RAM2, an essential gene of yeast, and RAM1 encode the two polypeptide components of the farnesyltransferase that prenylates a-factor and Ras proteins

(CAAX motifs/membrane targeting/post-translational modification/extragenic suppressors)

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Communicated by Leland Hartwell, September 23, 1991

ABSTRACT In the yeast Saccharomyces cerevisiae, mutations in either of two unlinked genes, RAM1 or RAM2, abolish the farnesyltransferase activity responsible for prenylation of Ras proteins and the a-factor mating pheromone. Here we report that the function of RAM1 and RAM2 genes is required for the membrane localization of Ras proteins and a-factor. The RAM2 gene was sequenced and can encode a 38-kDa protein. We examined the functional interaction of RAM2 and RAM1 by expressing the genes in Escherichia coli. Extracts derived from an E. coli strain that coexpressed RAM1 and RAM2 efficiently farnesylated a-factor peptide and Ras protein substrates. In contrast, extracts derived from E. coli strains that expressed either RAM gene alone were devoid of activity; however, when the latter extracts were mixed, protein farnesyltransferase activity was reconstituted. These results indicate that the yeast farnesyl-protein transferase is comprised of Ram1 and Ram2 polypeptides. Although Ram1 is a component of the enzyme, disruption of the RAM1 gene in yeast was not lethal, indicating that the Ram1-Ram2 farnesyltransferase is not essential for viability. In contrast, disruption of RAM2 was lethal, suggesting that Ram2 has an essential function in addition to its role with Ram1 in protein farnesylation.

The post-translational modification of proteins by the covalent attachment of isoprenoids plays an important role in the membrane targeting of various proteins (1–5). Three classes of prenylated proteins in eukaryotic cells have been described. The first class, represented by certain fungal mating pheromones, Ras proteins, and nuclear lamins, is initially synthesized with a C-terminal CAAX sequence, where X = Ser, Cys, Met, or Ala, and is modified by thioether linkage of a C15 farnesyl group to the cysteine residue (6–9). The second class of prenylated proteins, represented by the gamma subunits of certain heterotrimERIC G proteins and various Ras-like proteins, is initially synthesized with different C-terminal CAAX sequences, in which X = Leu or Phe, and is modified by the attachment of a C20 geranylgeranyl group to the cysteine residue (10–12). Members of both of these classes of prenylated proteins are subsequently processed by proteolytic removal of the three terminal amino acids and methylation of the newly exposed carboxyl group of the prenyl-cysteine (6, 10–15). The third class of prenylated proteins, represented by the Ypt1/Sec4 (Rab) family of Ras-like GTPases that terminate with the sequence C or CXC, is also geranylgeranylated at a C-terminal cysteine residue(s) (16–18).

The mammalian p21ras protein farnesyltransferase is a heterodimeric enzyme (19–21) that appears to be responsible for the modification of all proteins that have the first type of CAAX sequence noted above (22, 23). Geranylgeranyltransferase type I (GGTase I), which modifies proteins that terminate with the second type of CAAX sequence (23–25), is also a heterodimeric enzyme that appears to share a common a subunit with the p21ras protein farnesyltransferase (21, 23). A third enzyme has been identified, GGTase II, which geranylgeranylates the Ypt1/Sec4 class of proteins (21).

Genetic studies in yeast have identified three genes, each of which uniquely affects one type of prenyltransferase reaction. One of these genes, RAM1 (also called DPR1), was identified as a suppressor of RAS2val19, a mutationally activated RAS allele (26, 27). This same gene was identified based on its involvement in a-factor production (26, 28, 29) and as a suppressor of G protein function (30). ram1 extracts are defective in a-factor and Ras protein farnesyltransferase activity but not in other prenyltransferase activities (21, 23, 31, 32). Furthermore, Ram1 protein produced in Escherichia coli, although lacking enzyme activity on its own, biochemically complements ram1 extracts for a-factor peptide farnesyltransferase activity (31). This result suggests that the RAM1 gene product is a necessary but insufficient component of the protein farnesyltransferase. Mutants harboring lesions in CDC43/CAL1, a gene that shows homology to RAM1 (33), are specifically defective in GGTase I activity (21, 23, 34). Another yeast gene that is homologous to RAM1, called BET2, affects the membrane localization of Ypt1 and Sec4 proteins (35) and specifically affects yeast GGTase II activity (21).

In this report we characterize another yeast gene, RAM2, that was identified as an extragenic suppressor of RAS2val19. Previous reports have shown that ram2 mutants, like ram1 mutants, are defective in Ras protein farnesyltransferase activity (23, 32). Here we report the DNA sequence of RAM2 and present direct evidence that RAM1 and RAM2 encode the two essential subunits of yeast farnesyI-protein transferase.

MATERIALS AND METHODS

Yeast Strains and Materials. The ram2-1 mutants used in this study were derived from the original mutant STS39 by backcrosses to RS16-4C (26). Other yeast strains have been described (26, 29, 36). Recombinant p21ras was purified as described (37). Biotinylated KTSCVIM peptide (38) was obtained from Multiple Peptide Systems (San Diego). The a-factor peptides YIKGVFWD PAC and YIKGVFPWD PACVIA were synthesized by P. Shenbagamurthi (Johns

Abbreviations: CAAX, protein sequence where C = cysteine, A = usually an aliphatic amino acid, and X = any amino acid; FFP, farnesyl pyrophosphate; GGTase I, geranylgeranyltransferase type I; GGTase II, GGTase type II.

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Pram2::LEU2 was constructed by HindIII (Hopkins). \([^{3}H]\)Farnesyl pyrophosphate (\([^{3}H]FPP\); 20 Ci/mmol; 1 Ci = 37 GBq) was purchased from NEN.

Plasmid Constructions and Gene Disruptions. The integrative mapping plasmid pH43 contains the 1.85-kilobase-pair (kb) Sac I–Pst I RAM2 fragment inserted into pRS305 (39). pram2::LEU2 was constructed by insertion of the 1.5-kbp Pst I–Bgl II RAM2 fragment into pH43 and gene replacements were generated by digestion with Pst I and transformation into the diploid strain T10S1 (40). pram1::URA3 (pSM353) contains the 4.6-kbp BamHI–Bgl II fragment of the RAM1 locus inserted into the vector pUC4-k, with the URA3 HindIII fragment in place of the RAM1 HindIII fragment, such that codons 1–322 are deleted (41). Gene replacements were generated by digestion with EcoRI and transformation into the diploid strain SM1060 (36). To express RAM2 in E. coli, a RAM2 coding sequence cassette was generated by PCR utilizing two oligonucleotide primers that overlapped either the initiation codon or the termination codon shown in Fig. 3. The relevant sequence of the 5'-portion of the cassette is CTTCAGAGAAATTG, where the underlined ATG corresponds to the initiation codon of RAM2. The 3'-portion of the cassette contained an artificial BamHI site following the termination codon. This cassette was inserted into the chloramphenicol-resistance plasmid pBC-KS+ (Stratagene) that had been cleaved with Xho I and BamHI, to generate pBHS7, which utilizes the lac promoter to direct the synthesis of an N-terminal lacZ–RAM2 fusion protein. To express RAM1 in E. coli, the plasmid pH863 was constructed by inserting a Sal I–Kpn I RAM1 coding sequence cassette into the ampicillin-resistance plasmid pUC119. The RAM1 cassette was generated by PCR utilizing two oligonucleotide primers that overlapped either the initiation codon or the termination codon reported for Dp1 (41). The relevant sequence of the 5'-portion of the cassette is GTCTGACTAGT, where the underlined ATG indicates the initiation codon of RAM1.

Preparation of E. coli Extracts for Farnesyltransferase Assays. E. coli extracts were prepared by culturing JM110 double transformants to mid-logarithmic phase followed by induction with 0.5 mM isopropyl β-d-thiogalacitate and incubation for 1 hr. Cells were collected, washed in extraction buffer (50 mM sodium Mes, pH 6.5/0.1 mM EGTA/0.1 mM MgCl2/5% glycerol/2 mM phenylmethylsulfonyl fluoride), transferred to 0.5-mL tubes, pelleted, and lysed in 200 μl of extraction buffer with glass beads and Vortex mixing. Soluble fractions were prepared by centrifugation at 50,000 × g for 1 hr.

Other Methods. Fractionation of yeast and detection of a-factor and Ras2 proteins by immunoprecipitation were performed as described (26, 36). Yeast extracts were prepared as described (32). Farnesyltransferase assays using recombinant p21ras as substrate were performed and analyzed as described (32). Assays using synthetic a-factor peptides were carried out with 250 pmol of peptide essentially as described (31).

Experimental Results

Identification and Characterization of the ram2 Mutation. Several yeast mutations suppress ras2<sup>s1919</sup>, including ram1, cyr1, and cap1/srv2 (26, 27, 42, 43). Among the set of ras2<sup>s1919</sup> extragenic suppressor mutants derived by heat-shock selection from the MA7a ras<sup>i</sup> ras2<sup>s1919</sup> strain PT1-6 (26), some recessive mutations do not fall into these three known complementation groups. One of these suppressors, STS39, displayed two nonselected phenotypes: an inability to grow well at high temperatures (37°C) and a significant mating defect. The mating defect was determined to be MATa-specific and correlated with defective a-factor production. Based on cosegregation of all three of these phenotypes following a cross to a wild-type strain and subsequent analysis of 10 complete tetrads, these three phenotypes resulted from a single mutation (ram2-1). ram2-1 has been previously demonstrated to be unlinked to ram1 (32).

ram2 Mutants Are Defective in the Farnesylation and Membrane Targeting of Both Ras Proteins and the a-Factor Mating Pheromone. It has been shown that extracts prepared from ram2-1 mutants fail to farnesylate bacterially produced Ras proteins in vitro (23, 32). We determined that ram2-1 extracts, like ram1 extracts, are also defective in farnesylating a-factor in vitro (data not shown). To determine whether the lack of farnesylation measured in vitro was reflected in vivo by a defect in membrane targeting, we radiolabeled wild-type and mutant yeast cells and examined soluble and particulate fractions by immunoprecipitation using antibodies that recognized Ras proteins or a-factor (Fig. 1). In the wild-type strain, Ras2 was localized in the particulate fraction as an apparent 40-kDa species. In contrast, in the ram2-1 and the ram1 mutants, Ras2 was localized to the soluble fraction and migrated more slowly (Fig. 1A). Fig. 1B shows that a-factor was detected in the particulate fraction of wild-type cells as two precursor species (P1 and P2), whereas a-factor synthesized in ram1 or ram2-1 mutants was predominantly localized in the soluble fraction as a slower migrating form (P0). Thus, ram2-1, like mutations in RAM1, blocks the membrane targeting of Ras proteins and a-factor.

Cloning and Sequence Analysis of RAM2. We utilized the Ts<sup>−</sup> phenotype of ram-1 mutants to clone the wild-type RAM2 gene by complementation. A ram2-1 mutant strain was transformed with a yeast genomic library and two transformants were found that exhibited plasmid-dependent Ts<sup>−</sup> growth. Plasmids recovered from these two transformants restored normal growth and mating properties to ram2 mutant strains. Restriction enzyme mapping revealed that the two plasmids contained overlapping inserts with a common region of DNA of ≥11 kbp (Fig. 2). To determine whether the gene we cloned corresponded to the locus identified by the ram2-1 mutation, we integrated a LEU2<sup>+</sup>-marked plasmid (pBH43) containing a fragment of the cloned DNA into a RAM2<sup>s</sup> strain. Integration was targeted to the chromosomal locus corresponding to the cloned fragment by digestion with Sph I (see Fig. 2), and correctly targeted integration was

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**Fig. 1.** Localization of Ras proteins and a-factor in ram mutants. (A) SDS/PAGE analysis of immunoprecipitated Ras2 proteins from soluble (S) and particulate (P) fractions of extracts prepared from [35S]methionine-labeled wild-type and mutant yeast strains, all of which contained high-copy RAS2 plasmid (26). Lanes: 1, wild-type (S), 2, ram2-1 (STS39); 3, ram1-1 (STS11). The soluble precursor form of Ras2 (41 kDa) and the particulate mature form of Ras2 (40 kDa) are marked by arrows. (B) SDS/PAGE analysis of immunoprecipitated a-factor from total extracts (T) and particulate (P) and soluble (S) fractions of total extracts prepared from [35S]cysteine-labeled wild-type and mutant yeast strains, all of which contained high-copy MEA1 plasmid (29). Lanes: 1, wild-type (SM1229); 2, ram1::URA3 (SM1866); 3, ram2-1 (SM1863). Three of the intracellular forms of a-factor are marked by arrows (P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub>).
confirmed by DNA-blotted hybridization for the transformant BHYDC1. In a cross of a ram-1-1 leu2 strain with BHYDC1, the segregation pattern of LEU2+ and ram-1 indicated complete linkage (no recombinants in 25 tetrads), and we therefore conclude that the cloned gene is indeed RAM2.

Subclones of the two original plasmids were constructed and based on their ability to complement the ram-2 mutation we determined that the entire RAM2 gene (or at least a functional segment) resides within the 1.4-kbp Xba I–Sph I fragment shown in Fig. 2. The DNA sequence of this region was determined for both strands and it contains an open reading frame that can encode a protein of 316 amino acids if the first ATG is used to initiate translation (Fig. 3). The predicted molecular mass of the Ram2 protein is 38 kDa.

Reconstitution of Farnesyltransferase Activity. To investigate the possibility that Ram1 and Ram2 proteins are indeed structural subunits of the yeast farnesyl-protein transferase, we expressed the RAM1 and RAM2 genes in E. coli and assayed extracts for farnesyltransferase activity. Using a substrate peptide corresponding to the C-terminal sequence of K-rasB, control extracts and extracts prepared from bacterial strains that expressed either RAM1 or RAM2 gene alone were completely inactive (Table 1). In contrast, extracts prepared from a strain that coexpressed RAM1 and RAM2 exhibited high levels of farnesyltransferase activity (Table 1). We have determined that the specific activity of such E. coli extracts is at least 100-fold higher than that of similarly prepared wild-type yeast extracts (data not shown).

Extracts prepared from E. coli cells that coexpressed RAM1 and RAM2 also efficiently farnesylated synthetic α-factor substrate. We observed by SDS/PAGE analysis that control extracts derived from E. coli carrying plasmids without RAM inserts produced an unidentified product from [3H]FPP (Fig. 4A, lane 3) that is not related to α-factor since it was also observed in reactions in which the peptide was absent (Fig. 4A, lane 9). In contrast, using extracts from cells that coexpressed RAM1 and RAM2, a reaction product was observed (Fig. 4A, lane 1) that was not found in reactions utilizing extracts prepared from E. coli that expressed either the RAM1 or RAM2 gene alone (Fig. 4A, lanes 4 and 5). This product coimmigrated with authentic mature α-factor produced in vivo (Fig. 4B) and was not detected in control reactions containing a synthetic α-factor peptide from which the terminal-AXX residues (of the CAAX sequence) were missing (Fig. 4A, lane 8).

The E. coli host JM110 was transformed sequentially with two different plasmids to generate the strains used in this experiment. Each assay (25 μl) was performed in duplicate and contained 50 mM potassium phosphate (pH 7.4), 10 mM magnesium chloride, 5 mM dithiothreitol, 0.2% Tween 20, 50 μg of soluble extract from the indicated E. coli transformant, 90 pmol of biotinylated KTSCVIM peptide, and 25 pmol of [3H]farnesyl. After incubation for 30 min at 30°C, the amount of [3H]farnesyl transferred to peptide was measured as described (38). For the mixed extract, 50 μg of BHE-2 and BHE-3 E. coli extracts was mixed in a microcentrifuge tube and left on ice for 30 min before performing the assay. In a separate experiment, BHE-4 extract transferred 1.6 ± 0.1 pmol of [3H]farnesyl onto bacterially produced p21V, whereas control extracts listed transferred <6 fmol to Ras substrate.
were significantly defective for growth at low temperatures and completely unable to grow at 37°C (Fig. 5).

The viability of ral mutation null mutants is in marked contrast to the lethal effects of disrupting the RAM2 gene. This is not due to genetic variations in the strains employed since disruption of RAM2 was not lethal in the strains used to test the effects of RAM2 disruption (data not shown). To explore the possibility of residual protein farnesyltransferase activity in ral null mutants, we prepared soluble extract from a ral null mutant and assayed for farnesyltransferase activity. No reaction product was observed using the α-factor quindecapetide substrate, even after long exposures of the fluorogram (data not shown). The amount of [3H]farnesyl transferred to the K-rasB peptide using the ral null mutant extract was not significantly higher than blank values (<2 fmol), whereas parallel reactions with extract prepared from the wild-type strain SP1 transferred 284 ± 50 fmol. Since we did not detect residual protein farnesyltransferase activity in ral null mutants, we conclude that this activity is not required for yeast viability. This raises a paradox, since RAS function is essential in yeast. Possible resolution of the paradox, and explanations for the disparate effects of disrupting RAM1 and RAM2, are discussed below.

DISCUSSION

We have characterized a newly identified yeast gene, RAM2, and have shown by expression in E. coli that RAM1 and RAM2 encode the two required components of the yeast enzyme responsible for farnesylation of α-factor and Ras proteins. The finding that Ram1 and Ram2 proteins are required for reconstitution of protein farnesyltransferase activity parallels the finding that the mammalian Ras protein farnesyltransferase is comprised of two different polypeptide subunits (19–21). It is also noteworthy that both components of the mammalian enzyme, the β subunit and the α subunit, are structurally homologous to Ram1 and Ram2, respectively (21, 45, 46).

RAS function is essential for yeast, and mutant Ras proteins that cannot be farnesylated cannot supply essential RAS function unless significantly overexpressed (47–49). It is therefore somewhat surprising that RAM1, which is absolutely required for farnesylation of transferase activity, is not an essential gene. One possible explanation, based on the ability of CAAX peptides that are efficient substrates for the farnesyl-protein transferase to serve as less efficient substrates for GGTase I, is that in ral mutant cells a small but sufficient amount of Ras proteins is geranylgeranylated by yeast GGTase I (22, 23, 25). Accordingly, we propose that
ram2" null mutants are nonviable as a result of not only deficient farnesylation activity but also deficient GGTase I activity. This proposal is based in part on the finding that the mammalian α subunit of the farnesyl-protein transferase, which is homologous to Ram2, also appears to be a structural subunit of GGTase I (21, 24). In addition, ram2-1 mutants have been shown to be somewhat defective in GGTase I activity (23). Further analysis will be required to fully understand the role of Ram2 in other prenyltransferase reactions.

Two of the three yeast enzymes postulated to be involved in the post-translational modification of Ras proteins and α-factor have now been definitively assigned to particular genes in Saccharomyces cerevisiae and have been reconstituted by expression in E. coli. In addition to the Ram1-Ram2 farnesyl-protein transferase, it has been shown that STE14 encodes the methyltransferase that modifies α-factor and Ras proteins (36). In the absence of STE14 function, prior steps in Ras protein processing, presumably prenylation or proteolytic removal of the three C-terminal amino acids, were significantly delayed. This finding suggests that different steps in the modification of Ras proteins, although biochemically separable, may somehow be coupled in vivo.

S.P. thanks Michael Wigler, in whose laboratory the STS39 strain was isolated, for his continuing encouragement. We are grateful to N. Kohl and J. Gibbs for communicating results before publication, M. Kozak for critical reading of the manuscript, and S. Ferro-Novick for discussions. This work was supported by National Institutes of Health Grants GM41258 (S.P.) and GM41223 (S.M.), American Cancer Society Grant MG-1 (S.P.), and an American Heart Association–Maryland Affiliate Grant-in-Aid (S.M.).