomyces cerevisiae*. *EMBO J* **14**, 5931–5938.
- Ohya, Y., Goebl, M., Goodman, L. E., Peterson-Bjorn, S., Friesen, J. D., Tamanoi, F. & Anraku, Y. (1991). Yeast CAL1 is a structural and functional homologue to the DPR1 (RAM) gene involved in ras processing. *J Biol Chem* **266**, 12356–12360.
- Ohya, Y., Qadota, H., Anraku, Y., Pringle, J. R. & Botstein, D. (1993). Suppression of yeast geranylgeranyl transferase I defect by alternative prenylation of two target GTPases, Rho1p and Cdc42p. *Mol Biol Cell* **4**, 1017–1025.
- Ohya, Y., Caplin, B. E., Qadota, H., Tibbetts, M. F., Anraku, Y., Pringle, J. R. & Marshall, M. S. (1996). Mutational analysis of the

- β -subunit of yeast geranylgeranyl transferase I. *Mol Gen Genet* **252**, 1–10.
- Omer, C. & Gibbs, J. (1994). Protein prenylation in eukaryotic microorganisms: genetics, biology and biochemistry. *Mol Microbiol* **11**, 219–225.
- Park, H.-W. & Beese, L. S. (1997). Protein farnesyltransferase. *Curr Opin Struct Biol* **7**, 873–880.
- Park, H.-W., Boduluri, S. R., Moomaw, J. F., Casey, P. J. & Beese, L. S. (1997). Crystal structure of protein farnesyltransferase at 2.5 angstrom resolution. *Science* **21**, 1800–1804.
- Qadota, H., Python, C. P., Inoue, S. B., Arisawa, M., Anraku, Y., Zheng, Y., Watanabe, T., Levin, D. E. & Ohya, Y. (1996). Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3- β -glucan synthase. *Science* **272**, 279–281.
- Schafer, W. R. & Rine, J. (1992). Protein prenylation: genes, enzymes, targets, and functions. *Annu Rev Genet* **30**, 209–237.
- Schmalix, W. A. & Bandlow, W. (1994). *SWH1* from yeast encodes a candidate nuclear factor containing ankyrin repeats and showing homology to mammalian oxysterol-binding protein. *Biochim Biophys Acta* **1219**, 205–210.
- Sherlock, G., Gahman, A. M., Mahal, A., Shieh, J., Ferreira, M. & Rosamond, J. (1994). Molecular cloning and analysis of *CDC28* and cyclin homologues from the human fungal pathogen *Candida albicans*. *Mol Gen Genet* **245**, 716–723.
- Sherman, F., Fink, G. R. & Hicks, J. B. (1986). *Methods in Yeast Genetics: a Laboratory Course Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Thomas, B. & Rothstein, R. (1989). Elevated recombination rates in transcriptionally active DNA. *Cell* **56**, 619–630.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Trueblood, C. E., Ohya, Y. & Rine, J. (1993). Genetic evidence for in vivo cross-specificity of the CaaX-box protein prenyltransferases farnesyltransferase and geranylgeranyltransferase-I in *Saccharomyces cerevisiae*. *Mol Cell Biol* **13**, 4260–4275.
- Tuite, M. F. & Santos, M. A. (1996). Codon reassignment in *Candida* species: an evolutionary conundrum. *Biochimie* **78**, 993–999.
- Yalovsky, S., Trueblood, C. E., Callan, K. L., Narita, J. O., Jenkins, S. M., Rine, J. & Grussem, W. (1997). Plant farnesyltransferase can restore yeast Ras signaling and mating. *Mol Cell Biol* **17**, 1986–1994.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.
- Zaret, K. S. & Sherman, F. (1982). DNA sequence required for efficient transcription termination in yeast. *Cell* **28**, 563–573.
- Zhang, F. L. & Casey, P. J. (1996a). Protein prenylation: molecular mechanisms and functional consequences. *Annu Rev Biochem* **65**, 241–269.
- Zhang, F. L. & Casey, P. J. (1996b). Influence of metal ions on substrate binding and catalytic activity of mammalian protein geranylgeranyltransferase type-I. *Biochem J* **320**, 925–932.

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