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Genetic Evidence for In Vivo Cross-Specificity of the CaaX-Box Protein Prenyltransferases Farnesyltransferase and Geranylgeranyltransferase-I in *Saccharomyces cerevisiae*

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Two protein prenyltransferase enzymes, farnesyltransferase (FTase) and geranylgeranyltransferase-I (GGTase-I), catalyze the covalent attachment of a farnesyl or geranylgeranyl lipid group to the cysteine of a CaaX sequence (cysteine [C], two aliphatic amino acids [aa], and any amino acid [X]). In vitro studies reported here confirm previous reports that CaaX proteins with a C-terminal serine are farnesylated by FTase and those with a C-terminal leucine are geranylgeranylated by GGTase-I. In addition, we found that FTase can farnesylate CaaX proteins with a C-terminal leucine and can transfer a geranylgeranyl group to some CaaX proteins. Genetic data indicate that FTase and GGTase-I have the same substrate preferences in vivo as in vitro and also show that each enzyme can prenylate some of the preferred substrates of the other enzyme in vivo. Specifically, the viability of yeast cells lacking FTase is due to prenylation of Ras proteins by GGTase-I. Although this GGTase-I dependent prenylation of Ras is sufficient for growth, it is not sufficient for mutationally activated Ras proteins to exert deleterious effects on growth. The dependence of the activated Ras phenotype on FTase can be bypassed by replacing the C-terminal serine with leucine. This altered form of Ras appears to be prenylated by both GGTase-I and FTase, since it produces an activated phenotype in a strain lacking either FTase or GGTase-I. Yeast cells can grow in the absence of GGTase-I as long as two essential substrates are overexpressed, but their growth is slow. Such strains are dependent on FTase for viability and are able to grow faster when FTase is overproduced, suggesting that FTase can prenylate the essential substrates of GGTase-I when they are overproduced.

Protein prenylation is a posttranslational modification involving the covalent attachment of a prenyl lipid to a protein. In all known cases of prenylation, a 15-carbon farnesyl group or a 20-carbon geranylgeranyl group is attached via a thioether linkage to a cysteine at or near the C terminus of the protein. Protein prenylation was discovered over 10 years ago as a modification of peptide mating pheromones produced by two jelly fungi (46, 75) and has been studied extensively in recent years following the discovery that Ras proteins (including the oncogenic activated forms of Ras) require prenylation for membrane localization and efficient function. A number of reviews on protein prenylation are available (16, 22, 31, 79).

The 15-carbon farnesyl group is attached to mating pheromones from several fungi (3, 19, 46, 75), nuclear lamins (85, 89), Ras proteins (13, 36), gamma subunits of some heterotrimeric G proteins (28, 29, 53), and several proteins in the vertebrate visual system (2, 42, 43, 53, 68). All known farnesylated proteins share a C-terminal sequence feature known as a CaaX motif. The term CaaX was used originally to describe the sequence cysteine-aliphatic amino acid-aliphatic amino acid-any amino acid at the C termini of proteins. This term persists in the literature although it is not a completely accurate way to describe the C-terminal motifs that specify prenylation by farnesyltransferase (FTase) and geranylgeranyltransferase-I (GGTase-I). Prenylated proteins have a restricted set of amino acids in the C-terminal or X position, and some prenylated proteins have a nonaliphatic amino acid following the cysteine. In the case of farnesylated proteins, the C-terminal amino acid (X) is serine, methionine, cysteine, glutamine, or alanine. The processing steps have been well characterized for mammalian Ras proteins and for the *Saccharomyces cerevisiae* mating pheromone a-factor. These proteins undergo farnesylation of cysteine, proteolytic removal of the three C-terminal amino acids, and methylation of the newly formed C terminus (3, 24, 35). Complete processing is required for the biological activity of a-factor (3, 58, 59). In contrast, farnesylation is required for plasma membrane localization and transforming activity of Ras, but proteolysis and methylation are not (39, 49). Ras farnesylation is not sufficient to specify localization to the plasma membrane; the amino acid sequence adjacent to the farnesylated cysteine, which includes either a cysteine that is palmitoylated or a sequence rich in basic residues, is also required for plasma membrane localization (37). Farnesylation of Ras is required for growth of *S. cerevisiae* when Ras levels are normal but is not absolutely required for Ras function, since overproduction of a mutated form of the Ras2 protein lacking the CaaX cysteine can provide enough Ras function to sustain growth (23). However, prenylation (or a genetically engineered alternative mode of membrane association such as myristoylation) does appear to be required for the transformed phenotype produced by activated Ras in mammalian cells in culture (36) and for the activated Ras phenotypes in *S. cerevisiae* cells (23, 67, 78).

The 20-carbon geranylgeranyl group is attached to the gamma subunit of several heterotrimeric G proteins (56, 62, 90) and to a number of low-molecular-weight GTP-binding proteins in the Ras superfamily: G25K (90), smg21B (50),

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and Krev-1 (50), also known as Cdc42Hs, Rap1B, and Rap1A, respectively. These geranylgeranylated proteins have a CaaX motif in which the C-terminal amino acid is leucine or, in the case of placental G25K, phenylalanine. A second class of geranylgeranylated proteins consists of the low-molecular-weight G proteins that are involved in secretion: Ypt1 and Sec4 in *S. cerevisiae* (73), YPT protein in *Schizosaccharomyces pombe* (63), and the homologous mammalian Rab proteins. These proteins terminate with cysteine-cysteine or cysteine-X-cysteine, and at least some of these proteins have a geranylgeranyl group attached to both cysteines (26).

Mammalian cells and *S. cerevisiae* have at least three protein prenyltransferase enzymes that catalyze the attachment of farnesyl or geranylgeranyl groups to proteins: FTase, GGTase-I, and GGTase-II (61). Rat brain FTase, a heterodimer composed of an α subunit and a β subunit, farnesylates substrates with a CaaX motif in which the C-terminal amino acid is serine, methionine, cysteine, or glutamine (69–71). In *S. cerevisiae*, genetic and biochemical data demonstrate that the FTase that prenylates Ras and **a**-factor mating pheromone is composed of two subunits encoded by the *RAM1* and *RAM2* genes (34, 38, 67, 80). The Ram2 subunit (encoded by the *RAM2* gene) is homologous to the mammalian FTase α subunit (38), and the Ram1 subunit (encoded by the *RAM1* gene) is homologous to the mammalian FTase β subunit (15, 34). *RAM1* is also known as *DPR1* (34) and *STE16* (88). Genetic studies indicate that the Ram1-FTase prenylates Ras1, Ras2, Ste18, and **a**-factor (28, 67, 80), which have CaaX motifs that terminate with cysteine, serine, methionine, and alanine, respectively. In vitro prenylation assays have demonstrated that Ram1-FTase can prenylate **a**-factor (80), Ras2 (33), and H-Ras (80), which terminates with serine.

The second protein prenyltransferase, GGTase-I, geranylgeranylates CaaX substrates with leucine or phenylalanine as the C-terminal amino acid (14, 91). In vitro studies with bovine brain GGTase-I and FTase demonstrate that although CaaX-containing peptides with a C-terminal leucine are preferentially geranylgeranylated by GGTase-I and CaaX-containing peptides with a C-terminal serine are preferentially farnesylated by FTase, each enzyme appears to be able to prenylate some of the preferred substrates of the other enzyme (91). Since this in vitro cross-specificity was observed with partially purified enzymes and high peptide concentrations, it is unclear whether these enzymes exhibit cross-specificity in vivo. *S. cerevisiae* GGTase-I activity has been reconstituted from Ram2 and Cdc43 produced in *Escherichia coli* (60), demonstrating that the enzyme is composed of an α subunit encoded by the *RAM2* gene and a β subunit encoded by the *CDC43* gene. *CDC43* is also known as *CAL1* (64, 65). The two β subunits, Cdc43 and Ram1, are 32% identical and assemble with the same Ram2 α subunit to form the GGTase-I and FTase, respectively. Genetic data suggest that the Cdc43–GGTase-I is responsible for prenylation of the Ras-related GTP-binding proteins Cdc42, Rho1, and Rho2 (1, 8, 66), which have CaaX motifs terminating with leucine. In vitro studies have demonstrated that Cdc43 is required for geranylgeranylation of fusion proteins with C-terminal sequences that terminate with CVLL and CIIL (33).

The third class of protein prenyltransferase, GGTase-II, geranylgeranylates proteins terminating with cysteine-cysteine (CC) or cysteine-X-cysteine (CXC) (52, 61, 73). GGTase-II in mammalian cells has recently been shown to require three subunits for activity (82). Two of the subunits are shared by the GGTase-II enzymes that act on proteins with CC and CXC, but there may be multiple forms of the third subunit, which is known as component A. Patients with the disease choroideremia (which causes retinal degeneration) are deficient in the form of component A that is required for geranylgeranylation of a CXC protein but not a CC protein (81). In *S. cerevisiae*, two subunits of GGTase-II have been identified; these subunits are homologous to the α and β subunits of FTase and GGTase-I. The *BET2* gene encodes a protein that is 34% identical to Ram1. Bet2 is required for proper membrane localization of Ypt1 and Sec4 (73) and for in vitro geranylgeranylation of Ypt1 (52) and Sec4 (26a). The *MAD2* gene encodes a protein which is 28% identical to Ram2 (10) and has recently been shown to be required for prenylation of Ypt1 and Sec4 (53a). A yeast homolog of component A, Mrs6, has been identified recently (86). Hence, *S. cerevisiae* GGTase-II appears to be composed of a Mad2 α subunit and a Bet2 β subunit and may also require a component A homolog for function.

The intense interest in the protein prenylation field is due in part to the possibility that inhibitors of farnesyltransferase may provide a useful anticancer drug therapy. Activated Ras is found in many types of human cancers (6), and inhibition of prenylation can suppress activated Ras in mammalian cells in culture (18, 36, 40), *S. cerevisiae* (67, 80), and *Xenopus* oocytes (51, 80). A full understanding of the FTase and GGTase enzymes and their substrate specificities will aid in identification of speci... inhibitors of FTase that could suppress growth of certain tumors. In vitro studies from several laboratories have provided a wealth of information concerning the specificity requirements of the mammalian enzymes (49, 61, 71, 91). Knowledge about other substrates of FTase, substrate specificities of FTase and GGTase-I in vivo, and the sensitivity of normal Ras versus activated Ras to decreased prenylation will also be important in determining whether an inhibitor of FTase would be an effective anticancer agent.

To test the in vivo protein substrate specificities of FTase and GGTase-I and to evaluate the sensitivities of normal Ras and activated Ras to altered or attenuated prenylation, we have studied Ras prenylation in the genetically tractable yeast *S. cerevisiae*. Yeast strains that either lack or have increased levels of Ram1-FTase or Cdc43–GGTase-I were used to evaluate the effects of the C-terminal amino acid of Ras and Ras-related proteins on substrate selection by the Ram1-FTase or Cdc43–GGTase-I in vitro and in vivo.

MATERIALS AND METHODS

Genetic and molecular techniques. Growth of yeast strains on minimal medium (YM) and rich medium (YPD), mating, sporulation, and tetrad dissection were performed according to standard methods essentially as described previously (72). Lithium acetate yeast transformation was performed by using a modification (72) of the original method (44). Standard techniques (57) were used to construct plasmids and propagate them in *E. coli*.

Strains and plasmids. Strains are listed in Table 1, and plasmids are listed in Table 2. JRY2594, JRY2595, and JRY2593 are haploid strains derived from diploid strain JRY2582. As previously described (80), JRY2582 was constructed by replacing one chromosomal copy of the *RAM1* gene in diploid JRY120 with the *ram1*::*HIS3* allele (formerly called the *dpr1*::*HIS3* allele), which completely deleted the *RAM1* coding sequence. JRY120 was formed by mating two isogenic haploid strains, JRY1591 and JRY1594. Hence,

TABLE 1. Yeast strains used in this study

Strain	Genotype	Source or reference
CJ198-2B	*MATα his4 trp1 ura3 cdc43-2*	D. Johnson
CTX103	*MATα/a ADE2/ade2 HIS3/his3 his4/HIS4 LYS2/lys2 MET2/met2 trp1/TRP1 ura3/ura3 cdc43-2/CDC43 RAM1/ram1::HIS3* [pJR982 (low-copy *URA3, RAM1*)]	CJ198-2B × JRY3097
JRY120	*MATα/a ade2/ade2 his3/his3 lys2/lys2 met2/met2 ura3/ura3*	JRY1591 × JRY1594
JRY1591	*MATα ade2 his3 lys2 met2 ura3*	W. Schafer
JRY1594	*MATa ade2 his3 lys2 met2 ura3*	W. Schafer
JRY2582	*MATa/α ade2/ade2 his3/his3 lys2/lys2 met2/met2 ura3/ura3 RAM1/ram1::HIS3*	W. Schafer (78)
JRY2593	*MATa ade2 his3 lys2 met2 ura3 ram1::HIS3*	Segregant from JRY2582
JRY2594	*MATα ade2 his3 lys2 met2 ura3*	Segregant from JRY2582
JRY2595	*MATα ade2 his3 lys2 met2 ura3 ram1::HIS3*	Segregant from JRY2582
JRY3097	*MATa ade2 his3 lys2 met2 ura3 ram1::HIS3* [pJR982 (low-copy *URA3, RAM1*)]	Transformant of JRY2593
JRY3259	*MATα ade2 his3 lys2 met2 ura3* [pJR1039 (low-copy *URA3, RAS2*)]	Transformant of JRY2594
JRY3260	*MATα ade2 his3 lys2 met2 ura3* [pJR1040 (low-copy *URA3*, $RAS2^{Val-19}$)]	Transformant of JRY2594
JRY3262	*MATα ade2 his3 lys2 met2 ura3* [pJR1050 (low-copy *URA3*, $RAS2^{Val-19}$-Leu)]	Transformant of JRY2594
JRY3264	*MATα ade2 his3 lys2 met2 ura3 ram1::HIS3* [pJR1040 (low-copy *URA3*, $RAS2^{Val-19}$)]	Transformant of JRY2595
JRY3266	*MATα ade2 his3 lys2 met2 ura3 ram1::HIS3* [pJR1050 (low-copy *URA3*, $RAS2^{Val-19}$-Leu)]	Transformant of JRY2595
JRY3362	*MATα ade2 his3 lys2 met2 ura3* [pJR670 (high-copy *URA3, RAS2*)]	Transformant of JRY2594
JRY3366	*MATα ade2 his3 lys2 met2 ura3 ram1::HIS3* [pJR670 (high-copy *URA3, RAS2*)]	Transformant of JRY2595
JRY3383	*MATa ade2 his3 lys2 met2 ura3 ram1::HIS3* [YEp(43)2 (high-copy *URA3, CDC43*)]	Transformant of JRY2593
JRY3384	*MATa ade2 his3 lys2 met2 ura3 ram1::HIS3* [YEp24 (high-copy *URA3*)]	Transformant of JRY2593
JRY3385	*MATa ade2 his3 lys2 met2 ura3* [YEp(43)2 (high-copy *URA3, CDC43*)]	Transformant of JRY1594
JRY3386	*MATa ade2 his3 lys2 met2 ura3* [YEp24 (high-copy *URA3*)]	Transformant of JRY1594
JRY3395	*MATa ade2 his3 lys2 met2 ura3 ram1::HIS3* [pSEY8 (high-copy URA3)]	Transformant of JRY2593
JRY3396	*MATa ade2 his3 lys2 met2 ura3 ram1::HIS3* [pDL4hc (high-copy *URA3, BET2*)]	Transformant of JRY2593
JRY3397	*MATa ade2 his3 lys2 met2 ura3* [pJR670 (high-copy *URA3, RAS2*)]	Transformant of JRY1594
JRY3409	*MATα ade2 his4 his3? lys2 trp1 ura3 cdc43-2 ram1::HIS3* [pJR670 (high-copy *URA3, RAS2*)]	Transformant of JRY3412
JRY3410	*MATa his4 his3? trp1 ura3 cdc43-2 ram1::HIS3* [pJR670 (high-copy *URA3, RAS2*)]	Transformant of JRY3413
JRY3412	*MATα ade2 his4 his3? lys2 trp1 ura3 cdc43-2 ram1::HIS3* [pJR1051 (low-copy *TRP1, RAS2*)]	Transformant of JRY3571
JRY3413	*MATa his4 his3? trp1 ura3 cdc43-2 ram1::HIS3* [pJR1051 (low-copy *TRP1, RAS2*)]	Transformant of JRY3572
JRY3470	*MATa his3 lys2 met2 ura3 cdc43-2*	Segregant from CTX103
JRY3571	*MATα ade2 his4 his3? lys2 trp1 ura3 cdc43-2 ram1::HIS3* [pJR982 (low-copy *URA3, RAM1*)]	Segregant from CTX103
JRY3572	*MATa his4 his3? trp1 ura3 cdc43-2 ram1::HIS3* [pJR982 (low-copy *URA3, RAM1*)]	Segregant from CTX103
JRY3629	*MATa ade2 his3 leu2 lys2 trp1 ura3 cal1::ura3* (*cdc43::ura3*) [YEpT-RHO1 (high-copy *TRP1, RHO1*)] [YEpL-CDC42 (high-copy *LEU2, CDC42*)] [pJR1050 (low-copy *URA3*, $RAS2^{Val-19}$-Leu)]	Transformant of YOT35955
JRY3664	*MATa ade2 his3 leu2 trp1 ura3 cal1::ura3* (*cdc43::ura3*) *ram1::HIS3* [YEpU-CAL1 (high-copy *URA3, CAL1* {*CDC43*})] [YEpT(RHO1+CDC42) (high-copy *TRP1, RHO1, CDC42*)]	Derived from YOT35920 (see text)
JRY3709	*MATa ade2 his3 leu2 lys2 trp1 ura3 cal1::ura3* (*cdc43::ura3*) [YEpT(RHO1+CDC42) (high-copy *TRP1, RHO1, CDC42*)] [pJR1072 (high-copy *RAM1, LEU2*)]	Transformant of YOT35953
JRY3710	*MATa ade2 his3 leu2 lys2 trp1 ura3 cal1::ura3* (*cdc43::ura3*) [YEpT(RHO1+CDC42) (high-copy *TRP1, RHO1, CDC42*)] [YEplac181 (high-copy *LEU2*)]	Transformant of YOT35953
JRY3984	*MATa ade2 his3 leu2 lys2 trp1 ura3 cal1::ura3* (*cdc43::ura3*) *ram1::HIS3* [YEpU-CAL1 (high-copy *URA3, CAL1* {*CDC43*})] [YEpT(RHO1+CDC42) (high-copy *TRP1, RHO1, CDC42*)] [RAS2/AAH5 (high-copy *LEU2, RAS2*)]	Transformant of JRY3664
JRY3985	*MATa ade2 his3 leu2 lys2 trp1 ura3 cal1::ura3* (*cdc43::ura3*) *ram1::HIS3* [YEpU-CAL1 (high-copy *URA3, CAL1* {*CDC43*})] [YEpT(RHO1+CDC42) (high-copy *TRP1, RHO1, CDC42*)] [AAH5 (high-copy *LEU2*)]	Transformant of JRY3664

Continued on following page

TABLE 1—*Continued*

Strain	Genotype	Source or reference
JRY3986	MATa *ade2 his3 leu2 lys2 trp1 ura3 cal1*::*ura3* (*cdc43*::*ura3*) *ram1*::*HIS3* [YEpU-CAL1 (high-copy *URA2, CAL1* {*CDC43*}] [YEpT(RHO1+CDC42) (high-copy *TRP1 RHO1, CDC42*)] [pJR1072 (high-copy *LEU2, RAM1*)]	Transformant of JRY3664
JRY3987	MATa *ade2 his3 leu2 lys2 trp1 ura3 cal1*::*ura3* (*cdc43*::*ura3*) *ram1*::*HIS3* [YEpU-CAL1 (high-copy *URA3, CAL1* {*CDC43*})] [YEpT(RHO1+CDC42) (high-copy *TRP1, RHO1, CDC42*)] [YEplac181 (high-copy *LEU2*)]	Transformant of JRY3664
YOT35920	MATa *ade2 his3 leu2 lys2 trp1 ura3 cal1*::*ura3* (*cdc43*::*ura3*) [YEpU-CAL1 (high-copy *URA3, CAL1* {*CDC43*})] [YEpT(RHO1+CDC42) (high-copy *TRP1, RHO1, CDC42*)]	66
YOT35953	MATa *ade2 his3 leu2 lys2 trp1 ura3 cal1*::*ura3* (*cdc43*::*ura3*) [YEpT(RHO1+CDC42) (high-copy *TRP1, RHO1, CDC42*)]	66
YOT35955	MATa *ade2 his3 leu2 lys2 trp1 ura3 cal1*::*ura3* (*cdc43*::*ura3*) [YEpT-RHO1 (high-copy *TRP1, RHO1*] [YEpL-CDC42 (high-copy *LEU2, CDC42*)]	66

JRY2594, JRY2595, JRY2593, JRY1594, and transformants of these strains listed in Table 1 are isogenic.

CTX103 is a diploid strain formed by crossing a *cdc43*^{ts} strain (CJ198-2B) with a *ram1*::*HIS3* strain carrying a *RAM1* plasmid (JRY3097). Five of 24 haploid segregants of CTX103 from six tetrads appeared to be dependent on the *RAM1 URA3* plasmid for viability, as determined by their inability to grow on plates containing 5-fluoro-orotic acid (5-FOA), which inhibits growth of *URA3* cells but not *ura3* cells (9). Each of these five segregants carried the *cdc43*^{ts} mutation, as deduced from their inability to grow at 37°C. Although the presence of *ram1*::*HIS3* could not be directly determined from phenotypic analysis of these segregants (since *his3*, *his4*, and *ram1*::*HIS3* were all heterozygous in CTX103 and the *RAM1* plasmid was present in these strains), the two segregants that were analyzed further (JRY3571 and JRY3572; see below) were found to carry *ram1*::*HIS3*, as expected.

Two *cdc43*^{ts} segregants from CTX103 (JRY3571 and JRY3572) were transformed with a *TRP1 CEN* plasmid carrying *RAS2* (pJR1051) and plated on medium containing 5-FOA to select strains that had lost the *RAM1* plasmid (strains JRY3412 and JRY3413, respectively). Note that *CEN* plasmids are usually present at 1 to 2 copies per cell but can be present in excess of 10 copies per cell under selective conditions (74). The high-copy-number *RAS2 URA3* plasmid (pJR670) was transformed into *cdc43*^{ts} *ram1*Δ strains JRY3412 and JRY3413 and the resident *RAS2 TRP1 CEN* plasmid (pJR1051) was lost, creating JRY3409 and JRY3410, respectively. The inability of JRY3410 to mate confirmed that it carries the *ram1*Δ mutation, since *ram1*Δ mutations prevent maturation of a-factor, resulting in loss of mating ability of *MAT***a** cells. For JRY3409, the presence of the *ram1*Δ mutation was confirmed by complementation test.

To further investigate the observation that *cdc43*^{ts} *ram1*::*HIS3* segregants require a *RAM1* plasmid for viability, one of the *MAT***a** *his3 cdc43*^{ts} segregants of CTX103 (JRY3470) was crossed to a *MAT*α *his3 ram1*::*HIS3* strain (JRY2595) transformed with a *RAM1 URA3* plasmid (pJR982). The *ram1*::*HIS3* allele can be followed in all

TABLE 2. Plasmids

Plasmid	Characteristics	Parent vector	Source or reference
RAS2/AAH5	*ADHp-RAS2 LEU2* 2μm ori[a] (high copy)	AAH5	K. Tatchell (84)
pDL4hc	*BET2 URA3* 2μm ori (high copy)	pCGS40	S. Ferro-Novick
YEp(43)2	*CDC43 URA3* 2μm ori (high copy)	YEp24	D. Johnson (45)
YEpL-CDC42	*CDC42 LEU2* 2μm ori (high copy)	YEp352	66
YEpT-RHO1	*RHO1 TRP1* 2μm ori (high copy)	pYOT324	66
YEpT(RHO1+CDC42)	*RHO1 CDC42 TRP1* 2μm ori (high copy)	pYOT324	66
YEpU-CAL1	*CAL1* (*CDC43*) *URA3* 2μm ori (high copy)	pMK9	66
pJR670	*RAS2 URA3* 2μm ori (high copy)	pSEY8	W. Schafer (77)
pJR827	*RAS2 URA3*	pRS306	W. Schafer
pJR828	*RAS2*$^{Val-19}$ *URA3*	pRS306	W. Schafer
pJR856	*RAM1 URA3* 2μm ori (high copy)	pSEY8	W. Schafer (80)
pJR857	*RAM1*	pBluescript(KS)+	W. Schafer (80)
pJR868	*ram1*::*HIS3*	pBluescript(KS)+	W. Schafer (80)
pJR982	*RAM1 URA3 CEN6 ARSH4*	pRS316	W. Schafer
pJR1039	*RAS2 URA3 CEN6 ARSH4*	pRS316	This study (see text)
pJR1040	*RAS2*$^{Val-19}$ *URA3 CEN6 ARSH4*	pRS316	This study (see text)
pJR1042	*RAS2*$^{Val-19}$-Leu *URA3*	pRS306	This study (see text)
pJR1050	*RAS2*$^{Val-19}$-Leu *URA3 CEN6 ARSH4*	pRS316	This study (see text)
pJR1051	*RAS2 TRP1 CEN6 ARSH4*	pRS314	This study (see text)
pJR1052	*RAS2*$^{Val-19}$ *TRP1 CEN6 ARSH4*	pRS314	This study (see text)
pJR1054	*RAS2*$^{Val-19}$-Leu *TRP1 CEN6 ARSH4*	pRS314	This study (see text)
pJR1072	*RAM1 LEU2* 2μm ori (high copy)	YEplac181	W. Schafer

[a] ori, origin of replication.

haploid segregants from this diploid. Among 38 tetrads dissected, there were 18 tetrads with four viable segregants, 17 tetrads with three viable segregants, and 3 tetrads with two viable segregants. Each of the 15 viable *ram1*Δ *cdc43*^{ts} segregants retained the *RAM1 URA3* plasmid and was unable lose the plasmid (as determined by their inability to grow on 5-FOA medium). The 114 remaining viable segregants either lacked or were able to lose the plasmid, as expected. From the phenotypes of the viable segregants from each tetrad, 15 of the 23 inviable segregants could be definitively predicted to be *ram1*Δ *cdc43*^{ts}, and the other 8 inviable segregants are very likely to be *ram1*Δ *cdc43*^{ts} as well. In these inviable *ram1*Δ *cdc43*^{ts} segregants, the *RAM1 URA3* plasmid was presumably lost prior to or during meiosis. Thus, there were 15 *ram1*Δ *cdc43*^{ts} segregants that were dependent on the *RAM1 URA3* plasmid for viability and 23 inviable segregants that are deduced to be *ram1*Δ *cdc43*^{ts} segregants that had lost the *RAM1 URA3* plasmid. The number of *ram1*Δ *cdc43*^{ts} segregants observed (38 of 152, or 25%) is in agreement with the number expected, since *RAM1* and *CDC43* are unlinked genes.

Strains YOT35955, YOT35920, and YOT35953 are transformants of a *cdc43* (*cal1*::*ura3*) deletion strain (YOT359-12C) that carry high-copy-number plasmids with *RHO1*, *CDC42*, and/or *CDC43* (*CAL1*) (66). YOT35955 carries two high-copy-number plasmids, a *TRP1* plasmid with the *RHO1* gene (YEpT-RHO1) and a *LEU2* plasmid with the *CDC42* gene (YEpL-CDC42). The combined overexpression of *RHO1* and *CDC42* can suppress the inviability of a *cdc43* deletion strain (66). YOT35955 was transformed with a *URA3* *RAS2*$^{Val-19}$-Leu plasmid (pJR1050) to generate JRY3629. YOT35920 is YOT359-12C carrying two high-copy-number plasmids, a *TRP1* plasmid with the *RHO1* and *CDC42* genes [YEpT(RHO1+CDC42)] and a *URA3* plasmid with *CDC43* (*CAL1*) (YEpU-CAL1). YOT35953 is YOT359-12C carrying the *TRP1* plasmid with the *RHO1* and *CDC42* genes [YEpT(RHO1+CDC42)]. YOT35953 (YOT359-12C with a high-copy-number *RHO1+CDC42* plasmid) grows more slowly than YOT35920 (YOT359-12C with high-copy-number *RHO1+CDC42* and *CDC43* plasmids) (66). To test whether overproduction of Ram1 would suppress the slow-growth phenotype, YOT35953 (YOT359-12C with a high-copy-number *RHO1+CDC42* plasmid) was transformed with the high-copy-number *RAM1 LEU2* plasmid (pJR1072) and with the high-copy-number *LEU2* vector control (YEplac181), generating strains JRY3709 and JRY3710, respectively.

Toward the goal of generating a *ram1 cdc43* double-deletion strain, the *RAM1* gene in YOT35920 (YOT359-12C with high-copy-number *RHO1+CDC42* and *CDC43* plasmids) was disrupted with *ram1*::*HIS3* by transformation with pJR868 digested with *Sal*I and *Xba*I. Two His$^+$ transformants that were unable to grow at high temperature and unable to mate were analyzed by Southern blotting, which confirmed that the *RAM1* gene had been disrupted (data not shown). These strains, JRY3663 and JRY3664, were transformed with several high-copy-number plasmids. JRY3664 transformed with RAS2/AAH5 (carrying *RAS2*), AAH5 vector, pJR1072 (carrying *RAM1*), and YEplac181 vector generated strains JRY3984, JRY3985, JRY3986, and JRY3987, respectively. These strains were streaked to 5-FOA plates to select for segregants that were able to lose the *URA3 CDC43* plasmid. *ram1 cdc43* double-deletion mutants lacking the *CDC43* plasmid were obtained only when the *RAM1* plasmid was present.

YEp(43)2 is high-copy-number vector YEp24 (11) with the *CDC43* gene inserted between the *Bam*HI and *Sal*I sites (45). pDL4hc is high-copy-number vector pCGS40 (32) with the *BET2* gene inserted on a 2-kb *Eco*RI-*Cla*I fragment. pJR856 has the *RAM1* gene on a 4.8-kb *Sal*I-*Bam*HI fragment inserted between the *Sal*I and *Bam*HI sites of the high-copy-number vector pSEY8 (25). pJR982 has the *RAM1* gene on a 3-kb *Bam*HI-*Sal*I fragment inserted between the *Bam*HI and *Sal*I sites of the *CEN* vector pRS316 (83). pJR1072 is the high-copy-number *LEU2* plasmid YEplac181 (30) with the *RAM1* gene inserted into the polylinker on a 1.9-kb *Sal*I-*Xba*I fragment. pJR868 is pBluescript(KS)+ carrying the *ram1*::*HIS3* allele; as described previously (80), the *ram1*::*HIS3* allele was created in pJR857 [pBluescript(KS)+ with the 1.9-kb *Sal*I-*Xba*I *RAM1* fragment] by deleting the *Pst*I-*Hpa*I fragment that contains the entire *RAM1* coding region and replacing it with the *HIS3* gene. Note that the *Sal*I sites in these *RAM1* plasmids are not in the *RAM1* 5′ or 3′ untranslated region. In the cases of pJR856, pJR1072, and pJR868, the *Sal*I site is from the vector sequence 5′ to the *RAM1* gene in the original clone isolated by Goodman et al. (34). In pJR982, the *Sal*I site is from YCp50 vector sequence 3′ to the *RAM1* gene in a clone isolated by W. Schafer from the P. Novick YCp50 library. pJR827 and pJR828 carry the 1.9-kb *Cla*I-*Hin*dIII fragments of *RAS2* and *RAS2*$^{Val-19}$, respectively, between the *Cla*I and *Hin*dIII sites of the integrating plasmid pRS306 (83). pJR828 was used to generate single-stranded DNA for oligonucleotide-directed mutagenesis to change the last codon of *RAS2*$^{Val-19}$ from a serine codon to a leucine codon in order to create the *RAS2*$^{Val-19}$-Leu gene in plasmid pJR1042 (see below). The *RAS2*, *RAS2*$^{Val-19}$, and *RAS2*$^{Val-19}$-Leu genes were isolated (from pJR827, pJR828, and pJR1042) as 1.9-kb *Sal*I-*Eco*RI fragments and ligated to the corresponding sites of pRS316 and pRS314, which are *CEN* plasmids with the selectable markers *URA3* and *TRP1*, respectively (83). The resulting pRS316-based *CEN* plasmids carrying *RAS2*, *RAS2*$^{Val-19}$, and *RAS2*$^{Val-19}$-Leu are pJR1039, pJR1040, and pJR1050, respectively. The resulting pRS314-based *CEN* plasmids carrying *RAS2*, *RAS2*$^{Val-19}$, and *RAS2*$^{Val-19}$-Leu are pJR1051, pJR1052, and pJR1054, respectively. YEpT-RHO1, YEpL-CDC42, YEpU-CAL1, and YEpT(RHO1+CDC42) are high-copy-number 2μm plasmids with *TRP1 RHO1*, *LEU2 CDC42*, *URA3 CAL1* (*CDC43*), and *TRP1 RHO1 CDC42*, respectively (66). RAS2/AAH5 was provided by K. Tatchell (20) and has the *RAS2* gene under the control of the strong *ADH1* promoter in AAH5, a high-copy-number 2μm vector with the *LEU2* gene (21).

Mutagenesis to create *RAS2*$^{Val-19}$-Leu. Oligonucleotide-directed mutagenesis (41) of the *RAS2*$^{Val-19}$ gene was performed to change the last codon from a serine codon to a leucine codon. The oligonucleotide (5′CCTTTTTATTAAA GTATAATACAACAGC) was phosphorylated by using T4 polynucleotide kinase and was hybridized to single-stranded DNA template of *RAS2*$^{Val-19}$ generated from pJR828 in *E. coli* TG2 (76) after superinfection with R408 phage (Stratagene). The annealed oligonucleotide primer and template were incubated with T4 DNA polymerase (New England Biolabs) and then with T4 DNA ligase (U.S. Biochemical) to generate covalently closed double-stranded plasmids, which were transformed into *E. coli*. Mutant clones with the two base substitutions that change the last codon of *RAS2*$^{Val-19}$ from a serine codon (AGT) to a leucine codon (CTT) were identified by colony hybridization, using the end-labeled oligonucleotide as a hybridization probe. The sequence of the *RAS2*$^{Val-19}$-Leu gene was confirmed by double-strand dideoxy sequencing with Sequenase (U.S. Biochemical).

Growth rate measurements. Strains were inoculated into

minimal medium containing the required supplements and grown at the indicated temperature in a shaking water bath. The A_{600} was measured periodically and plotted against time. Doubling times were calculated by using the exponential curve fit function of CA-Cricket Graph (Computer Associates International, Inc.), on 6 to 11 absorbance readings taken during the exponential growth phase. Each doubling time presented (in Table 3 or Results) was calculated from growth data taken on a single growing culture but was representative of the doubling time observed in multiple (two to three) independent cultures. Growth rates for independent cultures of a particular strain (or for independent transformants of a given strain with a given plasmid) differed by less than 10% when grown under the same conditions. Although temperature influenced growth rates of the various strains to different degrees, suppression of growth defects by overexpression of Ras, Cdc43, or Ram1 was observed at a range of temperatures.

Cell fractionation and immunoblot analysis. Strains were grown at 25°C to an A_{600} of 0.4 to 1.0 in 100 ml of minimal medium supplemented with adenine, leucine, histidine, lysine, methionine, and tryptophan, harvested by centrifugation, washed in 10 ml of buffer B (50 mM potassium phosphate [pH 7.2], 150 mM sodium chloride, 1 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride), frozen on dry ice, and stored at −80°C. Cells were thawed, resuspended in buffer B at 170 A_{600} units/ml, and broken by vortexing with an equal volume of 0.45-mm-diameter glass beads in a conical glass tube at full speed as follows: six times for 30 s and two times for 1 min, with cooling on ice between vortexing bursts. Two sequential low-speed centrifugations at 4°C (10 min at 3,000 rpm in a Sorvall SS-34 rotor) were performed to pellet unbroken cells and cell wall debris. A portion of the low-speed supernatant was saved as the whole cell extract, and the remainder was fractionated by centrifugation at 4°C for 30 min at 100,000 × g. The supernatant (S100) was saved, and the pellet (P100) was washed in buffer B at 20 A_{600} units/ml and pelleted at 4°C for 30 min at 100,000 × g. The pellet was resuspended in buffer A (buffer B with 1% Triton X-100 and 1% deoxycholate) at 170 A_{600} units/ml, sonicated for two 20-s bursts with a Branson model 1200 bath sonicator, and incubated on ice for 30 min. Aliquots of whole cell extract, S100, and P100 were frozen on crushed dry ice and stored at −80°C.

Protein samples were electrophoretically separated on 10% polyacrylamide gels (30:0.8, acrylamide/bisacrylamide) and transferred to two layers of nitrocellulose. The nitrocellulose sheet closest to the gel during transfer was used to detect Ras protein immunologically by incubation with a 1:200 dilution of rat monoclonal antibody Y13-259 (Oncogene Science) and then with a 1:2,000 dilution of alkaline phosphatase-coupled goat anti-rat immunoglobulin G (Jackson Immunoresearch). The immunoblots were developed in a solution of 50 μg of nitroblue tetrazolium per ml and 25 μg of 5-bromo-4-chloro-3-indolylphosphate per ml in 0.1 M Tris (pH 9.5)–0.1 M NaCl–0.01 M $MgCl_2$ for 15 to 30 min. Contact prints of the immunoblots were made by using Kodak electrophoresis duplicating paper. The second sheet of nitrocellulose was washed in TBST (Tris-buffered saline [5 mM Tris {pH 7.6}, 150 mM sodium chloride] with 0.05% Tween 20) for 30 min, stained with India ink (0.4% in TBST) for more than 1 h, washed briefly in TBST, and blotted dry. The ink-stained blot served as a control for equivalence in gel loading and transfer of protein samples.

Heat shock sensitivity assay. Yeast strains were grown for 5 to 7 days on supplemented minimal plates lacking uracil and resuspended in water to an A_{600} of 25. Three-microliter drops of a 10-fold dilution series of each strain were spotted with a multiprong block onto supplemented minimal plates lacking uracil. After the liquid was absorbed (2 to 3 min), the plates were wrapped in Parafilm and placed, agar side down, into a 55°C water bath for 20 min or a 50°C water bath for 40 min. Afterwards, plates were incubated at 23°C for 3 to 5 days to allow growth of viable cells.

Protein prenyltransferase assays. The following peptide substrates were synthesized and purified by high-pressure liquid chromatography (HPLC) purified on a C_{18} column according to previously described methods (4): Ras2 (KKSGSGGSCIIS), Ras2-Leu (KKSGSGGSCIIL), Rsr1 (KKNASTCTIL), Rsr1-Ser (KKNASTCTIS), and **a**-factor (YIIKGVFWDPACVIA). The Ras2 peptide sequence, based on the last 11 amino acids of the Ras2 precursor prior to any C-terminal processing, differs from that of the Ras2 precursor in two respects: (i) an extra lysine has been added at the N terminus of the peptide to increase solubility, and (ii) the cysteine that is normally palmitoylated (immediately N terminal to the prenylated cysteine) has been changed to serine to avoid ambiguity as to which cysteine is prenylated. The Ras2-Leu peptide is identical to the Ras2 peptide except for the replacement of the C-terminal serine with leucine. The Rsr1 peptide has the sequence of the last 10 amino acids of the Rsr1 protein (8). The Rsr1-Ser peptide is identical to the Rsr1 peptide except for the replacement of the C-terminal leucine with serine. The **a**-factor peptide is the last 15 amino acids of the 36-amino-acid **a**-factor mating pheromone precursor. Fully processed, mature **a**-factor is a 12-amino-acid farnesylated peptide (YIIKGVFWDPAC-farnesyl) that is secreted by *MAT***a** cells (3). ^{3}H-farnesyl diphosphate (^{3}H-FPP) at 20 μCi/mmol was purchased from New England Nuclear, and unlabeled FPP was a gift from Dale Poulter. ^{3}H-geranylgeranyl diphosphate (^{3}H-GGPP) at 8 μCi/mmol and unlabeled GGPP were purchased from D. Kennedy (University of South Florida).

Soluble extracts (S100), isolated as described above, were incubated at 37°C with a peptide substrate and a lipid substrate (^{3}H-FPP or ^{3}H-GGPP) under the following reaction conditions: 50 mM potassium phosphate (pH 6.8), 50 mM KCl, 4 mM $MgCl_2$, 70 μM zinc sulfate, 1 mM dithiothreitol. Reactions were placed on ice and spotted onto Silica Gel 60 thin-layer chromatography plates (EM Science), which were developed in solvent B (pyridine-isoamyl alcohol-ethanol-water-acetic acid [70:70:20:20:5, vol/vol]) for 4 to 5 h. The plates were dried, sprayed with En^{3}Hance (New England Nuclear), and exposed to Kodak XAR film at −80°C. ^{3}H-FPP and ^{3}H-GGPP did not move significantly from the origin in solvent B. The peptide-dependent ^{3}H-labeled spots indicated by the arrows in Fig. 3 and 4 each represent a prenylated peptide species. For the Ras2 and Ras2-Leu peptides, multiple prenylated peptide spots were observed as a result of proteolysis of the full-length peptide substrate by proteases in the soluble extracts (see below). The prenylated peptide spot in the Rsr1 and Rsr1-Ser lanes was also a proteolytic derivative of the full-length peptide. Addition of protease inhibitors (5 μg of leupeptin per ml, 5 μg of aprotinin per ml, and 70 μM E64) to the prenylation reactions prevented the appearance of peptide-dependent ^{3}H-labeled spots, because ^{3}H-labeled intact peptides remained at the origin and were obscured by the ^{3}H-FPP and ^{3}H-GGPP. HPLC analysis of in vitro prenylation reactions (with ^{3}H-FPP and Ras2 peptide) showed a ^{3}H-labeled HPLC peak that coeluted with a chemically synthesized farnesylated Ras2 peptide standard. This ^{3}H-labeled HPLC peak was

present when protease inhibitors were present in the reaction but was not observed when protease inhibitors were absent.

For HPLC analysis, protein prenyltransferase assay samples were extracted with an equal volume of 1-butanol and dried under nitrogen. Reaction products were separated with a Hewlett-Packard 1090 HPLC and a Brownlee Lab RP300 C_8 reverse-phase column (2.1 mm by 25 cm) with a gradient of 35 to 65% acetonitrile over 20 min. Fractions were collected at 0.5-min intervals, and radioactivity in each fraction was determined with a Beckman LS 7500 scintillation counter after addition of 10 ml of Ecolume (ICN Biomedical Inc., Irvine, Calif.). Counting efficiency for ^3H was 13%.

RESULTS

Evidence for Ram1-independent prenylation of the Ram1-FTase substrates Ras1 and Ras2. In the course of work identifying Ram1 as a subunit of the farnesyltransferase that prenylates Ras proteins (80), several observations suggested that a second protein prenyltransferase could also act on Ras. First, strains with a complete deletion of the *RAM1* gene are viable; they grow slowly at 25°C but do not grow at 37°C. If Ras were completely dependent on Ram1-FTase for prenylation, a strain deleted for *RAM1* should be dead, since Ras function is required for growth of *S. cerevisiae* at all temperatures (47, 48, 84) and Ras2 lacking the CaaX-motif cysteine cannot support growth unless it is overproduced (23). Second, in strains deleted for *RAM1*, a small amount of Ras was membrane associated. It is unlikely that this Ram1-independent membrane localization of Ras is prenylation independent, since Ras2 lacking the CaaX-motif cysteine is not detected in the membrane fraction (23). Third, extracts from a strain deleted for *RAM1* exhibited a low amount of prenylation of H-Ras in vitro (80).

Overproduction of Ras2 or Cdc43–GGTase-I, but not Bet2–GGTase-II, partially suppresses the growth defect of *ram1*Δ strains. If the growth defects of *ram1*Δ strains were due primarily to a deficit of Ras function, overproduction of Ras2 or of a second protein prenyltransferase that can act on Ras would be expected to suppress the growth defects. The slow growth of *ram1*Δ strains at 24 and 35°C is substantially suppressed by overproduction of Ras2 (Table 3) (77). At 35°C, the doubling time of a *ram1*Δ strain carrying a *RAS2* high-copy-number plasmid (strain JRY3393) is 2.4 h, compared with a doubling time of 4.1 h for the *ram1*Δ strain carrying high-copy-number vector pSEY8 (JRY3395) and 1.8 h for *RAM1* strains (JRY3397, JRY3385, and JRY3386). These observations suggest that Ras is limiting for growth in *ram1*Δ strains and that no other functions required for growth at 35°C are eliminated in *ram1*Δ strains. Hence, the ability of *ram1*Δ strains to grow at various temperatures can be used as an assay for changes in Ras function in response to changes in prenylation.

As a first test of whether Cdc43–GGTase-I or Bet2–GGTase-II was responsible for the weak prenylation of Ras that allows the *ram1*Δ strain to grow, a *ram1*Δ strain (JRY2593) was transformed with high-copy-number plasmids carrying either *CDC43* or *BET2* (kindly provided by Douglas Johnson or Susan Ferro-Novick, respectively). The *ram1*Δ strain carrying the *CDC43* plasmid grew significantly better in minimal medium at 24 and 35°C than did the same strain carrying the control vector YEp24 or the *BET2* plasmid (Table 3). At 35°C, the doubling time of the *ram1*Δ strain carrying the *CDC43* plasmid (strain JRY3383) was 2.4 h, whereas the doubling times for the *ram1*Δ strains carrying the control vector YEp24 (strain JRY3384) and the *BET2* plasmid (strain JRY3396) were 4.6 h and 4.2 h, respectively. The doubling time of the isogenic *RAM1* strain carrying the high-copy-number *CDC43* plasmid (strain JRY3385) or the control vector YEp24 (strain JRY3386) was 1.8 h, indicating that suppression by the high-copy-number *CDC43* plasmid at 35°C is incomplete, perhaps because the amount of α subunit of the Cdc43-GGTase (Ram2) is limiting relative to the overproduced Cdc43 β subunit. The partial suppression of the *ram1*Δ strain growth defect by overproduction of Cdc43 is consistent with the hypothesis that the Cdc43–GGTase-I can prenylate Ras in vivo.

TABLE 3. Suppression of *ram1* growth defects

Strain[a]	High-copy-number plasmid[b]	Doubling time (h)[c]	
		24°C	35°C
*ram1*Δ	pSEY8	3.8	4.1
	YEp24	4.1	4.6
	RAS2	2.7	2.4
	CDC43	2.9	2.4
	BET2	4.1	4.2
RAM1	YEp24	2.9	1.8
	RAS2	2.7	1.8
	CDC43	3.0	1.8

[a] *ram1*Δ strains are transformants of JRY2593, and *RAM1* strains are transformants of JRY1594; JRY2593 and JRY1594 are isogenic (see Table 1 and Materials and Methods).

[b] pSEY8 and YEp24 are 2μm *URA3* vectors. The high-copy-number plasmids with *RAS2*, *CDC43*, and *BET2* are pJR670 (pSEY8 with *RAS2*), YEp(43)2 (YEp24 with *CDC43*), and pDL4hc (pCGS40 with *BET2*), respectively (see Table 2).

[c] One hundred milliliters of supplemented minimal medium was inoculated with 1 ml of a stationary-phase culture, and the A_{600} was measured at hourly intervals for 11 h. Doubling times were calculated by using the exponential curve fit function of CA-Cricket Graph on 7 to 11 readings taken during exponential growth phase.

CDC43* is required for Ras function and viability of *ram1*Δ strains.** If Cdc43–GGTase-I were responsible for prenylating Ras in a *ram1*Δ strain, reducing the level of Cdc43–GGTase-I in a *ram1*Δ strain could decrease the amount of functional Ras below the level required for viability. To test this possibility, we took advantage of a temperature-sensitive *cdc43* mutant (strain CJ198-2B), kindly provided by D. Johnson. The *cdc43*^{ts} mutation severely reduces Cdc43–GGTase-I activity even when strain CJ198-2B is grown and assayed at 25°C, the permissive temperature for growth (33, 84a). To determine whether a *ram1*Δ *cdc43*^{ts} haploid strain was viable, a *MATa** *his3 ram1*::*HIS3* strain (JRY2595) transformed with the *RAM1 URA3* plasmid pJR982 was crossed to a *MAT***a** *his3 cdc43*^{ts} strain (JRY3470), and the resulting diploid was subjected to sporulation and tetrad dissection. All viable *ram1*Δ *cdc43*^{ts} segregants that were recovered retained the *RAM1 URA3* plasmid and required it for viability. Moreover, tetrad analysis demonstrated that nearly all of the inviable segregants were *ram1*Δ *cdc43*^{ts} segregants. We infer that these segregants were inviable because they had lost the *RAM1 URA3* plasmid before or during meiosis (see Materials and Methods for more detail). We conclude from these data that *ram1*Δ *cdc43*^{ts} strains are inviable.

If a deficit of Ras function is primarily responsible for the inviability of the *ram1*Δ *cdc43*^{ts} strains, overproduction of *RAS2* should restore viability, since previous studies have shown that overproduction of Ras2$^{Ser\text{-}319}$ (which lacks the

CaaX cysteine) can overcome the requirement for prenylation and membrane localization (23). Introduction of a high-copy-number plasmid carrying *RAS2* allowed growth of the *ram1*Δ *cdc43*^{ts} strains in the absence of a *RAM1* plasmid (strains JRY3409 and JRY3410) but did not fully suppress the growth defects. At 25°C in minimal medium, JRY3409 and JRY3410 had doubling times of 5.0 and 5.2 h, whereas the *cdc43*^{ts} and *ram1*Δ parental strains transformed with the same high-copy-number *RAS2* plasmid had doubling times of 2.9 and 3.1 h. JRY3409 and JRY3410 were unable to grow in rich medium (YPD) at any temperature. The observation that extra copies of *RAS2* could restore viability to the *ram1*Δ *cdc43*^{ts} strains indicated that the inviability of the *ram1*Δ *cdc43*^{ts} strain was due to a deficit of functional Ras rather than to a nonspecific effect resulting from the combination of the two mutations. These results support the hypothesis that prenylation of Ras by Cdc43–GGTase-I enables *ram1*Δ strains to grow.

Cdc43 influences Ras membrane association in *ram1*Δ strains. If Cdc43–GGTase-I prenylates Ras in *ram1*Δ strains, decreasing the concentration of Cdc43 should decrease Ras membrane association and increasing the concentration of Cdc43 should increase Ras membrane association. The predicted changes in Ras membrane association were observed by immunoblot analysis (Fig. 1). Figure 1A is an immunoblot of Ras in membrane and soluble fractions from a *RAM1 CDC43* strain (JRY3362), a *ram1*Δ *CDC43* strain (JRY3366), and a *ram1*Δ *cdc43*^{ts} strain (JRY3409). Since viability of *ram1*Δ *cdc43*^{ts} strains requires extra copies of *RAS*, each of these strains carried a high-copy-number *RAS2* plasmid. The membrane fraction (P100) from the *ram1*Δ *cdc43*^{ts} strain had substantially less Ras2 than did the membrane fraction (P100) from the *ram1*Δ *CDC43* strain, indicating that Cdc43 plays a significant role in the membrane association of Ras in *ram1*Δ strains. In the converse situation, a *ram1*Δ strain overproducing Cdc43 from a high-copy-number *CDC43* plasmid had approximately fourfold more Ras1 and Ras2 in the membrane fraction (P100) than the same *ram1*Δ strain carrying the vector YEp24 (Fig. 1B). Taken together, the genetic and biochemical results presented above establish that Cdc43-GGTase-I promotes Ras membrane association and function in a *ram1*Δ strain, presumably by prenylating Ras in vivo.

In vitro specificities of Ram1-FTase and Cdc43–GGTase-I. To examine the importance of the C-terminal amino acid in substrate selection by Ram1-FTase and Cdc43–GGTase-I in vitro, we performed in vitro prenylation assays with two pairs of peptide substrates, Ras2 (KKSGSGGSCIIS)–Ras2-Leu (KKSGSGGSCIIL) and Rsr1 (KKNASTCTIL)–Rsr1-Ser (KKNASTCTIS). The Ras2 and Ras2-Leu peptide sequences are similar to the C-terminal sequence of the Ras2 protein (see Materials and Methods) and differ from each other only in whether they have a C-terminal serine or a C-terminal leucine. The Rsr1 peptide sequence is identical to the last 10 amino acids of Rsr1 (a Ras-related protein required for proper bud site selection), and Rsr1-Ser differs only in replacement of the C-terminal leucine with serine.

Ram1-FTase shows in vitro preference for CaaX substrates with C-terminal serine but can also prenylate substrates with leucine. The ability of the Ram1-FTase to farnesylate peptide substrates with a C-terminal serine versus leucine was evaluated by incubating soluble extracts from a wild-type strain (JRY2594) and a *ram1*Δ mutant strain (JRY2595) with ^{3}H-FPP and one of the four peptides (Ras2, Ras2-Leu, Rsr1, or Rsr1-Ser). Reaction products were separated by thin-layer chromatography and visualized by autoradiography.

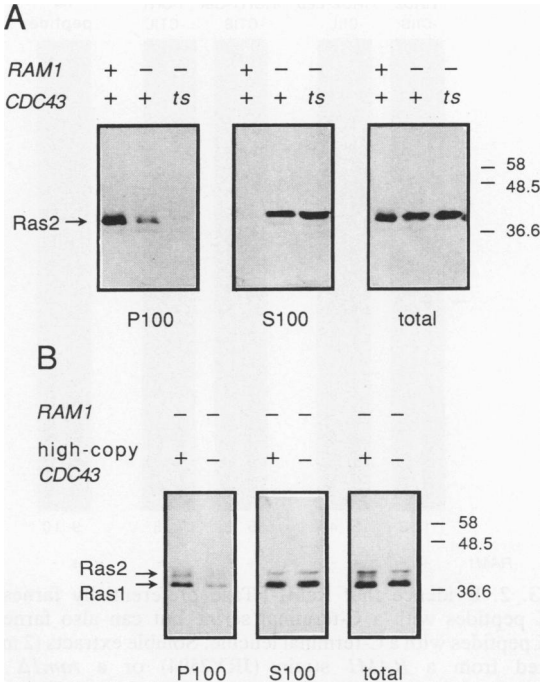

FIG. 1. Evidence that Cdc43 influences Ras membrane-association in *ram1*Δ strains. Immunoblot analysis was used to detect Ras protein in membrane fractions (P100), soluble fractions (S100), and whole cell extracts (total) (see Materials and Methods). (A) P100, S100, and whole cell extracts were isolated from a *RAM1 CDC43* strain (JRY3362), a *ram1*Δ *CDC43* strain (JRY3366), and a *ram1*Δ *cdc43*^{ts} strain (JRY3409). Each of these strains carries a high-copy-number *RAS2* plasmid (pJR670). Cell equivalents (based on measurements of optical density at 600 nm) of the fractions were loaded. (B) P100, S100, and whole cell extract were isolated from a *ram1*Δ strain carrying a high-copy-number *CDC43* plasmid (JRY3383) or the same *ram1*Δ strain carrying the high-copy-number vector YEp24 (JRY3384). Cell equivalents of P100 and S100 were loaded. Whole cell extract was loaded at one-half the cell equivalent of P100 and S100. Molecular mass standards are indicated in kilodaltons.

Peptide-dependent radiolabeled spots were observed for each of the four peptides, indicating that they were farnesylated by the wild-type soluble extract (Fig. 2, lanes 1, 3, 5, and 7). Multiple radiolabeled spots for the Ras2 and Ras2-Leu peptides resulted from proteolytic degradation (see Materials and Methods for more detail). The Ras2 and Rsr1-Ser peptides were farnesylated more efficiently than the Ras2-Leu and Rsr1 peptides, indicating that peptides with a C-terminal serine serve as better substrates for the Ram1-FTase than do peptides with a C-terminal leucine. The Ram1-FTase was responsible for the observed farnesylation of each of the four peptides, since soluble extracts from the *ram1*Δ mutant strain JRY2595, in which the *RAM1* gene is completely deleted, failed to exhibit any detectable farnesylation of the peptides (Fig. 2, lanes 2, 4, 6, and 8). Hence, the Ram1-FTase farnesylated substrates with a C-terminal serine with high efficiency and was able to farnesylate substrates with a C-terminal leucine with reduced efficiency. The relaxed specificity of the Ram1-FTase was observed at a peptide concentration of 50 μM (Fig. 2) but was not readily observed at a peptide concentration of 10 μM (data not shown).

Ram1-FTase can attach a geranylgeranyl group to peptide substrates in vitro. Ram1-FTase appeared to be able to

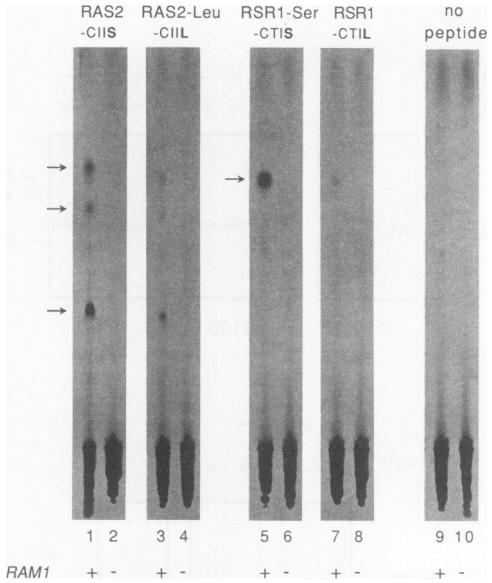

FIG. 2. Evidence that Ram1-FTase preferentially farnesylates CaaX peptides with a C-terminal serine but can also farnesylate CaaX peptides with a C-terminal leucine. Soluble extracts (2 mg/ml), isolated from a *RAM1* strain (JRY2594) or a *ram1*Δ strain (JRY2595), were incubated for 20 min at 37°C with ^{3}H-FPP (2 μM) and the indicated peptide substrate (50 μM): Ras2 (KKSGSG GSCIIS), Ras2-Leu (KKSGSGGSCIIL), Rsr1 (KKNASTCTIL), or Rsr1-Ser (KKNASTCTIS). Reaction products were separated by thin-layer chromatography, treated with En^{3}Hance spray, and exposed to film at −80°C for 34 h. ^{3}H-FPP did not move significantly from the origin. The peptide-dependent ^{3}H-labeled spots indicated by the arrows each represent a prenylated peptide species. Multiple prenylated peptide spots were observed as a result of proteolysis of the peptide substrates in the soluble extracts. See Materials and Methods for more detail.

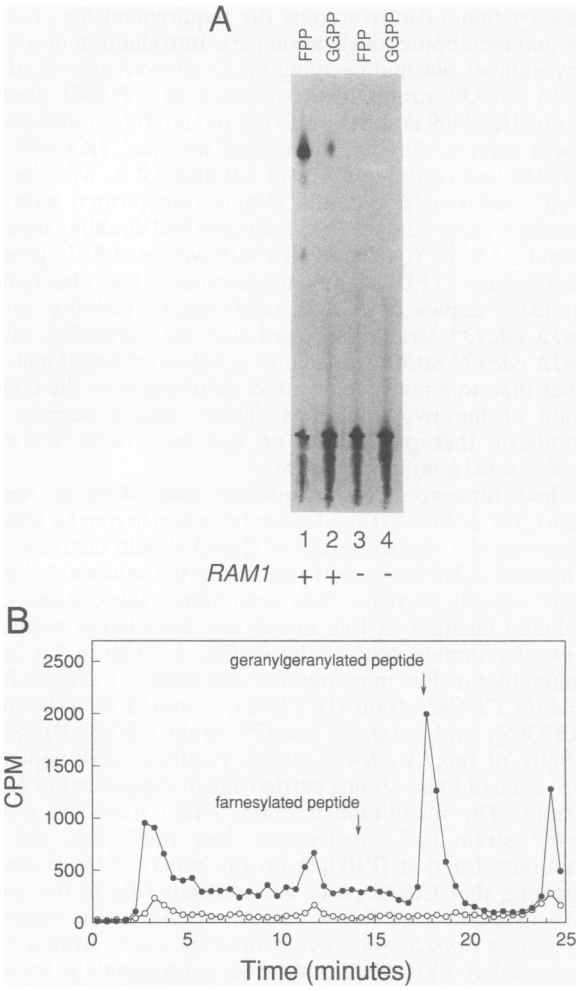

FIG. 3. Evidence that Ram1-FTase preferentially uses FPP as the prenyl lipid substrate but can also use GGPP. (A) Soluble extracts (1.6 mg/ml) isolated from a *RAM1* strain (JRY2594) or a *ram1*Δ strain (JRY2595) were incubated for 30 min at 37°C with 177 μM **a**-factor (YIIKGVFWDPACVIA) and 20 μM ^{3}H-FPP (0.75 Ci/μmol) or 20 μM ^{3}H-GGPP (1.5 Ci/μmol). Reaction products were separated by thin-layer chromatography, treated with En^{3}Hance spray, and exposed to film at −80°C for 18 h. (B) Butanol-extracted reaction products for the *RAM1* extract incubated with ^{3}H-GGPP and **a**-factor (●) or no peptide (○) were chromatographically separated on a C_8 reverse-phase HPLC column with a 35 to 65% acetonitrile gradient over 20 min. Fractions were collected at 0.5-min intervals, and radioactivity (counts per minute) was determined by scintillation counting. The arrows indicate the elution times of the chemically synthesized standards geranylgeranylated **a**-factor peptide (17.72 min) and farnesylated **a**-factor peptide (14.16 min).

geranylgeranylate the Ras2 and Rsr1-Ser peptides in an experiment which was identical to the one shown in Fig. 2 except that 10 μM ^{3}H-GGPP was substituted for 2 μM ^{3}H-FPP. Peptide-dependent ^{3}H-labeled spots were observed for Ras2 and Rsr1-Ser peptides in reactions with *RAM1* soluble extracts and ^{3}H-GGPP but not in reactions with *ram1* soluble extracts (data not shown). This Ram1-dependent geranylgeranylation of Ras2 and Rsr1-Ser was quite inefficient; the prenylated peptide products were faintly detected by thin-layer chromatography analysis after exposure of film for 5 days and were more obvious after 20 days.

To determine whether the Ram1-FTase was actually attaching a geranylgeranyl group to a peptide substrate, an **a**-factor peptide (YIIKGVFWDPACVIA) was incubated with *RAM1* and *ram1*Δ soluble extracts in the presence of either 20 μM ^{3}H-GGPP or 20 μM ^{3}H-FPP. **a**-factor peptide was chosen for the HPLC analysis since it is more resistant to proteolysis in the soluble extracts than are the Ras2 and Rsr1-Ser peptides. Thin-layer chromatography analysis (Fig. 3A) showed that *RAM1* extracts could prenylate the **a**-factor peptide when ^{3}H-GGPP was provided, although the extent of prenylation was approximately 10 times lower than when ^{3}H-FPP was provided. *ram1*Δ extracts failed to prenylate the **a**-factor peptide when either ^{3}H-GGPP or ^{3}H-FPP was provided, indicating that Ram1 is required for prenylation of the **a**-factor peptide by ^{3}H-GGPP or ^{3}H-FPP. To determine the nature of the lipid attached to the **a**-factor peptide after incubation with the *RAM1* extract and ^{3}H-GGPP, reaction products were analyzed by HPLC. Fractions eluted from a C_8 reverse-phase column with a 35 to 65% acetonitrile gradient were collected at 0.5-min intervals and subjected to liquid scintillation counting (Fig. 3B). The major peak of radioactivity eluted between 17.5 and 18 min, which corresponds well with the 17.72-min retention time observed for a chemically synthesized geranylgeranylated **a**-factor peptide standard. No radioactive peak was observed at 14.16 min, the retention time of a chemically synthesized farnesylated **a**-factor peptide standard. The three minor peaks of radioactivity have not been identified. They were not derived

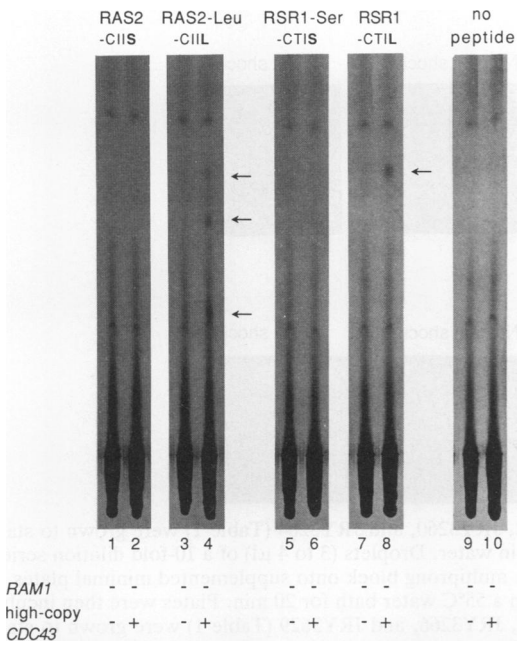

FIG. 4. Evidence that Cdc43-GGTase preferentially geranylgeranylates CaaX peptides with a C-terminal leucine. Soluble extracts (2 mg/ml), isolated from a *ram1*Δ strain transformed with a high-copy-number *CDC43* plasmid (strain JRY3383) or with a vector (strain JRY3384), were incubated for 20 min at 37°C with ^{3}H-GGPP (10 μM) and the indicated peptide substrate (50 μM): Ras2 (KKSGS GGSCIIS), Ras2-Leu (KKSGSGGSCIIL), Rsr1 (KKNASTCTIL), or Rsr1-Ser (KKNASTCTIS). Reaction products were separated by thin-layer chromatography, treated with En^{3}Hance spray, and exposed to film at −80°C for 122 h. ^{3}H-GGPP did not move significantly from the origin. The peptide-dependent ^{3}H-labeled spots indicated by the arrows each represent a prenylated peptide species. Multiple prenylated peptide spots were observed as a result of proteolysis of the peptide substrates in the soluble extracts. See Materials and Methods for more detail.

from the a-factor peptide since they were present in the control reaction (Fig. 3B, open circles), in which the *RAM1* extract was incubated with ^{3}H-GGPP in the absence of peptide. This experiment demonstrates that a Ram1-dependent protein prenyltransferase (presumably Ram1-FTase) can transfer a geranylgeranyl group to the a-factor peptide in vitro.

Cdc43–GGTase-I shows in vitro preference for CaaX substrates with C-terminal leucine. The ability of the Cdc43–GGTase-I to geranylgeranylate peptide substrates with a C-terminal leucine versus a C-terminal serine was evaluated by using the four peptide substrates, Ras2, Ras2-Leu, Rsr1, and Rsr1-Ser, incubated in the presence of 10 μM ^{3}H-GGPP. Soluble extracts were isolated from two strains that both have the *RAM1* gene deleted. One of these strains contains only the chromosomal copy of the *CDC43* gene (JRY3384), whereas the other strain carries a high-copy-number plasmid with the *CDC43* gene (JRY3383). Peptide-dependent ^{3}H-labeled spots were observed for Ras2-Leu (Fig. 4, lanes 3 and 4) and Rsr1 (Fig. 4, lanes 7 and 8), the two peptides terminating with leucine, but were not detected for Ras2 (Fig. 4, lanes 1 and 2) or Rsr1-Ser (Fig. 4, lanes 5 and 6), the two peptides terminating with serine. The dependence of Ras2-Leu and Rsr1 geranylgeranylation on the Cdc43–GGTase-I could be deduced from the dramatic increase in geranylgeranylation in soluble extracts from the strain that carries *CDC43* on a high-copy-number plasmid. The two peptides with a C-terminal leucine were clearly the preferred substrates for Cdc43-dependent geranylgeranylation. Our failure to detect geranylgeranylation of Ras2 and Rsr1-Ser peptides does not rule out the possibility that Ras2 and Rsr1-Ser can serve as inefficient substrates for Cdc43–GGTase-I. In fact, the in vivo experiments described above strongly suggested that Cdc43–GGTase-I was able to prenylate Ras2 protein in a strain deleted for the *RAM1* gene.

Effects of converting Ras2 C-terminal serine to leucine on prenylation in vivo. To investigate whether Ras2 with a C-terminal leucine could be prenylated in vivo by the Ram1-FTase and/or the Cdc43–GGTase-I, the function of Ras2^{Val-19}-Leu (an activated form of Ras2 with a C-terminal leucine) was analyzed. The alanine-to-valine substitution at codon 19 in yeast *RAS2* (*RAS2*$^{Val-19}$) is analogous to the mammalian *ras*$^{Val-12}$ mutation and results in a constitutively activated Ras, which results in low viability and pronounced heat shock sensitivity of stationary-phase cells. To determine whether Ras2^{Val-19}-Leu was functional, a heat shock sensitivity assay was performed on a wild-type strain (JRY2594) carrying a *CEN* plasmid with *RAS2*$^{Val-19}$-Leu (pJR1050) (Fig. 5A). As positive and negative controls, the same wild-type strain carrying plasmids with *RAS2*$^{Val-19}$ (pJR1040) or *RAS2* (pJR1039) was assayed. The wild-type strain carrying the *RAS2*$^{Val-19}$-Leu plasmid (JRY3262) exhibited a heat shock-sensitive phenotype like that observed for the wild-type strain carrying the *RAS2*$^{Val-19}$ plasmid (JRY3260), indicating Ras2^{Val-19}-Leu was functional. The wild-type strain carrying the *RAS2* plasmid (JRY3259) was heat shock resistant, as expected. A *ram1*Δ strain carrying the *RAS2*$^{Val-19}$ plasmid (JRY3264) was included to show the suppression of the heat shock-sensitive phenotype of Ras2^{Val-19} by a *ram1*Δ mutation, which reduces Ras membrane association and function, as previously shown for a *ram1* mutation (67). Note that the activated Ras phenotype was more dependent on Ram1-FTase than was the essential Ras function, since strains deleted for *RAM1* were viable (as a result of the weak prenylation by Cdc43–GGTase-I) but were not heat shock sensitive when *RAS2*$^{Val-19}$ was present.

Both Cdc43–GGTase-I and Ram1-FTase appear to prenylate Ras2^{Val-19}-Leu in vivo. If Cdc43–GGTase-I and Ram1-FTase can each independently prenylate and produce enough functional Ras2^{Val-19}-Leu to give an activated Ras phenotype in vivo, then neither a deletion of *CDC43* nor a deletion of *RAM1* would be expected to suppress the heat shock sensitivity conferred by Ras2^{Val-19}-Leu. To address this issue, we performed heat shock sensitivity assays to determine whether Ras2^{Val-19}-Leu was functional in a *ram1* deletion strain and in a *cdc43* deletion strain. The *cdc43* deletion strain is made viable by overproduction of Rho1 and Cdc42, which appear to be the only essential proteins that are dependent on Cdc43–GGTase-I for function (66). Both the *ram1*Δ and *cdc43*Δ strains carrying Ras2^{Val-19}-Leu were heat shock sensitive (Fig. 5B), indicating that Ras2^{Val-19}-Leu was functional in each strain. The dependence of Ras2^{Val-19}-Leu function on prenylation can be deduced from the observation that prenyl lipid depletion in the mevalonate auxotroph JRY1597 (*hmg1 hmg2*), which was previously shown to suppress the activated Ras phenotype of Ras2^{Val-19} (80), also suppressed the activated Ras phenotype of Ras2^{Val-19}-Leu (data not shown). Although Ras2^{Val-19}-Leu was functional in the *cdc43*Δ and *ram1*Δ strains, it was apparently less functional in these strains than in the wild-type strain, as shown by the heat shock survival of roughly 0.3% of viable cells in the *cdc43*Δ strain and

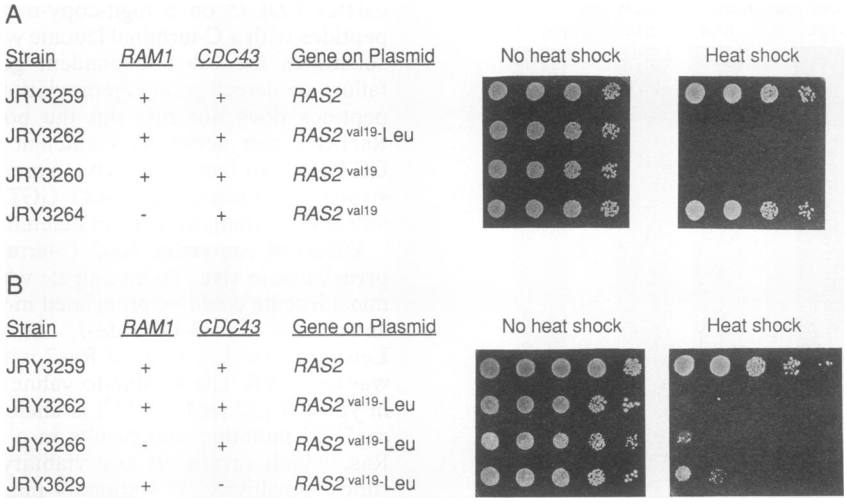

FIG. 5. Heat shock sensitivity assays. (A) Yeast strains JRY3259, JRY3262, JRY3260, and JRY3264 (Table 1) were grown to stationary phase (6 days) on supplemented minimal plates lacking uracil and resuspended in water. Droplets (3 to 4 μl) of a 10-fold dilution series (A_{600} cell densities of 2.5, 0.25, 0.025, and 0.0025) of each strain were spotted with a multiprong block onto supplemented minimal plates lacking uracil. One plate was not heat shocked, and the other plate was heat shocked in a 55°C water bath for 20 min. Plates were then incubated at 23°C for 3 days to allow growth of viable cells. (B) Strains JRY3259, JRY3262, JRY3266, and JRY3629 (Table 1) were grown to stationary phase on supplemented minimal lacking uracil and resuspended in water. Droplets of a 10-fold dilution series (A_{600} cell densities of 25, 2.5, 0.25, 0.025, and 0.0025) of each strain were spotted onto supplemented minimal plates lacking uracil. One plate was not heat shocked, and the other plate was heat shocked in a 50°C water bath for 40 min. Plates were then incubated at 23°C for 4 days to allow growth of viable cells.

roughly 0.01% of viable cells in the *ram1*Δ strain, compared with less than 0.002% in the wild-type strain. These observations are consistent with the hypothesis that both Cdc43–GGTase-I and FTase can prenylate and produce functional $Ras2^{Val\text{-}19}$-Leu and suggest that Cdc43–GGTase-I is roughly 30 times more effective than FTase in producing functional $Ras2^{Val\text{-}19}$-Leu.

Evidence for Cdc43-independent prenylation of Cdc43–GGTase-I substrates in vivo. The observation that simultaneous overproduction of Rho1 and Cdc42 can suppress the inviability of *cdc43* deletion mutants (66), together with the observations that Rho1 (66) and Cdc42 (93) require the CaaX-motif cysteine for function even when overproduced, suggests that a Cdc43-independent protein prenyltransferase can prenylate and provide functional Rho1 and Cdc42 in a *cdc43* deletion mutant. If Ram1-FTase were responsible for prenylating the overproduced Rho1 and Cdc42 proteins, an intact *RAM1* gene would be required for viability of a *cdc43*Δ strain. To address this issue, the *RAM1* gene was deleted in YOT35920, the *cdc43*Δ strain carrying a *RHO1 CDC42 TRP1* plasmid and a *CDC43 URA3* plasmid. The resulting strain (JRY3664) was transformed with high-copy-number *LEU2* plasmids carrying *RAM1* (pJR1072) or *RAS2* (RAS2/AAH5) and with the two high-copy-number *LEU2* vectors (YEplac181 and AAH5). When the *RAM1* plasmid was present (strain JRY3986), the *CDC43 URA3* plasmid could be segregated, which allowed growth on 5-FOA plates (Fig. 6). The strains transformed with the vectors (strains JRY3985 and JRY3987) or with the high-copy-number *RAS2* plasmid (strain JRY3984) were not able to lose the *CDC43* gene. A few Ura^- segregants of these strains were able to grow on 5-FOA plates (Fig. 6), but Southern blot analysis of 12 such Ura^- segregants demonstrated that they retained the *CDC43* gene (data not shown). Our inability to construct a strain lacking both the *CDC43* and *RAM1* genes is consistent with the hypothesis that Ram1-FTase is responsible for

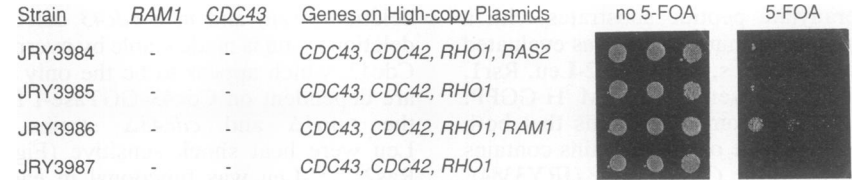

FIG. 6. 5-FOA resistance assays to detect the frequency of Ura^- cells, showing that *ram1*Δ *cdc43*Δ strains were able to lose *CDC43 URA3* plasmid when a *RAM1* plasmid was present but not when a high-copy-number *RAS2* plasmid or vector was present. The *ram1*Δ *cdc43*Δ strain carrying *CDC43*, *CDC42*, and *RHO1* on high-copy-number plasmids (JRY3664) was transformed with four high-copy-number plasmids: RAS2/AAH5 carrying *RAS2* (JRY3984), AAH5 vector (JRY3985), pJR1072 carrying *RAM1* (JRY3986), and YEplac181 vector (JRY3987). These strains were grown to stationary phase on supplemented minimal medium lacking uracil and resuspended in water. Droplets of a 10-fold dilution series (A_{600} cell densities of 5, 0.5, and 0.05) of each strain were spotted onto supplemented minimal plates lacking 5-FOA and onto supplemented minimal plates containing 5-FOA, as indicated. $Ura3^-$ and $Ura5^-$ yeast strains can form colonies on 5-FOA but Ura^+ yeast strains cannot. The presence of the high-copy-number *RAM1* plasmid allows the *CDC43 URA3* plasmid to be lost at a frequency of about 1 in 100. When the high-copy-number *RAS2* plasmid or either of the vectors was present, very few 5-FOA-resistant colonies (less than 0.01%) were observed. Southern blot analysis of eight 5-FOA-resistant colonies carrying RAS2/AAH5 and of four 5-FOA-resistant colonies carrying a vector revealed that each of these strains retains an intact copy of *CDC43* (data not shown).

prenylation of the essential proteins Rho1 and Cdc42 in a *cdc43Δ* strain.

If Ram1-FTase were responsible for prenylating the overproduced Rho1 and Cdc42 proteins, then the slow-growth phenotype of this strain (66) might be partially suppressed by overproduction of Ram1. The presence of the high-copy-number *RAM1* plasmid (pJR1072) did partially suppress the growth defect of the *cdc43Δ* strain overproducing Rho1 and Cdc42. In minimal medium at 30°C, the *cdc43Δ* strain carrying the *RAM1*, *RHO1*, and *CDC42* plasmids (strain JRY3709) had a doubling time of 3.0 h, whereas the *cdc43Δ* strain carrying the vector YEplac181 and the *RHO1+CDC42* plasmid (strain JRY3710) had a doubling time of 3.9 h. Isogenic *CDC43* strains carrying various plasmids had doubling times of 2.3 to 2.5 h, indicating that the high-copy-number *RAM1* plasmid was not able to completely suppress the growth defect of the *cdc43Δ* strain overproducing Rho1 and Cdc42. The high-copy-number *RAM1* plasmid was not able to allow a *cdc43Δ* strain to grow unless Rho1 and Cdc42 were overproduced, indicating that overproduction of Rho1 and Cdc42 is required for Ram1-FTase to substitute functionally for Cdc43–GGTase-I in vivo (data not shown). These observations are all consistent with the hypothesis that Ram1-FTase is responsible for prenylation of the essential proteins Rho1 and Cdc42 in a *cdc43Δ* strain.

DISCUSSION

The degree of protein substrate cross-specificity observed for FTase and GGTase-I varies among the published in vitro studies. Several studies (14, 61, 91) detect farnesylation of a substrate with a C-terminal leucine by partially purified FTase and detect geranylgeranylation of substrates with a C-terminal serine, methionine, or glutamine by partially purified GGTase-I, whereas other studies (92) conclude that the FTase and GGTase-I are unable to prenylate the substrates of the other enzyme. Since the in vitro cross-specificity of FTase and GGTase-I, when observed, occurs at high substrate concentrations with partially purified enzymes (which could potentially be cross-contaminated), the in vivo significance is unclear. In the in vitro studies reported here, potential cross-contamination of enzyme activities was avoided by using extracts that were isolated from genetically altered yeast strains. The farnesylation of peptide substrates was assayed in extracts from strains that have either an intact or deleted *RAM1* gene. The Ram1-FTase efficiently farnesylated the two peptide substrates with a C-terminal serine (Ras2 and Rsr1-Ser) but also farnesylated the two substrates with a C-terminal leucine (Ras2-Leu and Rsr1), albeit less efficiently (Fig. 2). The Ram1-FTase was clearly responsible for the farnesylation of each of the four peptides, since extracts from wild-type strains could farnesylate each peptide whereas extracts from the *ram1* deletion mutant could not. The geranylgeranylation of peptide substrates was assayed in *ram1* deletion strains that have a single copy of *CDC43* or have a high-copy-number *CDC43* plasmid. The Cdc43–GGTase-I was able to geranylgeranylate the two peptides with a C-terminal leucine (Ras2-Leu and Rsr1) (Fig. 4). We failed to detect in vitro prenylation of substrates with a C-terminal serine by Cdc43–GGTase-I, despite in vivo evidence that Cdc43–GGTase-I was able to prenylate Ras2 (see below).

Our in vivo genetic and immunoblot studies (Table 3, Fig. 1, and text) showed that Cdc43–GGTase-I, expressed from single-copy chromosomal *CDC43* and *RAM2* genes, was able to produce enough membrane-associated, functional Ras1 and Ras2 to keep a *ram1* deletion mutant alive. From the results of these studies, we deduced that Cdc43–GGTase-I (which efficiently prenylates substrates with a C-terminal leucine) was also able to prenylate substrates with a C-terminal serine (Ras2) or cysteine (Ras1), albeit with low efficiency. It is interesting to note that Cdc43–GGTase-I was able to prenylate enough Ras1 and Ras2 to allow *ram1Δ* strains to grow but was not able to prenylate enough $Ras2^{Val-19}$ to give an activated Ras phenotype. This result bolsters the idea that protein prenyltransferases may be effective pharmacological targets for treatment of cancers with activated Ras genes. Specifically, if activated Ras in mammalian cells is also more sensitive than normal Ras to a decrease in farnesylation, drugs that specifically inhibit the mammalian FTase could suppress the activated Ras function required for growth of certain cancers, without completely compromising the normal Ras function.

Are there Ram1-FTase substrates, in addition to Ras1 and Ras2, that can be prenylated and made functional by Cdc43–GGTase-I? The two other known substrates of Ram1-FTase, **a**-factor and Ste18, are required for mating but not growth. **a**-factor, which terminates with CVIA, is a mating pheromone produced by *MAT***a** cells that is required to arrest *MATα* cells in G_1 during the mating process. *MAT***a** *ram1Δ* mutants are completely unable to mate because of their inability to export functional **a**-factor. The overproduction of Cdc43 in a *MAT***a** *ram1Δ* strain did not restore mating ability (84a), suggesting that Cdc43–GGTase-I cannot geranylgeranylate enough **a**-factor to allow mating or that geranylgeranylated **a**-factor is not functional. This observation also indicates that the Cdc43–GGTase-I is not able to farnesylate **a**-factor in vivo. In vitro, no prenylation of **a**-factor by the Cdc43-GGTase was observed in the presence of either ^{3}H-FPP or ^{3}H-GGPP. Ste18, which terminates with CTIM, is the gamma subunit of the heterotrimeric G protein involved in the mating pheromone response pathway and is required for mating of both *MAT***a** and *MATα* cells (87). Mutations of *STE18* that convert the Ste18 CaaX-motif cysteine to serine abolish mating ability (28). *MATα ram1Δ* mutants exhibit a reduced mating efficiency, about 2 to 20% of wild-type mating efficiency (28). The ability of these strains to mate, contrasted with the severe mating defect conferred by the Ste18 cysteine-to-serine mutation, suggests that Ste18 may be prenylated and made functional by another protein prenyltransferase.

Are Ras1 and Ras2 the only essential substrates of Ram1-FTase that can be prenylated and made functional by Cdc43–GGTase-I in vivo? Ras2 overexpression from a high-copy-number plasmid was unable to restore wild-type growth rates either to the *ram1Δ* mutant at 35 or 37°C (Table 3) (77) or to the *ram1Δ* $cdc43^{ts}$ strain at 24°C, suggesting that the function of some other protein(s) may be partially compromised in these strains. The impaired ability of *ram1Δ* mutants to grow at high temperatures, even when Ras2 is overproduced, can be explained by the recent observation that a protein involved in the heat shock response is a substrate of Ram1-FTase (12). The yeast protein YDJ1, a DnaJ homolog, has a CaaX sequence terminating with CASQ and has been shown to be farnesylated by Ram1-FTase in vitro. Yeast strains in which the CaaX-motif cysteine of YDJ1 was changed to serine are viable but are unable to grow at 37°C. *YDJ1* is essential for growth at high temperatures but not for growth at low temperatures (5). The inability of high-copy *RAS2* to restore wild-type growth to $cdc43^{ts}$ *ram1Δ* strains at 25°C may indicate that additional essential or important substrates of Ram1-FTase are func-

tionally compromised in these strains. Nuclear lamins are logical candidates for essential substrates of the Ram1-FTase. Mammalian nuclear lamins A and B are farnesylated (7, 54, 89) and are thought to be essential proteins, since they are part of the nuclear envelope structure and are involved in the breakdown and reassembly of the nuclear envelope during each cell division cycle. Since lamins have not yet been identified in *S. cerevisiae*, this hypothesis cannot be tested directly.

What are the essential substrates of the Cdc43–GGTase-I and can they be prenylated and made functional by Ram1-FTase? Rsr1, Cdc42, Rho1, and Rho2 are GTP-binding proteins in the Ras superfamily that have CaaX sequences terminating with leucine: CTIL, CTIL, CVLL, and CIVL, respectively. Each of these proteins is implicated, by genetic (1, 8, 66) and/or in vitro prenylation (27) (Fig. 4) data, to be a Cdc43–GGTase-I substrate. Cdc42 (93) and Rho1 (55) are essential proteins, but Rsr1 (8) and Rho2 (55) are not. Cdc42 (93) and Rho1 (66) each require the CaaX-motif cysteine for function, and in contrast to the situation with Ras, overproduction of the Rho1 and Cdc42 proteins does not overcome the requirement for the cysteine.

cdc43 deletion strains are inviable (64), suggesting that Ram1-FTase cannot fully substitute for Cdc43–GGTase-I in the prenylation of its essential substrates. However, simultaneous overproduction of Rho1 and Cdc42 can suppress the temperature-sensitive growth defect of *cdc43*^{ts} mutants and the inviability of *cdc43* deletion mutants (66). The observation that Rho1 and Cdc42 functions are no longer dependent on Cdc43–GGTase-I when they are overproduced suggests that a Cdc43-independent protein prenyltransferase can prenylate and provide functional Rho1 and Cdc42. Our observation that the Ram1-FTase could prenylate the Rsr1 and Ras2-Leu peptides in vitro (Fig. 3) suggested that Ram1-FTase might prenylate Cdc42 and Rho1 in vivo, since the C termini of Cdc42 and Rsr1 are identical (CTIL) and the C termini of Rho1 (CVLL) and Ras2-Leu (CIIL) are similar.

Several in vivo experiments to assess whether Ram1-FTase could prenylate CaaX substrates that terminate with leucine were performed with the *cdc43* deletion strain overproducing Rho1 and Cdc42. Our observation that Ras2^{Val-19}-Leu was functional in this strain provides evidence that Ram1-FTase can prenylate CaaX substrates with C-terminal leucine in vivo. Additional genetic experiments, summarized below, implicate Ram1-FTase as the enzyme responsible for prenylating Rho1 and Cdc42 when they are overproduced in a *cdc43* deletion strain. The *cdc43* deletion strain overproducing Rho1 and Cdc42 was able to lose a *CDC43 URA3* plasmid and grow on 5-FOA medium when the *RAM1* gene was intact but not when it was deleted (Fig. 6) (66). Since the loss of Ras function alone would cause a *cdc43 ram1* double-deletion strain to be inviable, Ras2 was overproduced to bypass the requirement for Ras prenylation. The *cdc43 ram1* deletion strain overproducing Ras2, Rho1, and Cdc42 was not able to lose the *CDC43 URA3* plasmid (Fig. 6), suggesting that Ram1-FTase is required for prenylation of Rho1 and Cdc42 in a *cdc43* deletion strain. In addition, we have observed that the slow-growth phenotype of the *cdc43* deletion strain overproducing Rho1 and Cdc42 was partially suppressed by the overproduction of Ram1, again suggesting that Ram1-FTase can prenylate Rho1 and Cdc43 in vivo. However, the overproduction of Rho1 and Cdc42 appears to be required for effective prenylation of these proteins by Ram1-FTase, since Ram1 overproduction alone was unable to restore viability to a *cdc43* deletion strain with single-copy *RHO1* and *CDC42*. The incomplete nature of the suppression may be due in part to limiting amounts of the α subunit of Ram1-FTase (Ram2). There is also evidence suggesting that farnesylated Rho1 and Cdc42 are less functional than geranylgeranylated Rho1 and Cdc42. Ohya et al. (66) observe that the slow-growth phenotype of the *cdc43* deletion strain overproducing Rho1 and Cdc42 can be suppressed by substitution of the C-terminal leucines of Rho1 and Cdc42 with methionine, which is expected to convert these proteins to preferred substrates of the Ram1-FTase. This suppression appears to be complete when *RHO1*-Met and *CDC42*-Met are on high-copy-number plasmids but is not observed when *RHO1*-Met and *CDC42*-Met are present as single-copy chromosomal genes. Thus, Rho1 and Cdc42 appear to function less effectively with a farnesyl group than with a geranylgeranyl group.

The ability of Ram1-FTase and Cdc43–GGTase-I to substitute for each other in the prenylation of their essential substrates in vivo may appear to indicate that either a farnesyl or geranylgeranyl group can make Ras, Rho1, and Cdc42 functional. However, such a conclusion is premature since in vitro data indicate that Ram1-FTase and Cdc43–GGTase-I can use their nonpreferred lipid substrates, albeit at low efficiency. Our in vitro results (Fig. 3) show that Ram1-FTase, supplied with ^{3}H-GGPP as the only prenyl lipid substrate, can geranylgeranylate **a**-factor, one of its preferred protein substrates. In addition, weak prenylation of Ras2 and Rsr-Ser peptides with ^{3}H-GGPP was observed in *RAM1* extracts but not in *ram1*Δ extracts (data not shown), suggesting that Ram1-FTase can also geranylgeranylate Ras2 and Rsr1-Ser, albeit inefficiently. Mayer et al. (60) have shown that purified Cdc43–GGTase-I can prenylate Ras proteins terminating with CVVL and CAIL when ^{3}H-FPP is the only prenyl lipid provided, but the efficiency is about 10-fold lower than prenylation with ^{3}H-GGPP. We detected weak prenylation of Ras2-Leu (-CIIL) and Rsr1 (-CTIL) peptides with ^{3}H-FPP in reactions with extracts from a *ram1*Δ strain overproducing Cdc43 from a high-copy-number plasmid but not in reactions with extracts from a *ram1*Δ strain carrying the high-copy-number vector (data not shown). Unfortunately, *S. cerevisiae* cells do not take up radiolabeled intermediates of the prenyl lipid biosynthetic pathway well enough to allow radiochemical detection of the lipid attached to Ras2 by Cdc43–GGTase-I or to Rho1 and Cdc42 by Ram1-FTase in vivo. Most if not all of the Ras that was prenylated by Cdc43–GGTase-I would be expected to be geranylgeranylated. However, without direct measurement of the lipid attached to the Ras proteins, we cannot exclude the possibility that function was provided by a small subpopulation of Ras that may have been farnesylated by Cdc43–GGTase-I in vivo. Thus, the observations that Ras1, Ras2, and Ras2^{Val-19}-Leu were prenylated and made functional by Cdc43–GGTase-I in a *ram1*Δ strain suggest, but do not prove, that geranylgeranylated Ras1, Ras2, and Ras2^{Val-19}-Leu are functional. Likewise, most if not all of the Rho1 and Cdc42 prenylated by Ram1-FTase would be expected to be farnesylated, but we cannot exclude the possibility that this function was provided by a small subpopulation of Rho1 and Cdc42 that may have been geranylgeranylated by the Ram1-FTase in vivo.

Our observation that Ras2^{Val-19}-Leu was functional in wild-type yeast strains is similar to the previously published observation that Ras(61L)CVLL, a mutant form of activated H-Ras with the C-terminal serine changed to a leucine, is functional in mammalian cells. Activated Ras(61L)CVLL produces transformed foci in NIH 3T3 and Rat-1 cells that are indistinguishable in number and morphology from those

produced by activated Ras(61L)CVLS (17). The latter result has been interpreted to indicate that geranylgeranylated Ras(61L)CVLL is functional. Studies to determine whether the Ras(61L)CVLL is exclusively geranylgeranylated were unsuccessful because labeling of Ras(61L)CVLL was very low compared with that of Ras(61L)CVLS. If intracellular GGPP pools are substantially higher than FPP pools, the low labeling itself may indicate that Ras(61L)CVLL is predominantly geranylgeranylated rather than farnesylated, but these data cannot rule out the possibility that a small proportion of the Ras(61L)CVLL is farnesylated. Our observation that $Ras2^{Val-19}$-Leu was functional in a *cdc43* deletion strain, together with the in vitro data demonstrating farnesylation of Rsr1 and Ras2-Leu peptides, strongly suggests that Ram1-FTase can farnesylate Ras2 with a C-terminal leucine in vivo. These results emphasize the importance of determining the identity of the lipid group(s) attached to a protein before drawing firm conclusions about whether or not a geranylgeranyl group can functionally substitute for a farnesyl group.

There is emerging evidence that the difference in length between the 15-carbon farnesyl group and the 20-carbon geranylgeranyl group can affect Ras function. Cox et al. (17) recently reported that H-Ras with the C-terminal serine replaced with leucine [Ras(WT)CVLL] inhibits growth of NIH 3T3 cells unless it is overexpressed to the level required for cellular transformation. This dominant growth-inhibitory effect has been attributed to the geranylgeranyl group, since the lipid group would be the only difference, assuming that proteolysis and methylation occur properly. The possibility that a small amount of Ras(WT)CVLL is farnesylated rather than geranylgeranylated is less relevant in this case, since Ras(WT)CVLL has a dominant-negative phenotype that cannot be due to properly processed farnesylated Ras. The growth-inhibitory effect of Ras(WT)CVLL is not understood, but the reversal of the phenotype by coexpression of activated H-Ras suggests that Ras(WT)CVLL antagonizes endogenous Ras function (17). Preliminary results from our laboratory also suggest that Ras2-Leu prenylated by Cdc43–GGTase-I may function differently from farnesylated Ras2 (84a).

Future studies examining interactions of Ras (and Ras-related proteins) with membrane proteins and lipids should reveal more about the role of the farnesyl and geranylgeranyl lipid groups in membrane localization and function of Ras and Ras-related GTP-binding proteins and about the degree to which the two lipids are functionally interchangeable.

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