as well as the yeast enzyme and to inhibit the growth of *ras*-transformed fibrosarcoma,[9] the inhibitors isolated could act as anticancer drugs. The assays could also be used to evaluate synthetic inhibitors such as peptidomimetics because the yeast system provides simple *in vivo* assays. It is possible, however, that the effects of inhibitors are different between the yeast and mammalian enzymes and that the penetration and/or degradation of inhibitors are different between yeast and mammalian cells. Detailed comparison of the effects of farnesyltransferase inhibitors on yeast and mammalian cells needs to be carried out.

Acknowledgments

We thank Dr. Hirofumi Nakano (Kyowa Hakko Kogyo Co. Ltd.) for helpful comments on the *gpa1* assay. This work is supported by National Institutes of Health Grant CA41996. F. T. is an Established Investigator of the American Heart Association.

[6] Mutagenesis and Biochemical Analysis of Recombinant Yeast Prenyltransferases

By BRIAN E. CAPLIN and MARK S. MARSHALL

Introduction

A common posttranslational event occurring in eukaryotic cells is the prenylation of proteins.[1,2] The first indication that proteins could be prenylated came from the study of fungal mating factors.[3] The mating factors are covalently linked to the isoprenoid farnesyl. Studies of the **a**-factor mating peptide of *Saccharomyces cerevisiae* demonstrated that prenylation of the peptide was essential for function.[4] Since these early studies a growing number of proteins modified with geranylgeranyl and farnesyl have been identified in both mammalian cells and *S. cerevisiae*.[4,5] The modification of many of these proteins is essential for cell growth as has been demonstrated using genetic approaches for several *S. cerevisiae* proteins, including farnesylation of the RAS proteins and geranylgeranylation of the CDC42 and

[1] J. A. Glomset, M. H. Gelb, and C. C. Farnsworth, *Trends Biochem. Sci.* **15,** 139 (1990).
[2] J. B. Gibbs, *Cell (Cambridge, Mass.)* **65,** 1 (1991).
[3] Y. Kamiya, A. Sakurai, S. Tamura, and N. Takahashi, *Biochem. Biophys. Res. Commun.* **83,** 1077 (1978).
[4] W. R. Schafer and J. Rine, *Annu. Rev. Genet.* **30,** 209 (1992).
[5] P. J. Casey, *J. Lipid Res.* **33,** 1731 (1992).

RHO1 proteins.[6,7] Interest in the prenylation of proteins has increased with the discovery that farnesylation of oncogenic mammalian ras p21 is strictly required for cellular transformation.[8] Understanding the enzymology of the enzymes responsible for protein prenylation has become a key component of many anticancer drug development programs.[2]

Protein prenylation occurs via a thioether linkage to a cysteine residue near the carboxyl terminus of substrate proteins.[9] One protein farnesyltransferase (FTase) and two distinct protein geranylgeranyltransferases have been identified which catalyze these modifications.[4,5] The protein farnesyltransferase adds the 15-carbon isoprenoid farnesyl to substrate proteins which end in the sequence -CaaX, where C is Cys, a is usually an aliphatic amino acid, and X is Ala, Ser, Gln, Cys, or Met.[10,11] The type I geranylgeranyltransferase (GGTase I) modifies proteins which end in a -CaaL sequence, where L is Leu.[10,12] The type II geranylgeranyltransferase (GGTase II) recognizes substrate proteins which end in -CC or -CaC.[10,13]

Clues to the identity of the subunits of these transferases arose early as a result of genetic studies in *S. cerevisiae.* Mutations in the *RAM1/DPR1* and *RAM2* genes were found to affect the FTase, reducing the farnesylation of **a**-factor and RAS protein.[4] Similarly, mutations in the *CDC43/CAL1* and *RAM2* genes reduced the activity of the GGTase I, whereas mutations in *BET2* and *MAD2* affected GGTase II.[4] Biochemical characterization and molecular cloning of the genes and mammalian counterparts have shown that the genes are the structural genes for the various protein prenyltransferase subunits. The methods used to identify the biochemical nature of the gene products will in part be described in this chapter. The *RAM1/DPR1, CDC43/CAL1,* and *BET2* genes respectively encode the β subunits of the FTase and the type I and type II geranylgeranyltransferases.[14–16]

[6] S. Powers, S. Michaelis, D. Broek, S. S. Anna-A, J. Field, I. Herskowitz, and M. Wigler, *Cell (Cambridge, Mass.)* **47,** 413 (1986).

[7] C. E. Trueblood, Y. Ohya, and J. Rine, *Mol. Cell. Biol.* **13,** 4260 (1993).

[8] J. F. Hancock, A. I. Magee, J. E. Childs, and C. J. Marshall, *Cell (Cambridge, Mass.)* **57,** 1167 (1989).

[9] C. C. Farnsworth, M. H. Gelb, and J. A. Glomset, *Science* **247,** 320 (1990).

[10] S. L. Moores, M. D. Schaber, S. D. Mosser, E. Rands, M. B. O'Hara, V. M. Garsky, M. S. Marshall, D. L. Pompliano, and J. B. Gibbs, *J. Biol. Chem.* **266,** 14603 (1991).

[11] Y. Reiss, J. L. Goldstein, M. C. Seabra, P. J. Casey, and M. S. Brown, *Cell (Cambridge, Mass.)* **62,** 81 (1991).

[12] M. C. Seabra, Y. Reiss, P. J. Casey, M. S. Brow, and J. L. Goldstein, *Cell (Cambridge, Mass.)* **65,** 429 (1991).

[13] H. Horiuchi, M. Kawata, M. Katayama, Y. Yoshida, T. Musha, S. Ando, and Y. Takai, *J. Biol. Chem.* **266,** 16981 (1991).

[14] B. He, P. Chen, S. Y. Chen, K. L. Vancura, S. Michaelis, and S. Powers, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 4448 (1991).

[15] M. Mayer, B. E. Caplin, and M. S. Marshall, *J. Biol. Chem.* **267,** 20589 (1992).

The *RAM2* gene encodes an α subunit common to both FTase and GGTase I, whereas *MAD2* encodes the unique α subunit of the GGTase II.[15,17] The yeast prenyltransferase subunits are highly homologous to one another and to mammalian counterparts.[16] The *S. cerevisiae* enzymes are also mechanistically similar to the mammalian enzymes.[18] Because of this similarity, the yeast protein prenyltransferases present an attractive model system for investigating the structure–function aspects of each transferase and their subunits as well as the biological significance of this unique class of enzymes.

Experimental Rationale

This chapter presents a variety of protocols for characterizing protein prenyltransferase function in *S. cerevisiae* or recombinant transferases produced in bacteria. These methods have allowed assignment of functions to yeast gene products involved in protein prenylation, determination of transferase substrate requirements, and structure–function analysis of the β subunits of FTase and GGTase I. *Saccharomyces cerevisiae* strains containing conditional mutations affecting protein prenylation in the cell can be readily cultivated under permissive conditions which allow growth while retaining significant impairment of transferase activity. Cells can be easily lysed and the extracts quickly assayed for multiple protein prenyltransferases. In addition, the substrate specificity of each transferase in extracts from conditional mutant strains can be accurately assessed by using yeast RAS1 protein modified at the carboxyl terminus to encode any desired CaaX substrate sequence. The isoprenoid moieties attached to each substrate can then be determined by chemical cleavage and high-performance liquid chromatography (HPLC) analysis.

The *S. cerevisiae RAM2* and *CDC43/CAL1* genes were identified as the structural genes of the α and β subunits of the GGTase I by functional reconstitution of enzyme activity by coexpressing each gene in *Escherichia coli*.[15] The *RAM2* gene was subcloned by Scott Powers (Onyx Pharmaceuticals, Richmond, CA) into the chloramphenicol-selectable expression plasmid pBH57 and used with permission.[14] The *CDC43* gene was subcloned into the ampicillin-selectable pUC18 using the polymerase chain reaction (PCR). Cotransformation of the two plasmids results in the expression of authentic yeast GGTase I. Sufficient transferase can be purified from

[16] N. E. Kohl, R. E. Diehl, M. D. Schaber, E. Rands, D. D. Soderman, B. He, S. L. Moores, D. L. Pompliano, S. Ferro-Novick, S. Powers, K. A. Thomas, and J. B. Gibbs, *J. Biol. Chem.* **266,** 18884 (1991).

[17] M. S. Brown and J. L. Goldstein, *Nature* (*London*) **366,** 14 (1993).

[18] B. E. Caplin, L. A. Hettich, and M. S. Marshall, *Biochim. Biophys. Acta* **1205,** 39 (1994).

expressing *E. coli* cells to be used for quantitative analysis of substrate specificity. This technique has also proved useful for the biochemical characterization of genes encoding subunits of the yeast FTase and GGTase II. The yeast *RAM1* gene can be coexpressed with *RAM2* in *E. coli* to produce authentic FTase.[18] As with the recombinant GGTase I, sufficient FTase can be easily purified for quantitative analysis of substrate specificity.

The ease with which both the FTase and the GGTase I can be made and functionally analyzed in *E. coli* extracts provides a rapid and convenient method for structure–function analysis based on mutation of the component genes. The *RAM1* gene can be mutated using site-directed mutagenesis of a *RAM1* expression plasmid and the effects on FTase activity determined by coexpression with *RAM2*. The crude bacterial lysates can be rapidly analyzed for FTase activity or changes in substrate specificity. The carboxyl terminus of the RAM1 protein has been modified with an epitope tag for easy detection using a commercially available monoclonal antibody.

This chapter outlines the following techniques: growth and extract preparation of yeast strains containing temperature-sensitive mutations in the subunit genes of the FTase and GGTase I, protein prenyltransferase assays using yeast extracts, the use of alternative CaaX substrates in the assays, chemical cleavage and identification of protein prenyl modification, production of recombinant protein prenyltransferases in *E. coli*, analysis of transferase activity in bacterial extracts, purification of recombinant FTase and GGTase I from *E. coli*, and protein prenyltransferase assays using purified recombinant enzymes.

Materials

Chemicals

[^{3}H]Farnesyl diphosphate (FPP; 15 Ci/mmol) and [^{3}H]geranylgeranyl diphosphate (GGPP; 15 Ci/mmol) are purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Chemical reagents are obtained from United States Biochemical Co. (Cleveland, OH) and Sigma (St. Louis, MO). Restriction endonucleases and Vent polymerase are purchased from New England Biolabs (Beverly, MA). GF/F filters are from Whatman (Clifton, NJ). Bradford protein assay reagents are from Bio-Rad (Richmond, CA). Biosafe II scintillation cocktail and Fluorohance are obtained from Research Products International. Hyperfilm MP is purchased from Amersham (Arlington Heights, IL). Glass beads (425–600 μm, acid washed) are from Sigma. Construction and purification of the Ras-CaaX proteins are described elsewhere[10] (see also [10] by Cox in this volume). The Ras proteins are referred to in this chapter as Ras plus the attached tetrapeptide CaaX sequence (i.e., Ras-CVLS). Polypropylene Falcon tubes (17 × 25

mm) are purchased from Fisher. Anti-Glu-Glu-Phe antibody, YL1/2, for detection of the epitope-tagged RAM1 protein, is purchased from Serotec. Transformer site-directed mutagenesis kit and Trans Oligo *Afl*III/*Bgl*II are from Clonetech (Palo Alto, CA). The enhanced chemiluminescence detection kit (ECL kit) and horse-radish peroxidase (HRP)-labeled anti-mouse immunoglobulin G (IgG) antibodies are from Amersham.

Strains

The *S. cerevisiae* strains used are obtained from Scott Powers and Douglas Johnson.[6,14,19]

RS16-4C: *MATα his3 ade2 ade8 trp1 ura3 can1*
RS40-4C: *MATa ram1-1 his3 de8 trp1 ura3 can1*
RS51-3A: *MATα ram2-1 his3 ade8 trp1 ura3 can1*
CJ198-2B: *MATα cdc43-2 ura3 trp1 gal2*

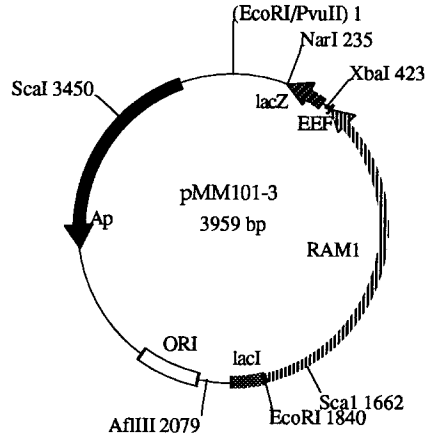
Media

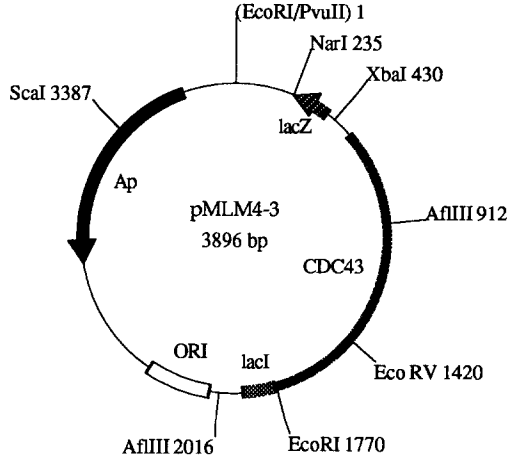
YPD: 2% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) glucose
Luria broth (LB): 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl
LBA: LB with 100 mg/ml ampicillin, sodium salt
LBC: LB with 10 mg/ml chloramphenicol
LBAC: LB with 100 mg/ml ampicillin sodium salt, 10 mg/ml chloramphenicol
Plates: 2% (w/v) Bacto-agar

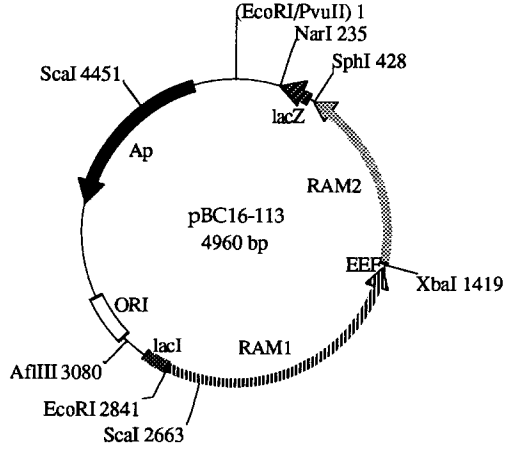
Buffers

Transferase buffer (10×): 500 m*M* sodium HEPES, pH 7.5, 50 m*M* dithiothreitol (DTT), 200 m*M* $MgCl_2$
Resuspension buffer: 50 m*M* sodium HEPES, pH 7.5, 5 m*M* DTT
Lysis buffer: 50 m*M* sodium HEPES, pH 7.5, 5 m*M* DTT, 1 m*M* EDTA, 1 m*M* EGTA, 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.1 m*M* pepstatin A, 0.1 m*M* leupeptin
Dialysis buffer: 20 m*M* Tris-HCl, pH 7.5, 1 m*M* DTT, 20 μM $ZnCl_2$
Mono Q buffer A: 50 m*M* Tris-HCl, pH 7.5, 1 m*M* DTT, 20 μM $ZnCl_2$
Mono Q buffer B: 50 m*M* Tris-HCl, pH 7.5, 1 m*M* DTT, 20 μM $ZnCl_2$, 1.0 *M* $NaCl_2$
Transferase storage buffer: 50 m*M* HEPES, pH 7.5, 1 m*M* DTT, 20 μM $ZnCl_2$, 20% glycerol, 0.1% polyethylene glycol 6000 (PEG 6000)

[19] A. A. Finegold, D. I. Johnson, C. C. Farnsworth, M. H. Gelb, S. R. Judd, J. A. Glomset, and F. Tamanoi, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 4448 (1991).


(EcoRI/PvuII) 1
NarI 235
XbaI 423
ScaI 3450
lacZ
EEF1
pMM101-3
3959 bp
Ap
RAM1
ORI
lacI
ScaI 1662
AflIII 2079
EcoRI 1840


(EcoRI/PvuII) 1
NarI 235
XbaI 430
ScaI 3387
lacZ
Ap
pMLM4-3
3896 bp
AflIII 912
CDC43
ORI
lacI
Eco RV 1420
AflIII 2016
EcoRI 1770


(EcoRI/PvuII) 1
NarI 235
SphI 428
ScaI 4451
lacZ
Ap
RAM2
pBC16-113
4960 bp
EEF1
XbaI 1419
ORI
lacI
RAM1
AflIII 3080
EcoRI 2841
ScaI 2663

HPLC solvent A: 50% CH_3CN, 25 m*M* H_3PO_4
HPLC solvent B: 95% CH_3CN, 25 m*M* H_3PO_4
2× SDS sample buffer: 130 m*M* Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol
Fixing solution: 40% methanol, 10% concentrated acetic acid
Towbin buffer: 190 m*M* glycine, 25 m*M* Tris-HCl, pH 8.3, 1.3 m*M* SDS, 20% methanol
TBS-T: 20 m*M* Tris-HCl, pH 7.6, 137 m*M* NaCl, 0.1% Tween 20
Western blocking buffer: TBS-T plus 5% swine skin gelatin

Plasmids

Figure 1 gives the restriction maps for plasmids made specifically for these methods.

pBH57: originally described by He *et al.*[14] Expresses *RAM2* from the *lac* promoter, chloramphenicol resistant
pMLM4: *CDC43 E. coli* expression plasmid constructed by inserting the *CDC43* gene (generously provided by Douglas Johnson) as an *Eco*RI–*Xba*I cassette into the *Eco*RI–*Xba*I sites of pUC18. The cassette was generated by polymerase chain reaction using Vent polymerase and oligonucleotide primers (5′-CGCGAATTCTGGAAAAATGTGTCAAG-3′ and 5′-CGCTCTAGATTAAAATTCTTCAAAACAGCACCTTTTAC-3′) using YEp(43)2 as the template[19]
pMM101: *RAM1 E. coli* expression plasmid constructed by inserting the *RAM1* gene (a gift from Scott Powers) as an *Eco*RI–*Xba*I cassette into the *Eco*RI–*Xba*I sites of pUC18. The cassette was generated by polymerase chain reaction using Vent polymerase and oligonucleotide primers (5′-CGCGAATTCAAAGCGTATGCGACAG-3′; 5′-CGCTCTAGATTAAAATTCTTCACTTGAGAAGATAATTG-3′) using pBH53 as a template. The 3′ primer introduced the amino acids Glu-Glu-Phe at the C terminus of the gene product to be used as an epitope tag
pBC16-113: *E. coli* FTase integrated expression plasmid which expresses both *RAM1* and *RAM2* as an operon. Constructed by insertion of an oligonucleotide cassette (top strand 5′-CTAGAGGAGAACAGATGGTACCTTTGGATCCGCATG-3′; bottom strand 5′-CGGATCCAAAGGTACCATCTGTTCTCCT-3′) containing a

FIG. 1. Restriction maps of expression plasmids specifically designed in this laboratory for prenyltransferase subunit expression.

Shine–Dalgarno Box and a translation start codon into the *Xba*I–*Sph*I sites of pMM101. The *RAM2* gene from pBH57 was subcloned into the cassette, adjacent to *RAM1* in-frame with the new initiation codon, as a *Bam*HI–*Kpn*I fragment

Growth and Lysis of *Saccharomyces cerevisiae* Strains

Reagents. YPD media, resuspension buffer, lysis buffer, and glass beads are required.

Saccharomyces cerevisiae Strains. Yeast strains RS16-4C, RS40-4C, RS51-3M, and CJ198-28 are used.

Procedure. Inoculate 150 ml YPD with an appropriate yeast strain. Grow cells to an OD_{660} of 0.25–0.75 at 24°. Pellet cells at 3000 *g* for 10 min at 4°. Remove the supernatant and wash cells in ice-cold resuspension buffer. Pellet cells again at 4°. Resuspend cells in 0.8 ml lysis buffer and transfer to a polypropylene microcentrifuge tube. Add glass beads to 50–70% of the final volume. Lyse the cells by alternate high-speed vortexing for 30 sec followed by cooling on ice. After the fourth round of vortexing, monitor the degree of cell lysis by light microscopy. Repeat vortexing as necessary to achieve greater than 90% cell lysis. Transfer the lysate to a fresh microcentrifuge tube and pellet the cell debris by centrifugation at 10,000 *g* for 10 min at 4°. Transfer the cleared lysate to a fresh microcentrifuge tube and determine the protein concentration by the Bio-Rad assay. Dilute each lysate to a final protein concentration of 2 μg/μl.

Prenyltransferase Assay with Crude Yeast Extracts

Reagents. Required reagents include cleared *S. cerevisiae* lysates, Ras-CVLS, -CVIM, -CAIL, and -SVLS proteins, 10× transferase buffer, [^{3}H]FPP, [^{3}H]GGPP, 1.0 *M* HCl in ethanol, GF/F filters, 100% ethanol, and Biosafe II scintillation cocktail.

Procedure. Transfer 25 μl of each lysate (50 μg protein) to fresh microcentrifuge tubes. Add 5 μl of 10× transferase reaction buffer. Add 5.25 μg of Ras-CaaX protein to yield a final concentration of 5 μ*M*. Bring the volume to 49 μl and prewarm the tubes at 30° for 2 min. Add 1.0 μl of a 15 Ci/mmol solution of [^{3}H]FPP or [^{3}H]GGPP (67 pmol) and allow the reaction to proceed for 40 min. Terminate each reaction by adding 0.95 ml of 1.0 *M* HCl in ethanol. This precipitates the Ras protein. Protein precipitation in acid/ethanol proceeds at room temperature for 15 min. The precipitation mixture is transferred to ethanol-wetted GF/F filters and filtered very slowly (~5 ml/min) using a vacuum manifold. The filters are washed four times with 2-ml volumes of 100% ethanol by slow filtration.

The filters are then blotted dry for 10 min and counted in 5 ml of Biosafe II liquid scintillation cocktail. When desired, fluorography of the transferase reactions can be performed by removing a small aliquot of the reaction mix prior to the termination step (see fluorography protocol below).

Table I presents the results of a typical determination of prenyltransferase activity in extracts made from yeast strains defective in the β subunits of the FTase (*RAM1*) and GGTase I (*CDC43*). Each extract was assayed for the ability to transfer either FPP or GGPP to Ras-CVLS, -CVIM, or -CAIL and compared to a wild-type extract. By comparing the substrates prenylated by extracts from cells that possess only functional FTase (*cdc43*) or GGTase I (*ram1*), it can be observed that both transferases share some common specificities (Table I). The *RAM1*-dependent FTase is responsible for the majority of farnesylation reactions, including weak farnesyl modification of Ras-CAIL in the *cdc43* strain. The FTase present in the *cdc43* strain is also quite capable of attaching geranylgeranyl to both Ras-CVIM and -CAIL substrates as well. The *CDC43*-dependent GGTase I of the *ram1* strain, while less active in this assay than the FTase, is observed to geranylgeranylate Ras-CAIL and to farnesylate Ras-CVIM.

Fluorography of Isoprenyltransferase Reactions

Reagents. Prenyltransferase reactions, 2× SDS sample buffer, 12% SDS–polyacrylamide gel, fixing solution, and Fluorohance are needed.

Procedure. Prior to terminating isoprenyltransferase reactions (see above) with acid/ethanol, transfer a 10-μl aliquot of the reaction mixture

TABLE I
PRENYLATION OF REPRESENTATIVE CaaX SUBSTRATES IN MUTANT *Saccharomyces cerevisiae* CELL EXTRACTS[a]

	FPP (pmol/mg)			GGPP (pmol/mg)		
CaaX substrate	WT	*ram1*	*cdc43*	WT	*ram1*	*cdc43*
CVLS	25.0	1.0	35.0	2.0	0.8	1.4
CVIM	38.9	7.9	35.6	21.9	2.2	36.5
CAIL	5.7	0.3	7.2	13.4	6.0	1.0

[a] Wild-type (WT), *ram1*, and *cdc43* yeast strains were grown to mid-logarithmic phase at 25°, lysed, and soluble extracts prepared. Ras-CaaX substrates were added to 5 μM with 67 pmol of either [^{3}H]FPP or [^{3}H]GGPP. Addition of isoprenoid to the protein substrates was detected by the filter binding assay. Counts from Ras-SVLS were subtracted from each sample as background. Results are representative of three experiments and are expressed as picomoles of isoprenoid incorporated per milligram yeast extract. From Caplin *et al.*[18]

to a fresh microcentrifuge tube, containing 10 μl of 2× SDS sample buffer. Boil each sample for 5 min. Load samples on a 12% Laemmli gel and electrophorese according to standard protocols until the dye front has reached the bottom of the gel. (Note: Free [^{3}H]FPP and [^{3}H]GGPP migrate with the dye front and may be kept on the gel for lane reference.) Transfer the gel to fixing solution, shaking slowly for 30 min. Wash the gel in distilled water, for 30 min. Remove the water and cover the gel with Fluorohance and continue shaking for 30 min. Lay the gel flat on blotting paper and dry under vacuum for 90 min at 80°. Expose to X-ray film for 1 week at −80°.

Chemical Cleavage and Identification of Isoprenoid Moieties

Reagents. Required reagents include prenylated Ras protein product, 15% trichloroacetic acid (TCA), HPLC-grade acetone (Fisher), HPLC-grade acetonitrile, HPLC solvent A, HPLC solvent B, formic acid, methanol, chloroform, farnesol, geranylgeraniol, Biosafe II scintillation cocktail, N_2 gas, Na_2CO_3, methyl iodide (CH_3I), and a 4.6 × 250 mm C_{18} reversed-phase HPLC column (Beckman, Palo Alto, CA).

Procedure. The protocol is performed basically as previously described by Casey *et al.*[20] Prenylated Ras-CaaX protein is precipitated with 0.5 ml of 15% TCA on ice for 15 min. Recover the precipitate by centrifugation at 10,000 *g* for 10 min at 4°. Delipidate the sample with three 6-h washes in HPLC-grade acetone and two 12-h washes in chloroform/methanol (1:2, v/v), each at 0°. End-over-end rotation works well for these washes followed by brief centrifugation to recover the pellet. Pellets are air-dried and resuspended in 0.6 ml of 0.5% formic acid (Aldrich, Milwaukee, WI).

Cleavage reactions are initiated by the addition of 0.1 ml of CH_3I. Reactions proceed at room temperature for 48 hr in the dark. Cleavage reactions are terminated by the addition of 0.03 ml 35% Na_2CO_3, followed by an additional 12 hr in the dark. The sample is then extracted three times with 0.4 ml of chloroform/methanol (9:1, v/v). The organic phases are combined and dried under a stream of N_2 gas at 4°. Resuspend the sample in 0.2 ml of HPLC solvent A. One microgram each of farnesol and geranylgeraniol are added to the sample as standards. Samples are analyzed with a linear gradient of 0–15% HPLC solvent B, over 22.5 ml, and 15–100% of HPLC solvent B over 58.5 ml at a flow rate of 1 ml/min. Wash the column with 20 ml of solvent B followed by 10 ml of solvent A. One-milliliter fractions are collected and dried to 0.1 ml in a fume hood to remove CH_3CN, then counted in 5 ml of Biosafe II scintillation cocktail.

[20] P. J. Casey, P. A. Solski, C. J. Der, and J. E. Buss, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 8323 (1991).

The elution times of farnesol and geranylgeraniol standards are detected by measuring the absorption of 210 nm light.

Figure 2 shows the results of a similar assay using Ras-CTIL protein modified in a wild-type yeast extract in the presence of both [^{3}H]FPP and [^{3}H]GGPP. As predicted from the experiment shown in Table I, the CTIL sequence is dually modified with both farnesyl and geranylgeranyl, with geranylgeraniol as the major cleavage product.

Production of Recombinant Protein Prenyltransferases in *Escherichia coli* Using Subunit Coexpression

Cotransformation and Coselection of Transformants

Reagents. Plasmids pUC18, pBH57, pMM101-3, and pMLM4-3 along with LB, LBAC agar plates, and transformation-competent *E. coli* HB101 are needed.

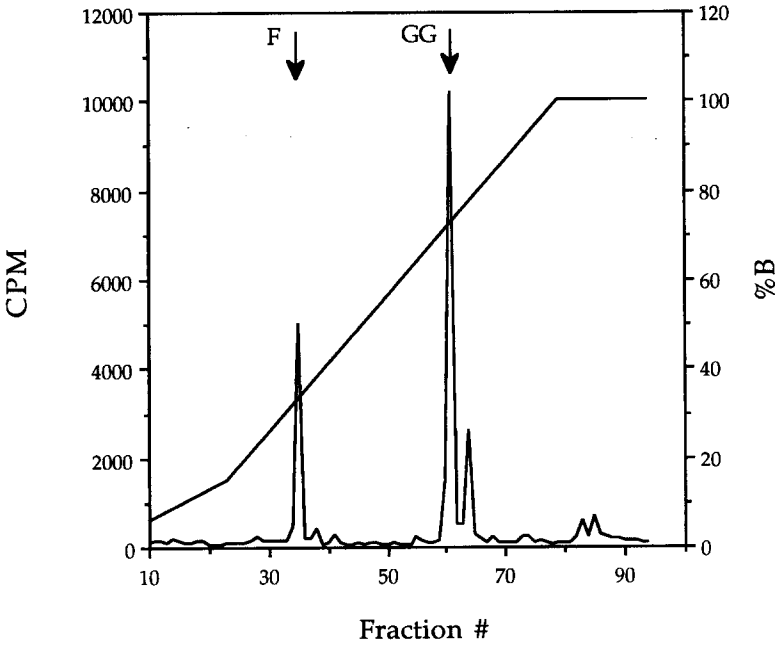


FIG. 2. Determination of the isoprenoid moiety attached to Ras-CTIL in *S. cerevisiae* cell extracts. Ras-CTIL (5 μM) was incubated together with 67 pmol each of [^{3}H]FPP and [^{3}H]GGPP in a soluble *S. cerevisiae* cell extract. The amount of attached farnesyl and geranylgeranyl was determined by chemical cleavage and product identification using reversed-phase HPLC. Standards are indicated by F (farnesol) and GG (geranylgeraniol). (From Caplin *et al.*[18])

Procedure. One to two hours prior to transformation prewarm and dry the plates to be used in a 37° incubator. To fresh 17 × 25 mm polypropylene tubes (Fisher) add 0.5 μg of pBH57 plasmid DNA with 0.5 μg of pMM101-3 to make FTase, pMLM4-3 for GGTase I, or pUC18 as a negative control. Tubes are precooled on ice, 0.1 ml of competent HB101 *E. coli* is added, and tubes are held on ice for 30 min. The transformations are then transferred to 42° for 45 sec and subsequently held on ice for 2 min. Following heat shock add 0.8 ml of fresh Luria broth to each transformation sample and incubate the cells at 37° for 1 hr, shaking at 225 rpm. Following the incubation, plate 0.2 ml cells per LBAC plate. Incubate the plates overnight at 37°.

Induction of Recombinant Protein Prenyltransferases

Reagents. For protein induction use freshly cotransformed *E. coli* HB101. Sterile inoculation loops, LBAC, 15-ml polystyrene conical tubes, isopropylthio-β-D-galactoside (IPTG), ice-cold resuspension buffer, and ice-cold lysis buffer are also needed.

Procedure. Transfer 10 fresh colonies from each cotransformation plate into 5 ml of LBAC using a sterile inoculating loop. Grow cells at 37°, shaking at 300 rpm, to an OD_{660} of around 0.4. Add IPTG to a final concentration of 0.5 m*M* and continue the incubation for another 4 hr. Pellet the cells at 4° and 3000 *g* for 15 min. Remove the supernatant and resuspend the cells in ice-cold resuspension buffer. Pellet the cells again at 4° (3000 *g*, 15 min). Remove the supernatant and resuspend the cells in 0.25 ml of ice-cold lysis buffer in a microcentrifuge tube. Keep the cells ice-cold at all times. Alternatively, the induced cell pellets can be stored for several weeks at −80°.

Isoprenyltransferase Assays in *Escherichia coli* Extracts

Reagents. Required reagents include induced *E. coli* expressing either FTase or GGTase I, 10× transferase buffer, Ras-CaaX proteins, [^{3}H]FPP, [^{3}H]GGPP, 1.0 *M* HCl in ethanol, Whatman GF/F filters, 100% ethanol, Biosafe II scintillation cocktail, and Bio-Rad protein assay reagents.

Procedure. Lyse cells in 0.25 ml of lysis buffer at 4° by sonication with two 30-sec bursts at 35% power using a Fisher Model 300 sonicator with a micro tip. Make certain cells are kept cold during sonication. Pellet cell debris by centrifugation at 4° and 10,000 *g* for 10 min. Transfer the cleared lysate to a fresh microcentrifuge tube and measure the protein concentration by the Bio-Rad assay. Dilute the lysate to a final concentration of 2 μg/μl. Transfer 34 μg of protein to one microcentrifuge tube for each reaction, add 5 μl of 10× transferase buffer, 5 μ*M* of Ras-CaaX protein,

and sufficient double-distilled water to bring the reaction volume to 50 μl. Prewarm the reactions at 30° for 1 min, followed by the addition of 34–67 pmol of [^{3}H]FPP or [^{3}H]GGPP. Farnesyl transferase reactions proceed for 10 min, and geranylgeranyl transferase reactions proceed for 40 min. Terminate the reactions by the addition of 0.95 ml of 1.0 *M* HCl in ethanol. Precipitation of proteins, filtering, and washing are as for assays using crude *S. cerevisiae* extracts (see above). A comparison of the activity and specificity of recombinant FTase and GGTase I is shown in Table II.

Purification of Recombinant Protein Prenyltransferases

Large-Scale Growth and Induction

Reagents. Escherichia coli HB101 cotransformed with either pMM101-3/pBH57, pMLM4-3/pBH57, or the *RAM1 RAM2* operon plasmid pBC16-113 is needed, along with LBA, LBAC, IPTG, resuspension buffer, and lysis buffer.

Procedure. One fresh, confluent plate of HB101 *E. coli* transformed with protein prenyltransferase subunit expression plasmids is scraped into 1 liter of appropriate medium. The cells are grown at 37° while shaking at

TABLE II
PRENYLATION OF REPRESENTATIVE CaaX SUBSTRATES BY RECOMBINANT *Saccharomyces cerevisiae* PROTEIN PRENYLTRANSFERASES[a]

	FPP (pmol/mg)		GGPP (pmol/mg)	
CaaX substrate	FTase	GGTase I	FTase	GGTase I
CVLS	18,844	30	88	14
CVIM	40,482	218	10,341	5103
CAIL	2041	596	34	11,046
CIIC	37,076	111	40	371
CIIS	53,267	119	252	39
CIIL	4627	1446	2313	14,371
CTIL	4966	1300	82	12,548

[a] Recombinant FTase and GGTase I were incubated with 5 μ*M* each of Ras-CaaX substrates in the presence of 67 pmol [^{3}H]FPP or 34 pmol [^{3}H]GGPP. The Ras-CIIS, -CIIC, -CTIL, and CIIL are naturally occurring CaaX sequences in yeast. Isoprenylation of Ras-Caax substrates was measured by the filter binding assay. Counts obtained from Ras-SVLS were subtracted from each sample as background. Results are representative of at least three experiments and are expressed as picomoles of isoprenoid incorporated per milligram enzyme preparation.

300 rpm. When the cells reach an OD_{660} of 0.4, IPTG is added to a final concentration of 0.5 m*M*. The cells are then cultured for an additional 4 hr. After induction, chill the culture on ice. During the purification keep all reagents and cell products on ice. Harvest cells by centrifugation at 3000 *g* for 10 min (4°). Wash cells in ice-cold resuspension buffer, pellet cells again, and resuspend in 15 ml of ice-cold lysis buffer.

Cell Lysis and Extract Preparation

Reagents. Induced *E. coli* cells, prepared as above and resuspended in cold lysis buffer, enzyme-grade ammonium sulfate, dialysis tubing, 8 liters ice-cold dialysis buffer, and Bio-Rad protein assay reagents are required.

Procedure. The following method for partial purification of recombinant prenyltransferases is based on an original protocol by Reiss *et al.*[21] Lyse cells with four 30-sec bursts at 70% power using a Fisher Model 300 sonicator (large tip). Pellet cell debris by centrifugation at 10,000 *g* for 15 min (4°), then transfer the cleared lysate to a 50-ml polypropylene tube. Add cold lysis buffer to bring the volume to 20 ml. While stirring at 4°, slowly add solid ammonium sulfate to make a 30% solution. Continue stirring for 30 min in the cold. Pellet the precipitated material by centrifugation at 12,000 *g* for 20 min at 4°. Transfer the supernatant to a fresh 50-ml tube. Add solid ammonium sulfate, as above, to make a final concentration of 50%. Maintain stirring for 30 min. Pellet the precipitated material as above and remove the supernatant. The majority of the prenyltransferase activity should be in the 50% pellet. Dissolve the 50% pellet in 10 ml of resuspension buffer. Transfer each sample to dialysis tubing and dialyze against 4 liters of ice-cold dialysis buffer for 4 hr. Change to fresh buffer and dialyze an additional 12 hr. Analyze 1–5 μl of dialyzate for transferase activity as described for crude cell extracts. Determine protein concentration by the Bio-Rad assay.

Ion-Exchange Chromatography

Reagents. The protocol requires dialyzed active fractions from ammonium sulfate-precipitated *E. coli* extracts from cotransformants expressing recombinant FTase or GGTase I, 1.0 *M* stock Tris-HCl, pH 7.5, ice-cold Mono Q buffers A and B, a 10-ml Mono Q column, and transferase storage buffer.

Procedure. The dialyzed fraction is brought to 50 m*M* Tris-HCl, pH 7.5, by the addition of 1.0 *M* Tris-HCl, pH 7.5. The sample is applied to an HR10/10 Mono Q column equilibrated in Mono Q buffer A using a

[21] Y. Reiss, J. L. Goldstein, M. C. Seabra, P. J. Casey, and M. S. Brown, *Cell* (*Cambridge, Mass.*) **62,** 81 (1991).

Pharmacia (Piscataway, NJ) FPLC (fast protein liquid chromatography) system at a flow rate of 0.5 ml/min. Following application, the column is washed with 10 column volumes of the same buffer. Elution of prenyltransferase is accomplished using a linear gradient of 0–100% Mono Q buffer B (1.0 *M* NaCl) over 140 ml at a flow rate of 1 ml/min. Fractions are collected with a 2 ml volume. The FTase activity elutes at around 0.3 *M* NaCl, whereas GGTase I activity elutes at approximately 0.4 *M* NaCl. Assay every other fraction for prenyltransferase activity and protein concentration. Dialyze active fractions against 1 liter of Mono Q buffer A. Store active fractions in transferase storage buffer at −80°. Additional purification can be accomplished by size-exclusion chromatography using a Superose 12 column (Pharmacia).

Using this basic protocol approximately 28,000 units of FTase and 8800 units of GGTase I are obtained per liter of initial culture. The unit definition of prenyltransferase activity was set by Reiss *et al.*[21] as picomoles [^{3}H]isoprenoid transferred to Ras-CaaX per milligram of prenyltransferase per hour at 37°. Our unit determination uses the physiologically optimum temperature for yeast growth of 30°. Usually 12 units of purified prenyltransferase is sufficient for most experimental assays.

Site-Directed Mutagenesis of *RAM1*

Reagents. Required reagents include pMM101-3, mutagenic oligonucleotide, selection oligonucleotide (converts the unique *Afl*III site of pMM101-3 to *Bgl*II), and transformer site-directed mutagenesis kit.

Procedure. The protocol of Deng and Nickoloff[22] is used to introduce specific mutations into the *RAM1* gene in the pMM101-3 expression plasmid, eliminating the need for further subcloning of the mutated gene. Basically the target plasmid is denatured and annealed with a mixture of the mutagenic oligonucleotide and a specifically designed selection oligonucleotide. The selection oligonucleotide removes a restriction enzyme site unique to the plasmid, replacing it with a new restriction site. After an initial passage through a *mutS* bacterial strain, wild-type plasmids can be selected against by digesting the DNA with the restriction enzyme for the original site. Mutated plasmids will no longer have the recognition site. With the pMM101-3 plasmid and the *Afl*III/*Bgl*II Trans Oligo, the frequency of introducing the desired *RAM1* codon change along with the new selection site is near 100%. Mutants are identified by DNA sequencing. Mutagenic oligonucleotides should be designed as recommended by Clonetech. When using the *Afl*III/*Bgl*II Trans Oligo (5′-CAGGAAAGAAGATCGAGC-

[22] W. P. Deng and J. A. Nickoloff, *Anal. Biochem.* **200,** 81 (1992).

AAAG-3′) and pMM101-3, mutagenic oligonucleotides should be designed to hybridize with the coding strand of *RAM1*. That is, they should have an antisense sequence. Typical mutagenic oligonucleotides used are as follows (the mutated amino acids are given in brackets): *RAM1*[F261N262], 5′-CTGTAGCACAGAAATTAAAGCCTCCGTGTG-3′; *RAM1*[Y263], 5′-CTGTAGCACACAGTAAGTATAGCCTCCGTGTG-3′; *RAM1*[S309], 5′-CCAAAAACTATAGGAACCGTCAAC-3′.

Mutated variants of the *RAM1* gene can be rapidly analyzed for functional reconstitution of FTase on coexpression with *RAM2* in *E. coli* as described above. In Table III a comparison is given of the activities and substrate specificities of the *RAM1*[F261N262], *RAM1*[Y263], and *RAM1*[S309] mutants. RAM1 protein expression can be confirmed by immunoblotting as described below.

Immunoblotting of RAM1 Protein Containing EEF Epitope

Reagents. Standard SDS–polyacrylamide gel electrophoresis (SDS–PAGE) reagents, 10% Laemmli gel, nylon-backed nitrocellulose, Towbin buffer, Western blocking buffer, TBS-T, YL1/2 antibody, HRP-coupled anti-mouse IgG antibody, and ECL kit are needed.

Procedure. The RAM1 protein made from the pMM101-3 plasmid has a tripeptide Glu-Glu-Phe (EEF) tag at the carboxyl terminus which is

TABLE III
PRENYLATION OF REPRESENTATIVE CaaX SUBSTRATES BY MUTANT *Saccharomyces cerevisiae* PROTEIN FARNESYLTRANSFERASE[a]

	FPP (cpm)		GGPP (cpm)	
RAM1 mutant	CIIS	CIIM	CIIL	CIIM
WT	291,292	333,975	233,061	223,365
F261N262	279,330	391,703	197,230	257,807
Y263	349,371	368,253	210,721	194,200
S309	−83	1886	−619	389

[a] Wild-type and mutant *RAM1* genes were coexpressed with *RAM2* in *E. coli*, and crude extracts were prepared and analyzed for FTase activity as described in the text. Ras-CaaX substrates were chosen as the most suited to detect changes in substrate specificity. Isoprenylation of Ras-CaaX substrates was measured by the filter binding assay. Counts obtained from pUC8/*RAM2* extracts were subtracted from each sample as background. Results are the average of duplicate data points and are expressed as counts per minute (cpm) to demonstrate the magnitude of raw counts observed with this assay.

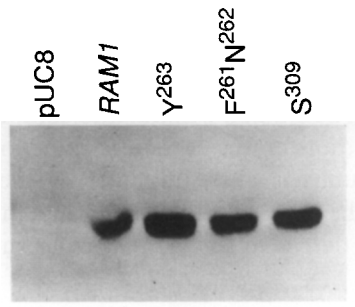


FIG. 3. Immunoblot analysis of wild-type and mutant RAM1 proteins tagged with the EEF epitope. *RAM1* genes were coexpressed with the *RAM2* gene, and soluble extracts made. Ten micrograms of protein was loaded in each lane and analyzed by SDS–PAGE followed by electrotransfer to nitrocellulose and immunodetection with the YL1/2 antibody. A negative control was made by IPTG induction of bacteria cotransformed with pUC8 and the pBH53 *RAM2* plasmid.

readily detected by a commercially available monoclonal antibody.[23] To detect the protein, boil 5 μl of the bacterial extracts used for prenyltransferase assays in 2× SDS sample buffer. Include appropriate control extracts as shown in Fig. 3. Separate proteins by Laemmli SDS–PAGE on a 10% gel. Following electrophoresis, transfer proteins to nitrocellulose by semidry transfer with Towbin buffer for 2 hr at 0.8 mA/cm^2 of gel. After transfer, block the nitrocellulose membrane with Western blocking buffer for 1 hr at room temperature on an orbital shaker. Rinse the membrane with two changes of TBS-T followed by one 15-min and two 5-min washes with TBS-T. At room temperature incubate the membrane in a 0.5 μg/ml dilution of the YL1/2 antibody in TBS-T for 1 hr. Wash as above. Incubate the membrane for 1 hr with a 1:1000 dilution of HRP-conjugated anti-mouse IgG antibody in TBS-T. Wash with one 15-min and four 5-min changes of TBS-T. Expose the blot with ECL kit detection reagents according to the manufacturer's specifications. Figure 3 shows a typical immunoblot detecting soluble RAM1 proteins in *E. coli* extracts.

Summary

The use of the *S. cerevisiae* protein prenyltransferases as a model system for general prenyltransferase study is justified by the similarity of mechanism, substrate specificity, and evolutionarily conserved substrates with the mammalian prenyltransferases. Genetic identification of potential struc-

[23] R. H. Skinner, S. Bradley, A. L. Brown, N. J. E. Johnson, S. Rhoades, D. K. Stammers, and P. N. Lowe, *J. Biol. Chem.* **266,** 14163 (1991).

tural genes involved in prenyltransferase activity can be easily confirmed with biochemical assays using recombinant enzyme reconstitution. Yeast FTase and GGTase I produced in *E. coli* are indistinguishable from the native proteins and can be studied without interference from contaminating cellular protein prenyltransferases. Structure–function analysis of the yeast prenyltransferase subunits is also simplified by the rapidity with which mutant enzymes can be analyzed in *E. coli* and their biological activity characterized in yeast defective for the particular subunit gene.

Acknowledgments

The authors thank Scott Powers and Douglas Johnson for providing all original DNA clones and yeast strains and Charlie Mantel for assistance with HPLC. We would also like to acknowledge original protocols by Y. Reiss for partial purification of FTase and P. Casey for identification of attached isoprenoid species which were adapted to the study of the yeast prenyltransferases. This study was supported in part by the American Cancer Society.

[7] Characterization of Protein Prenylation in *Saccharomyces cerevisiae*

By DAVID A. MITCHELL and ROBERT J. DESCHENES

Introduction

A growing number of membrane-localized proteins require posttranslational modification of a C-terminal sequence motif called the CaaX box, where C is Cys, a is generally an aliphatic residue, and X is the C-terminal residue.[1–3] The steps in the modification pathway include prenylation of the CaaX box cysteine, proteolytic removal of the -aaX residues, methyl esterification of the newly exposed α-carboxyl group, and palmitoylation of a second cysteine often found within 1–6 residues of the prenylated cysteine. The importance of these steps has been established by studying the effect of mutating the CaaX box,[4–6] isolating mutations in genes encod-

[1] S. Clarke, *Annu. Rev. Biochem.* **61,** 355 (1992).
[2] W. R. Schafer, and J. Rine, *Annu. Rev. Genet.* **26,** 209 (1992).
[3] A. D. Cox and C. J. Der, *Curr. Opin. Cell Biol.* **4,** 1008 (1992).
[4] K. Kato, A. D. Cox, M. M. Hisaka, S. M. Graham, J. E. Buss, and C. J. Der, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6403 (1992).
[5] R. J. Deschenes and J. R. Broach, *Mol. Cell. Biol.* **7,** 2344 (1987).
[6] B. M. Willumson, K. Norris, A. G. Papageorge, N. L. Hubbert, and D. R. Lowy, *EMBO J.* **3,** 2581 (1984).