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Mutational analysis of the β-subunit of yeast geranylgeranyl transferase I

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Abstract The gene *CAL1* (also known as *CDC43*) of *Saccharomyces cerevisiae* encodes the β subunit of geranylgeranyl transferase I (GGTase I), which modifies several small GTPases. Biochemical analyses of the mutant enzymes encoded by *cal1-1*, and *cdc43-2* to *cdc43-7*, expressed in bacteria, have shown that all of the mutant enzymes possess reduced activity, and that none shows temperature-sensitive enzymatic activities. Nonetheless, all of the *cal1/cdc43* mutants show temperature-sensitive growth phenotypes. Increase in soluble pools of the small GTPases was observed in the yeast mutant cells at the restrictive temperature in vivo, suggesting that the yeast prenylation pathway itself is temperature sensitive. The *cal1-1* mutation, located most proximal to the C-terminus of the protein, differs from the other *cdc43* mutations in several respects. An increase in soluble Rho1p was observed in the *cal1-1* strain grown at the restrictive temperature. The temperature-sensitive phenotype of *cal1-1* is most efficiently suppressed by overproduction of Rho1p. Overproduction of the other essential target, Cdc42p, in contrast, is deleterious in *cal1-1* cells, but not in other *cdc43* mutants or the wild-type strains. The *cdc43-5* mutant cells accumulate Cdc42p in soluble pools and *cdc43-5* is suppressed by overproduction of Cdc42p. Thus, several phenotypic differences are observed among the *cal1/cdc43* mutations, possibly due to alterations in substrate specificity caused by the mutations.

Key words Prenylation · Geranylgeranyl transferase I · *CAL1* · *CDC43*

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Introduction

Protein prenylation, farnesylation and geranylgeranylation are posttranslational reactions which require the covalent attachment of a hydrophobic tail, isoprenoid (C15 or C20), to the C-terminal cysteine residue of substrate proteins (Clarke 1992; Schafer and Rine 1992). Prenylation is necessary for many proteins to interact with membranes and be localized at proper intracellular sites. Many lines of evidence have been accumulated to show that small GTPases require prenylation for full function (Clarke 1992; Omer and Gibbs 1994; Schafer and Rine 1992).

Genetic and biochemical studies of protein prenylation in rat and yeast have revealed three distinct protein prenyltransferases (Chen et al. 1991a, b; Kohl et al. 1991), including farnesyl transferase (FTase), geranylgeranyl transferase I (GGTase I) and geranylgeranyl transferase II (GGTase II). FTase (Chen et al. 1991b; Dolence and Poulter 1995; Reiss et al. 1990, 1991b; Seabra et al. 1991) and GGTase I (Moomaw and Casey 1992; Moores et al. 1991; Seabra et al. 1991; Yokoyama et al. 1991) prenylates proteins ending with a Cys-Ali-Ali-Xaa (Ali, aliphatic; Xaa, any amino acid residue) sequence, whereas GGTase II modifies proteins ending

with Gly-Gly-Cys-Cys or Cys-Xaa-Cys (Seabra et al. 1992, 1993). Substrate preference for FTase and GGTase I is affected by the last amino acid residues in the Cys-Ali-Ali-Xaa sequence, such that GGTase I most frequently geranylgeranylates proteins ending with Leu (Finegold et al. 1991; Kinsella et al. 1991; Yokoyama and Gelb 1993). However, cross-specificity is observed to some extent between FTase and GGTase I, both in vitro (Armstrong et al. 1995; Caplin et al. 1994; Yokoyama et al. 1991) and in vivo (Adamson et al. 1992; Trueblood et al. 1993), and can be explained by the similarity in subunit composition between the two enzymes. FTase and GGTase I both consist of an α/β heterodimer (Seabra et al. 1991; Reiss et al. 1991a) and share a common α subunit (Mayer et al. 1992; Moomaw and Casey 1992; Seabra et al. 1991). In contrast, no cross-specificity has been reported between GGTase II and the other prenyltransferases. GGTase II has a different subunit composition, consisting of component A and an α/β heterodimer (Seabra et al. 1992, 1993). Recently, yeast FTase mutants that show altered substrate specificity were isolated and characterized (Mitsuzawa et al. 1995).

Genes encoding subunits of each prenyltransferase have been cloned in the yeast *Saccharomyces cerevisiae*. The genes *CAL1* (Ohya et al. 1991), also known as *CDC43* (Johnson et al. 1991), and *DPR1* (Goodman et al. 1988), also known as *RAM1*, encode β subunits of the yeast GGTase I and FTase, respectively, and *RAM2* encodes the common α subunit (He et al. 1991). The α subunit, β subunit and component A of the yeast GGTase II are encoded by *BET4*, *BET2* and *MSI4*, respectively (Fujimura et al. 1994; Rossi et al. 1991). An alignment of the homologous regions of the three β subunit sequences (positions 159–350 of the Cal1/Cdc43 sequence) reveals 32–40% identity between pairs (Ohya et al. 1991). This region contains novel repeat motifs (Boguski et al. 1992). These repeats have a length of 44–45 residues and there are three repeats in the Cal1p/Cdc43p sequence. The repeats show a conserved central Gly-Gly-Phe-Gly-Gly sequence region. The α subunit of isoprenyl transferases also possesses distinct internally repetitive sequences containing tryptophan. Hydrophobic bonds between the side chains of the conserved tryptophan and phenylalanine may be important for forming heterodimers (Boguski et al. 1992).

Yeast GGTase I has been well characterized biochemically by using the recombinant prenyltransferase expressed in *E. coli*. The yeast GGTase I is a Mg^{2+}-requiring, Zn^{2+} metalloenzyme (Mayer et al. 1992). This is consistent with the results obtained with the mammalian FTase (Reiss et al. 1991b) and GGTase I (Moomaw and Casey 1992; Yokoyama et al. 1995), which require Mg^{2+} for isoprenoid transfer and Zn^{2+} for binding of protein substrate. Yeast GGTase I differs from FTase, since GGTase I can also function with Ca^{2+} as the only divalent cation (Mayer et al. 1992). Ca^{2+} probably binds to both Mg^{2+} and Zn^{2+} binding sites of the yeast GGTase I to mediate both isoprenoid transfer and substrate binding.

Among prenyltransferase mutants, a mutation in the GGTase I β subunit gene was the first to be isolated and characterized: *cal1-1* was identified originally as a mutation resulting in a Ca^{2+}-dependent phenotype (Ohya et al. 1984). The *cal1-1* mutant exhibits a homogeneous terminal phenotype with a G2/M nucleus and a small bud at 37° C (Ohya et al. 1984). Independent screening of yeast cell cycle mutants which accumulated as enlarged unbudded cells identified six other alleles, *cdc43-2* to *cdc43-7* (Adams et al. 1990). Yeast GGTase I is essential for yeast cell growth, since deletions in the *CAL1* gene result in a lethal phenotype (Ohya et al. 1991). However, GGTase I is no longer essential when the dosage of the two GTPases, Rho1p (Madaule et al. 1987; Qadota et al. 1994) and Cdc42p (Johnson and Pringle 1990), are artificially elevated (Ohya et al. 1993). Since the yeast GGTase I prenylates these two GTPases, Cdc42p and Rho1p are implicated genetically as the only two essential substrates of GGTase I (Ohya et al. 1993). *CAL1/CDC43* is necessary not only for the function of the small GTPases, but also for membrane localization of these proteins. An increase in soluble Cdc42p is observed in the *cdc43-2* strain grown at the restrictive temperature (Ziman et al. 1993).

This study was undertaken to elucidate the molecular lesions caused by the seven mutations available in the *CAL1/CDC43* gene. All of the mutation sites were determined at the nucleotide level. We examined, the activities of mutant proteins expressed in *E. coli*. We also examined the effect of the two essential GTPases on each mutant to determine whether the genetic interaction is allele specific.

Table 1 Yeast strains used in this study

Strain	Genotype	Reference
YPH500	*MATα ade2 his3 leu2 lys2 trp1 ura3*	Sikorski and Hieter (1989)
YOT159-3C	*MAT***a** *ade2 leu2 trp1 ura3 cal1-1*	Ohya et al. (1991)
CJ198-2D	*MATα his4 trp1 ura3 cdc43-2*	Adams et al. (1990)
YOT433-2C	*MATα leu2 lys2 trp1 ura3 cdc43-3*	This study
YOT434-2B	*MATα lys2 trp1 ura3 cdc43-4*	This study
YOT435-1A	*MATα ade2 his3 leu2 lys2 trp1 ura3 cdc43-5*	This study
YOT436-3C	*MAT***a** *ade2 his3 trp1 ura3 cdc43-6*	This study
YOT437-2C	*MAT***a** *ade2 his3 trp1 ura3 cdc43-7*	This study

Materials and methods

Materials

[^{3}H]Farnesyl diphosphate (15 Ci/mmol) and [^{3}H]geranylgeranyl diphosphate (15 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, Mo.). Chemical reagents were purchased from United States Biochemical (Cleveland, Ohio) and Sigma (St. Louis, Mo.). Restriction endonucleases were purchased from New England Biolabs (Beverly, Mass.). GF/F filters were obtained from Whatman. BioSafe II scintillation cocktail was from Research Products International (Mount Prospect, Ill.) Hyperfilm-MP was purchased from Amersham. Construction of Ras-CaaX mutants and Ras protein purification is described elsewhere (Moores et al. 1991).

YPD medium contained 1% Bacto-yeast extract (Difco Laboratories, Detroit, Mich.), 2% polypeptone (Nihon Chemicals, Osaka), and 2% glucose (Wako Chemicals, Tokyo). YPD supplemented with 100 mM or 300 mM $CaCl_2$ was used as Ca^{2+}-rich medium. Other standard media are described elsewhere (Rose et al. 1990).

Bacterial and yeast strains

E. coli strains DH5α (Bethesda Research Laboratories, Gaitherburg, Md.) and SCS1 (F^-, *recA1*, *endA1*, *gyrA96*, *thi*, *hsd17*, *supE44*, *relA1*, $lambda^-$; an uncharacterized mutation improves transformation efficiency) (Stratagene, San Diego, Calif.) were used for propagation of plasmids. Strain XL1-Blue (Stratagene) and helper phage M13KO7 (Vieira and Messing 1987) were used for preparation of single-stranded DNAs (Sambrook et al. 1989). Yeast strains used in this study are listed in Table 1. The alkaline method (Ito et al. 1983; Schiestl and Gietz 1989) was used for yeast transformation.

DNA manipulations

DNA fragments containing the *cdc43* mutations were cloned by gap repair (Orr-Weaver et al. 1983). The pCAL-F9 plasmid containing the 2.8-kb *Sph*I-*Pst*I fragment of the *CAL1/CDC43* gene was digested with *Nsp*(7524)V and *Eco*T22I and introduced into the *cdc43* strains (*cdc43-2* to *cdc43-7*). Transformation of the plasmid containing the *Nsp*(7524)V-*Eco*T22I gap resulted in gap repair yielding plasmids in which the gap had been repaired by gene conversion with the chromosomal sequences. The gap-repaired plasmids were recovered from yeast, and the *Nsp*(7524)V-*Eco*T22I fragments were subcloned into the *Nsp*(7524)V-*Eco*T22I gap of pCAL-F9. The resulting plasmid (YCpT-cdc43-2 to YCpT-cdc43-7 were introduced into the *cdc43* strains. Because the transformants showed a temperature-sensitive phenotype, we concluded that all of the *cdc43* mutations resided within the region between the *Nsp*(7524)V and *Eco*T22I sites. Nucleotide sequencing of the 1.0-kb *Nsp*(7524)V-*Eco*T22I fragment from the plasmids YCpT-cdc43-2 to YCpT-cdc43-7 revealed that each of the *cdc43* mutants had a single basepair change within the ORF.

The β subunit of the yeast GGTase I enzyme, Cal1p/Cdc43p, was expressed in *E. coli* using the plasmid pMLM4-3 (Mayer et al. 1992). The alpha subunit, Ram2p, was expressed from the chloramphenicol-resistant plasmid pBH57 (He et al. 1991).

pMLM4-3 (Term257) was made by eliminating the unique *Nco*I site in pMLM4-3. The plasmid DNA was digested with the restriction enzyme *Nco*I, followed by treatment with T4 DNA polymerase and dNTPs to fill-in the overhangs. The blunted plasmid was resealed with T4 DNA ligase, resulting in a frame shift in the *CAL1* reading frame causing premature termination after codon 257.

The *cal1-1* and *cdc43-2*, *3*, *4*, *5*, *6* and *7* mutations were introduced into the pMLM4-3 (-Term257) plasmid by replacement of the *Asu*II–*Nsi*I fragment of the wild-type *CAL1/CDC43* gene with the same fragment from each mutant. The absence of the *Nco*I site in the vector allowed identification of the subcloned mutations.

Epitope tagging of the Ram2p α subunit facilitated purification of GGTase I upon coexpression of the respective β subunit genes. A polyhistidine tag (6 × His) was introduced into the 5′-region of the *RAM2* gene by the insertion of the synthetic linker sequence 5′-CTCGAGTGGTGATGGTGATGGTGCGGTACC-3′ into the *Kpn*I and *Xho*I sites of pBH57.

pYO920 is an expression plasmid for haemagglutamin (HA)-tagged versions of Cdc42p. A DNA fragment encoding HA-tagged Cdc42p was amplified by PCR with 5′-GGGGAATTCATGTACCC ATACGACGTC-3′ and 5′-GGAAGATCTCTACAAAATTGTA CATTT TTT-3′ as primers, and pRS316-HA^2-CDC42 (a gift of Kazuma Tanaka) as a template. After digestion with *Eco*RI and *Bgl*II, the amplified fragment was inserted into the *Eco*RI–*Bgl*II gap of pYO773 (Qadota et al. 1994) to make pYO920.

GGTase I protein expression

Functional GGTase I was made by co-expression of the Ram2p subunit with the Cal1p/Cdc43p subunit in *E. coli* (Mayer et al. 1992). Aliquots (0.5 μg) of pBH57 and pMLM4 were co-transformed into the *E. coli* strain HB101. Freshly transformed cells were scraped into Luria Broth containing 100 μg/ml ampicillin and 10 μg/ml chloramphenicol, to give a starting OD_{660} of 0.2. The culture was incubated at 37° C with vigorous aeration to an OD_{660} of 0.4, at which time protein expression was induced by the addition of 0.5 mM IPTG. After an additional 4-h incubation, the culture was harvested by centrifugation at 3000 × *g*. The cell pellet was resuspended in ice-cold lysis buffer [50 mM TRIS-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA. 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM leupeptin, 0.1 μM pepstatin A] and lysed by sonication with 4 × 30-s bursts using a Fisher Model 300 sonicator at 35% power. Lysates were clarified by centrifugation at 14 000 × *g*, after which the supernatants were assayed for geranylgeranyltransferase activity.

Enzyme purification

The *CDC43* and *cdc43-6* GGTase I enzymes were purified using affinity chromatography specific for the polyhistidine tag on the Ram2p subunit of the heterodimer. The presence of the polyhistidine tract allows purification on Ni-NTA agarose. The *CDC43* and *cdc43-6* β subunits were co-expressed in *E. coli* with the 6 × His-Ram2p alpha subunit expressed from pBH57-6 × His. The cells were lysed as described above in 50 mM NaH_2PO_4 pH 7.0, 300 mM NaCl and the protease inhibitors listed above. The lysate was clarified by centrifugation at 14 000 × *g* and the supernatant removed to a 50-ml polypropylene conical tube. To the supernatant fraction (5 ml) was added 4 ml of Ni-NTA agarose beads and the mixture slowly rotated for 2 h at 4° C. The beads were washed in batch twice with 50 ml of buffer and transferred to a 10-ml Biorad Econo-column. Using a peristaltic pump, the beads were washed with an additional 5 ml of buffer at a flow rate of 0.2 ml/min. The GGTase I enzymes were eluted with a 0–250 mM imidazole gradient in 24 ml. Because a number of metal-binding *E. coli* proteins co-purify with the enzyme on Ni-NTA agarose, it was not possible to quantitate the amount of GGTase purified, other than by activity measurements.

Enzyme assays

Geranylgeranyltransferase assays were carried out as previously described (Mayer et al. 1992; Moomaw and Casey 1992; Seabra et al. 1991). Each individual assay consisted of 50 mM HEPES pH 7.5, 20 mM $MgCl_2$, 5 mM DTT, 5 μM Ras protein and 34 or 67 pmol [^{3}H]geranylgeranyl diphosphate or 67 pmol [^{3}H]farnesyl diphosphate in a reaction volume of 0.05 ml. After equilibrating the

reaction mixture at 29° C for 2 min, 12 units of geranylgeranyltransferase protein or 50 μg of *E. coli* lysate were added. For the standard assay the reaction was terminated after 40 min by the addition of 1 ml of 1 M HCl in ethanol. The quenched reactions were allowed to stand for 15 min at room temperature prior to slow filtration through Whatman GF/F filters. Filters were washed four times with 2 ml of 100% ethanol dried, mixed with 5 ml of Biosafe II scintillation fluid, and then assayed for radioactivity in a Beckman LS3801 scintillation counter. When necessary, reactions were analyzed by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis; labeled proteins were visualized by fluorography following gel treatment with Fluorohance (Research Products International).

Production of the anti-Rho1p antibody

The purified GST-Rho1p (64–209) which consists of GST fused to residues 64 to 209 of Rho1p was minced and emulsified with R-700 (RIBI ImmunoChem Research, Hamilton, Mont.) and the resulting emulsion was used to immunize four guinea pigs. Five booster injections were administered at three-week intervals; three weeks later blood was collected from the animals and one of the immune sera was used in this study. The anti-Rho1p antibody specifically recognized Rho1p. Western blotting analysis showed that there no other protein band was detectable with the antibody in the lysates of cells expressing human *rhoA* instead of *RHO1*.

Cell fractionation experiments

Cell fractionation experiments were performed using techniques described by Ziman et al. (1993). Briefly cells were grown at 23° C to mid log phase, and approximately 5×10^8 cells were collected, washed with water, resuspended in 0.1 ml of lysis buffer (0.8 M sorbitol, 1 mM EDTA, 10 mM HEPES pH 7.0) with 0.5 mM PMSF, and lysed on ice by vortexing with acid-washed glass beads (400–500 μm in diameter; Sigma). Greater than 80% lysis was verified by light microscopy. After addition of 0.4 ml of lysis buffer, cell lysates were spun at $390 \times g$ for 1 min at 4° C. The supernatant was then spun at $436\,000 \times g$ for 20 min at 4° C, and the pellets were resuspended in the same volume of lysis buffer. To assess the relative amounts of Rho1p and Cdc42p in each fraction, equal volumes of each fraction were loaded onto a sodium dodecyl sulfate-12.5% polyacrylamide gel for immunobloting analysis. Guinea pig polyclonal antibody against Rho1p and mouse monoclonal antibody against HA (12CA5; Boehringer, Mannheim Germany) were used at 1 : 500 and 1 : 100, respectively. Alkaline phosphatase-conjugated goat anti-guinea pig IgG and anti-mouse IgG were used at 1 : 5000. Antibody-antigen complexes were detected with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium.

Results

Localization of the mutations *cdc43-2* to *cdc43-7*

DNA fragments containing the mutations *cdc43-2* to *cdc43-7* were cloned by the gap-repair method (Orr-Weaver et al. 1983) to yield YCpT-cdc43-2 to YCpT-cdc43-7. Based on the subcloning analysis (see Materials and methods), we concluded that all of the *cdc43* mutations reside within the 1.0-kb region between the restriction sites *Nsp*(7524)V and *Eco*T22I, which encompasses nearly the entire coding region of *CAL1/CDC43*. Nucleotide sequencing of the 1.0-kb *Nsp*(7524)V-*Eco*T22I fragment from the YCpT-cdc43-2 to YCpT-cdc43-7 revealed that each of the *cdc43* mutants had a single basepair change within the ORF. Figure 1 shows the amino acid alterations in the *cdc43* sequences. *cdc43-4* and *cdc43-6* resulted from identical nucleotide changes, and are hereafter referred to as *cdc43-6*. *cdc43-5* had a change at the same position as *cdc43-4* and *cdc43-6*, but resulted in a different amino acid. Figure 1 shows that the four *cdc43/cal1* mutations (*cdc43-5*, *cdc43-6*, *cdc43-7*, *cal1-1*) mapped within the domain homologous to the β-subunits of other protein isoprenyltransferases (positions 159–350). Interestingly enough, these mutations affect amino acid residues that are conserved among the β subunits of GGTase I from four different species (Diaz et al. 1993; Ohya et al. 1991; Zhang et al. 1994). The other two *cdc43* mutations (*cdc43-2* and *cdc43-3*) mapped outside of the homologous domain.

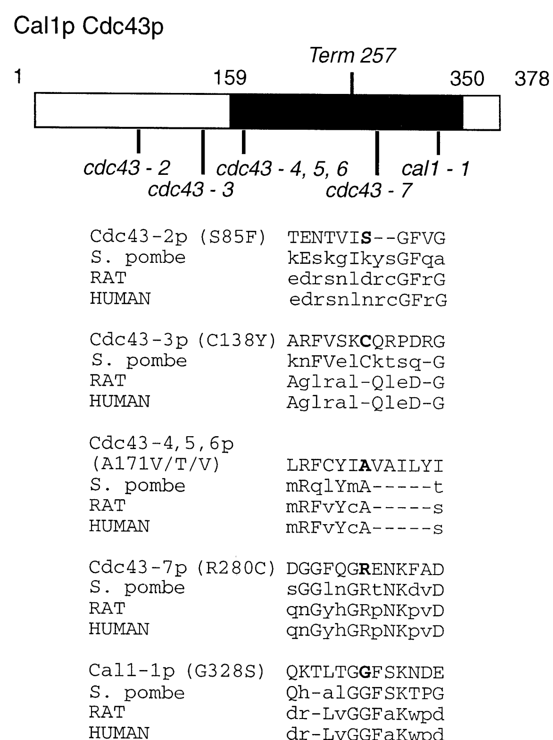

Fig. 1 Alignment of the β-subunits of GGTases I, showing positions of *cal1/cdc43* mutations. Positions of the *cal1/cdc43* mutations are shown *under the bar* representing the *CAL1/CDC43* coding region. The *filled bar* represents the homologous region common to the β-subunits of protein isoprenyltransferases. The CLUSTER program was used to align Cal1p with the β-subunits of the *S. pombe*, rat and human GGTase Is near the *cal1/cdc43* mutation points

Relative GGTase I activity of *cdc43/cal1* mutants

In order to elucidate the biochemical effects caused by the *cal1/cdc43* mutations, we examined the relative GGTase I activity of each of the *cal1/cdc43* mutants. The effects of the *cal1-1* and *cdc43* mutations upon

GGTase I activity were initially assessed by reconstituting the holoenzyme by co-expression of Ram2p with each mutant Cal1p/Cdc43p subunit in *E. coli* at the permissive temperature. The ability of each mutant GGTase I, in crude extracts, to transfer tritiated geranylgeranyl to Ras-CIIL was measured (Fig. 2). As determined by fluorography, each enzyme was severely impaired in its ability to specifically geranylgeranylate the protein substrate, even at the permissive temperature. Weak activity was also detected for the GGTase I reconstituted with the codon-257 termination mutant of *CDC43*. However, the activity of the truncated Cal1p/Cdc43p subunit was too weak to be measured by the filter binding assay.

The *cal1-1* mutation was originally isolated using a screen for mutants requiring high calcium for growth. We have observed that calcium can substitute for the zinc requirement of the GGTase I, suggesting that the *cal1-1* transferase might have been sufficiently altered as to require calcium in place of zinc as a cofactor. To test this hypothesis, 5 and 20 mM $CaCl_2$ was included in the transferase assays containing the *cal1-1* GGTase I. No detectable activity was measured for the *cal1-1* enzyme in the presence or absence of $CaCl_2$.

Effects of expression and assay temperature on mutant GGTase-I activity

The initial activity measurements for each *cal1/cdc43* GGTase I were made on crude extracts obtained from *E. coli* cells expressing each mutant gene at the permissive temperature. In order to determine if the activity of each mutant GGTase I was temperature sensitive, each mutant protein was expressed in *E. coli* grown at both 25° C and 35° C. Crude extracts made from each expressing culture were assayed for GGTase I activity at both 25° C and 37° C (see Table 2). As suggested by the initial characterization, each *cal1/cdc43* mutant synthesized an impaired GGTase I, regardless of whether expression was at the permissive or restrictive temperature. In every case, enzymatic activity was increased when the assay temperature was increased from 25 to 35° C. The results of the filter binding assay were confirmed by the analysis of a portion of each assay mixture by SDS-PAGE and fluorography to confirm weak geranylgeranylation of Ras by the *cdc43-Nco*(*term 257*), *cdc43-2*, *cdc43-3*, *cdc43-4*, *cdc43-5* and *cdc43-7* mutants. No geranylgeranyltransferase activity was ever observed for the *cal1-1* GGTase I.

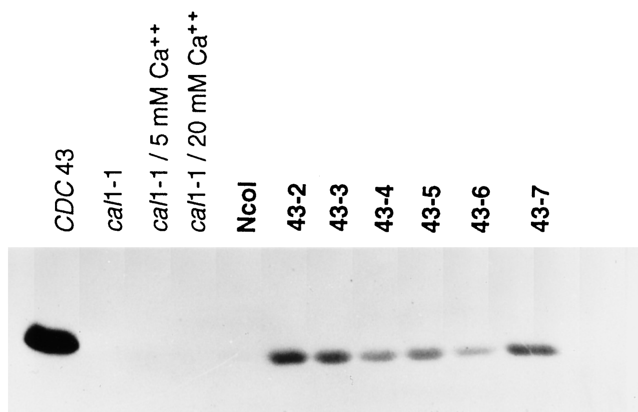

Fig. 2 Autoradiograph showing the ability of wild-type or each mutant GGTase I to transfer tritiated geranylgeranyl to Ras-CIIL. The wild-type or mutant β subunit of yeast GGTase I was expressed in *E. coli*, together with the wild-type α-subunit. Each recombinant enzyme was assayed for geranylgeranyltransferase activity in crude cell lysates. The product of the *cal1-1* mutant was assayed in the presence of added calcium. *Nco*I indicates the codon-257 termination mutant, which shows weak activity

Comparison of partially purified wild-type and Cdc43-6 GGTase I

To facilitate comparison of the mutant and wild-type GGTase I, both the Cdc43p::Ram2p and Cdc43-6p::Ram2p enzymes were partially purified. Each GGTase-I was then assayed for the ability to geranylgeranylate Ras-CIIL as a function of time and temperature (see Fig. 3A, B). Other than a twenty-fold difference in the quantity of isoprenoid transferred, the wild-type *CDC43* and mutant *cdc43-6* enzymes were nearly identical in their temperature optima and time dependence. This is additional evidence that, in the case of *cdc43-6*, no temperature-dependent inactivation of the enzyme is observed. Similar results were obtained for the partially purified *cdc43-2* enzyme.

Overproduction of Rho1p in *cdc43* mutants

Rho1p is one of the essential substrates of GGTase I. We have previously shown the functional interaction between *RHO1* and *CAL1* based on the observation that

Table 2 Activities of mutant *cal1/cdc43* GGTases I expressed in *E. coli*

Expression temperature	25° C		35° C	
	Assay temperature			
Mutant	25° C	35° C	25° C	35° C
CDC43 (wild-type)	523	1529	995	1787
cal1-1	0	21	9	13
cdc43-term 257	0	74	39	120
cdc43-2	20	85	34	78
cdc43-3	36	111	18	133
cdc43-5	0	153	123	425
cdc43-6	0	122	57	160
cdc43-7	0	122	23	158

Activities are expressed as fmol geranylgeranyl transferred to the substrate Ras-CIIL

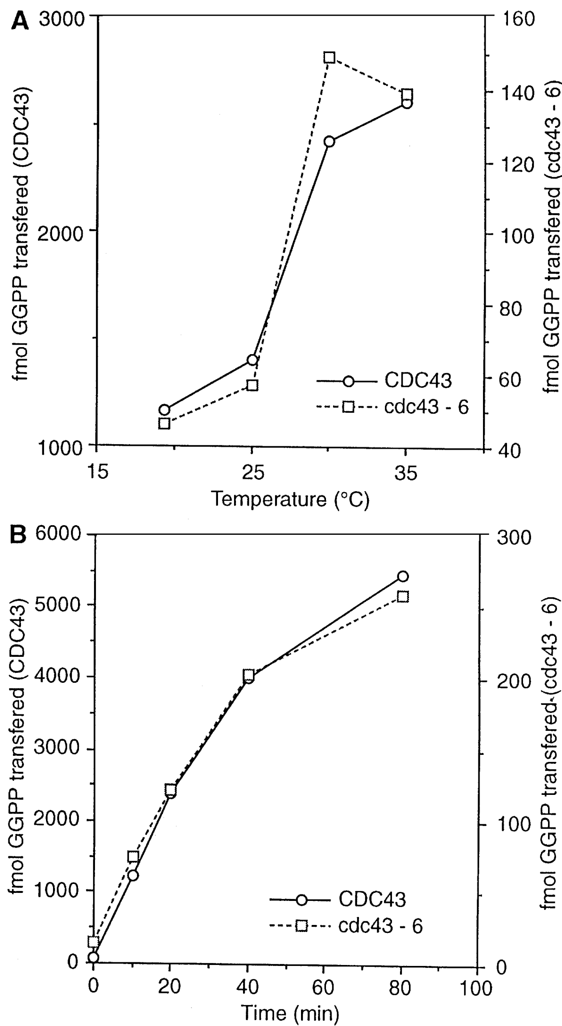

Fig. 3A, B Geranylgeranyltransferase activity of the wild-type and *cdc43-6* enzymes as a function of time (**A**) and temperature (**B**)

overproduction of Rho1p suppressed the temperature sensitivity of *cal1-1* (Qadota et al. 1992). In order to determine whether this suppression by overproduction of Rho1p is seen only with the *cal1-1* allele, we examined the effect of overproduction of Rho1p on the phenotypes of the *cdc43* mutations. Since the restrictive temperatures for the *cdc43* mutants differ, effects of the Rho1p overexpression were examined at five different temperatures (23° C, 28° C, 30° C, 33° C and 37° C). We found that the *cdc43* mutations were not suppressed effectively by overproduction of Rho1p (Table 3). None of the *cdc43* mutations was suppressed at 37° C, while *cal1-1* was suppressed at this temperature. The *cdc43-2* and *cdc43-7* strains carrying multicopy *RHO1* grew slightly faster than those with vector alone at 30° C, while *cal1-1* was suppressed completely at this temperature. Slight improvement in growth of *cdc43-5*, on overproduction of Rho1p, was observed only at 23° C. These results indicate that among the *cal1/cdc43* mutations so far isolated, *cal1-1* is unique in that is effectively suppressed by overproduction of Rho1p.

Overproduction of Cdc42p in the *cal1/cdc43* mutants

Since overproduction of Rho1p suppressed a mutation of the *CAL1/CDC43* gene, we next attempted to examine multicopy suppression of the *cdc43* mutations by overproduction of another essential substrate of GGTase I, Cdc42p. We found that overproduction of Cdc42p suppressed the temperature-sensitive phenotype of *cdc43-5* (Table 3); the *cdc43-5* mutant with multiple copies of *CDC42* grew well at 37° C. Among the *cdc43* mutations, *cdc43-5* was most effectively suppressed by overproduction of Cdc42p; *cdc43-3*, *cdc43-6* and *cdc43-7* were suppressed slightly by overproduction of Cdc42p at the intermediate temperature, and *cdc43-2* was not suppressed at all at any temperature examined.

Several attempts to introduce multiple copies of *CDC42* into the *cal1-1* strain were unsuccessful (data not shown). Reasoning that overexpression of Cdc42p might be lethal to the *cal1-1* strain, we attempted to increase the levels of Cdc42p by placing its expression under the control of the *GAL1* promoter, which is induced by galactose in the medium. The *cal1-1* strain with pGAL-CDC42 could grow on solid media containing glucose but did not grow on media containing galactose (Fig. 4). This growth inhibition was observed at all temperatures examined (23° C, 30° C and 37° C). Since pGAL-CDC42 was not toxic to the wild-type strain or to many other *cdc43* mutants (Fig. 4), we concluded that the lethality caused by the overexpression of Cdc42p is specific to the *cal1-1* mutant. Although *CDC42* on a multicopy plasmid is not toxic in *cdc43-7*, pGAL-CDC42 is dereterious in *cdc43-7* (Fig. 4). This may be due to the fact that the expression level of Cdc42p from pGAL-CDC42 is higher than that attained by expression from multiple copies of *CDC42*.

We have previously shown that co-overexpression of Rho1p and Cdc42p suppresses the lethality caused by disruption of the *cal1/cdc43* gene required for most prenylation of Rho1p and Cdc42p. Co-overexpression of Rho1p and Cdc42p also suppressed the temperature-sensitive phenotype of all *cdc43* mutations (data not shown). Since multiple copies of *CDC42* are toxic in *cal1-1* cells, we examined whether increased levels of Rho1p suppress toxicity caused by overproduction of Cdc42p in the *cal1-1* mutant. Rho1p was overexpressed under the control of the inducible *GAL1* promoter. The *cal1-1* strain containing pGAL-RHO1 and multiple copies of *CDC42* grew on solid media containing galactose, but not glucose (Fig. 5), indicating that lethality of *cal1-1* caused by overexpression of Cdc42p is suppressed by overproduction of Rho1p.

Fractionation of Rho1p and Cdc42p in mutant strains

cal1-1 was suppressed most effectively by overexpression of Rho1p, while *cdc43-5* was suppressed by

Table 3 Effect of overproduction of Rho1p and Cdc42p in the *cal1/cdc43* mutants

Growth medium		YPD					YPD + Ca[a]	
Strain	Plasmid	23° C	28° C	30° C	33° C	37° C	33° C	37° C
cal1-1	pYO324	+	+	±	−	−	+ +	+
	YCpT-CAL1	+ +	+ +	+ +	+ +	+ +	+ +	+ +
	YEpT-RHO1	+ +	+ +	+ +	+ +	+	+ +	+G
	YEpT-CDC42	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
cdc43-2	pYO324	+ +	+	±	−	−	−	−
	YCpT-CAL1	+ +	+ +	+ +	+ +	+ +	+ +	+ +
	YEpT-RHO1	+ +	+	+	−	−	−	−
	YEpT-CDC42	+ +	+	±	−	−	−	−
cdc43-3	pYO324	+ +	+ +	+ +	±	−	−	−
	YCpT-CAL1	+ +	+ +	+ +	+ +	+ +	+ +	+ +
	YEpT-RHO1	+ +	+ +	+ +	±	−	−	−
	YEpT-CDC42	+ +	+ +	+ +	±	−	+	−
cdc43-5	pYO324	+	±	−	−	−	−	−
	YCpT-CAL1	+ +	+ +	+ +	+ +	+ +	+ +	+ +
	YEpT-RHO1	+ +	±	−	−	−	−	−
	YEpT-CDC42	+ +	+ +	+ +	+ +	+	+ +	+
cdc43-6	pYO324	+ +	+	±	−	−	−	−
	YCpT-CAL1	+ +	+ +	+ +	+ +	+ +	+ +	+ +
	YEpT-RHO1	+ +	+	±	±	−	−	−
	YEpT-CDC42	+ +	+	±	−	−	−	−
cdc43-7	pYO324	+ +	+	±	−	−	±	−
	YCpT-CAL1	+ +	+ +	+ +	+ +	+ +	+ +	+ +
	YEpT-RHO1	+ +	+	+	−	−	±	−
	YEpT-CDC42	+ +	+	±	−	−	+	−

[a] YPD medium supplemented with 100 mM $CaCl_2$
n.d., not determined

Table 4 Summary of the effect of the GTPases Cdc42p and Rho1p on the *cal1/cdc43* mutants

Phenotype	Protein overproduced	
	Cdc42p	Rho1p
Suppression	*cdc43-5* (*cdc43-3*, *4*, *7*)	*cal1-1* (*cdc43-2*, *5*, *7*)
Enhancement	*cal1-1* (*cdc43-7*)	

overexpression of Cdc42p. To test the possibility that the allele-specific suppression is due to the substrate specificity of the mutant GGTase I, we examined the partitioning of Rho1p and Cdc42p in the *cal1-1* and *cdc43-5* mutant strains. It has already been shown that soluble Cdc42p levels increase in the *cdc43-2* strain grown at the restrictive temperature (Ziman et al. 1993), suggesting that membrane localization of small GTPases is dependent on the geranylgeranyl modification. We found that the proportion of Rho1p in the soluble fraction of *cal1-1* increases dramatically after the temperature shift (Fig. 6). Rho1p in the *cdc43-5* strain grown at 37° C for 2 h was present almost exclusively in the particulate fraction, indicating that the increase in soluble Rho1p is specific to *cal1-1*. The proportion of HA-tagged Cdc42p found in the soluble fraction of *cdc43-5* increased after a 2-h incubation at 37° C, while *cal1-1* did not affect partitioning of HA-tagged Cdc42p (Fig. 6). The temperature shift itself did not affect the partitioning of these GTPases in the wild-type control strain. These results suggested that *cal1-1* and *cdc43-5* specifically impair geranylgeranylation of Rho1p and Cdc42p, respectively.

Crude preparations of recombinant *cal1-1* and *cdc43-5* geranylgeranyl transferase I were tested for altered specificity using Ras proteins containing the isoprenylation sequences of Cdc42p (CTIL) and Rho1p (CVLL). To mimic the growth conditions under which genetic complementation was tested, each enzyme was expressed in *E. coli* and assayed at the restrictive growth temperature. Under conditions where wild-type geranylgeranyl transferase I modified Ras-CTIL and Ras-CVLL, little detectable geranylgeranylation of these substrates by the mutant proteins was observed.

Discussion

Seven mutations have been isolated in the gene encoding the β subunit of the geranylgeranyl transferase I,

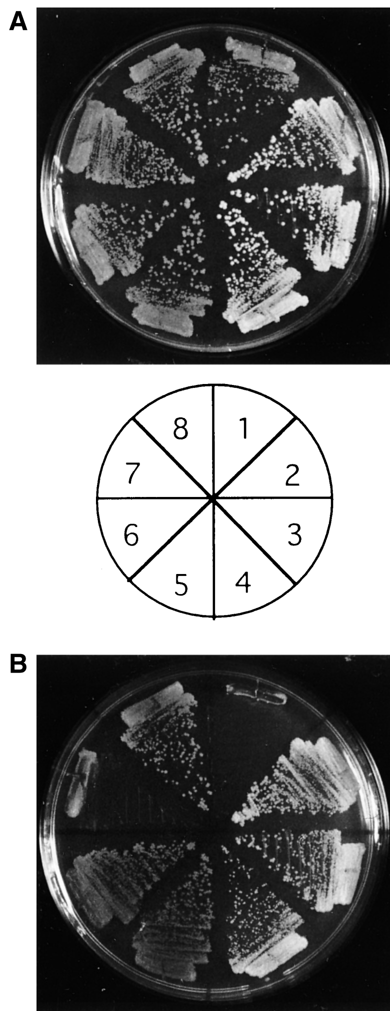

Fig. 4A, B Overproduction of *CDC42* is toxic to *cal1-1* cells. *cal1-1* (sector 1), *cdc43-2* (2), *cdc43-3* (3), *cdc43-4* (4), *cdc43-5* (5), *cdc43-6* (6), *cdc43-7* (7) and the wild-type strain (8) harboring pGAL-CDC42 were streaked on a plate containing glucose (**A**) or galactose (**B**), and incubated at 23° C for 1 week

resulting in two different phenotypes; the *cal1-1* mutation arrests in the cell cycle at G2/M with a small bud (Ohva et al. 1984), whereas all of the other temperature-sensitive *cdc43* mutants (*cdc43-2* to *cdc43-7*) undergo arrest as enlarged unbudded cells (Adams et al. 1990). We present a molecular basis for the phenotypes caused by the *cal1/cdc43* mutations. First, the positions of all the *cdc43* mutations were mapped in the *CAL1/CDC43* sequence. Second, the biochemical properties of the mutant forms of GGTase I were analyzed using recombinant prenyltransferases expressed in *E. coli*. Third, in vivo interactions of GGTase I with its protein substrates were assessed by a genetic approach. Fourth, the membrane localization of the protein substrates was analyzed in the GGTase I mutants by cell fractionation.

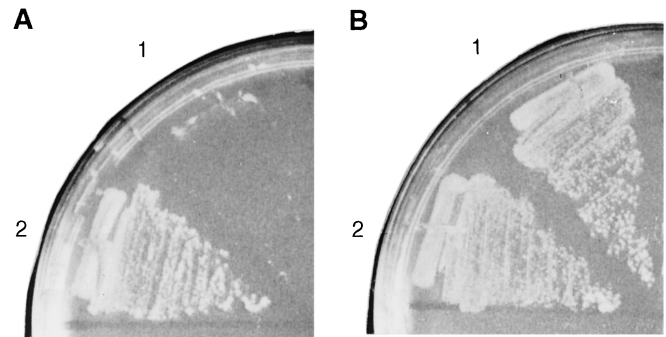

Fig. 5A, B Suppression of *cal1-1* by co-overexpression of *CDC42* and *RHO1*. *cal1-1* cells harboring pGAL-RHO1 plus YEpT-CDC42 (sector 1) and YEp-CAL1 (2) were streaked on a plate containing glucose (**A**) or galactose (**B**), and incubated at 23° C for 1 week

Examination of GGTase I in crude extracts revealed that all of the mutant enzymes showed a reduced activity to geranylgeranylate a protein substrate, even at the permissive temperature. Unfortunately, we have not been successful in obtaining an antibody sensitive enough to determine the relative expression levels of each GGTase I. Hence, the decreases in activity, noted might be due to protein instability rather than to inactivation of the active site. Nonetheless, it is of interest to note that *cdc43-5*, *cdc43-6*, *cdc43-7* and *cal1-1* mutations change amino acid residues conserved among β subunits of GGTase I from different sources. Therefore, these amino acid residues appear to be important for protein folding or for the enzymatic activity of the β subunit of prenyltransferases. The *cdc43-6* GGTase I itself in fact shows dramatically reduced transferase activity, based on the analysis of partially purified enzyme. These biochemical results with recombinant enzymes are consistent with data from experiments in which we examined GGTase I activity in *cdc43-2* yeast grown at permissive temperature and found no detectable activity.

Although the growth of *cal1-1* and other *cdc43* mutants is temperature sensitive, our biochemical results have indicated that the activities of the GGTase mutants are not heat labile. The enzymatic activities of the *cdc43* GGTase I, as well as the wild-type GGTase I, increased when the assay temperature was shifted from 25 to 35° C. In addition, the relative activities and *CaaX* specificities of the GGTase I mutants did not change with the temperature at which the *E. coli* cells were cultured. This indicates that neither enzymatic activity nor protein folding of the mutant GGTase I is heat sensitive. Nonetheless, the proportion of the GTPases found in the soluble fractions increased in the GGTase I mutant yeast cells, suggesting temperature-sensitive geranylgeranylation of the substrate GTPases in vivo (Fig. 6). Taking all, into account, we suggest

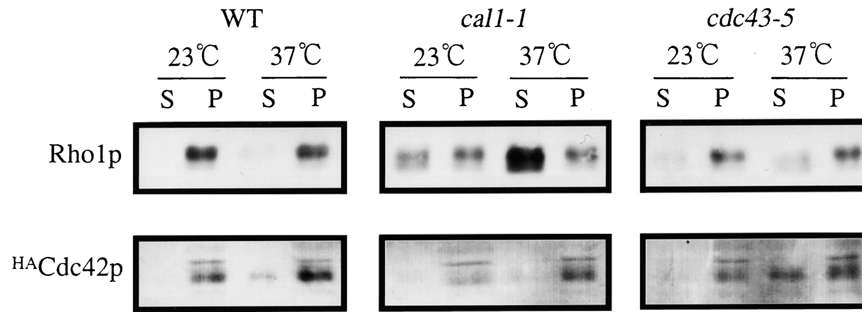

Fig. 6 Subcellular localization of Rho1p and Cdc42p in wild-type and mutant strains. Yeast strains were grown to mid-log phase at the permissive temperature (23° C), shifted to the restrictive temperature and collected after 2 hr at 37° C; then cell lysates were prepared. Rho1p was detected by Western blotting analysis with a polyclonal guinea pig antibody against Rho1p. In order to express a HA-tagged version of Cdc42p, yeast strains transformed with pYO920 were incubated at 23° C in 2% galactose-containing medium for 6 h before the temperature shift. The HA-tagged version of Cdc42p was detected by Western blotting analysis with the antibody 12CA5. WT, YPH500; *cal1-1*, YOT159-3C; *cdc43-5*, YOT435-1A

that the yeast prenylation pathway itself is temperature-sensitive, although other mechanisms could still account for the temperature-sensitive growth of the *cdc43* mutants.

Multiple copies of either Rho1p or Cdc42p phenotypically suppressed specific alleles of *cal1/cdc43* (Table 4): *cal1-1* was suppressed effectively by multicopy *RHO1*, while *cdc43-5* was suppressed effectively by multicopy *CDC42*. Given that Rho1p and Cdc42p accumulate in the soluble fraction in the *cal1-1* strain and the *cdc43-5* strain, respectively, the substrate specificity of the mutant GGTase I probably accounts for the allele-specific suppression. Our inability to detect variations in substrate specificity for these mutants in vitro may be a function of either low enzyme expression levels, or reflect requirement for additional recognition sequences not present in Ras (Kalman et al. 1995). In our current model, *cal1-1* and *cdc43-5* selectively impair the in vivo geranylgeranylation of Rho1p and Cdc42p, respectively. This is consistent with observation of the mutant phenotypes; the terminal phenotypes of *cdc43-5* and *cdc42* are indistinguishable, and those of *cal1-1* and temperature-sensitive *rho1* strains are somewhat similar. This is also consistent with our observation that overexpression of Cdc42p is specifically lethal in the *cal1-1* strain, because over-expression of Cdc42p probably leads to sequestration of the *cal1-1* GGTase I, further impairing geranylgeranylation of Rho1p. Taken together, our genetic results suggest that the *CAL1/CDC43* GGTase I has an ability to prenylate the substrate GTPases by some domain-specific and substrate-specific recognition mechanisms.

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