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# *RAM*, a Gene of Yeast Required for a Functional Modification of *RAS* Proteins and for Production of Mating Pheromone a-Factor

**Scott Powers,* Susan Michaelis,† Daniel Broek,* Sonia Santa Anna-A.,† Jeffrey Field,* Ira Herskowitz,† and Michael Wigler***
* Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724
† Department of Biochemistry and Biophysics
University of California, San Francisco
San Francisco, California 94143

## Summary

**We have identified a gene (*SUPH*) of S. cerevisiae that is required for both *RAS* function and mating by cells of a mating type. *supH* is allelic to *ste16*, a gene required for the production of the mating pheromone a-factor. Both *RAS* and a-factor coding sequences terminate with the potential acyltransferase recognition sequence Cys-A-A-X, where A is an aliphatic amino acid. Mutations in *SUPH-STE16* prevent the membrane localization and maturation of *RAS* protein, as well as the fatty acid acylation of it and other membrane proteins. We propose the designation *RAM* (*RAS* protein and a-factor maturation function) for *SUPH* and *STE16*. *RAM* may encode an enzyme responsible for the modification and membrane localization of proteins with this C-terminal sequence.**

## Introduction

The membrane targeting of certain proteins does not follow the classical localization pathway involving the transfer of newly synthesized protein across the membrane of the endoplasmic reticulum (Walter et al., 1984; Novick et al., 1981). Two mammalian genes that regulate cell proliferation, H-*ras* and *src*, encode proteins that do not contain signal sequences and are synthesized on free cytoplasmic ribosomes (Shih et al., 1982; Levinson et al., 1981), yet are localized to the inner surface of plasma membranes (Courtneidge et al., 1980; Willingham et al., 1980; Papageorge et al., 1982). Both of these proteins are covalently modified by the addition of fatty acid (Sefton et al., 1982). For H-*ras*, this modification occurs near the C-terminus (Willumsen et al., 1984a). Eukaryotic *ras* proteins share a common C-terminal sequence Cys-A-A-X, where A is an aliphatic amino acid, and X is the C-terminal amino acid (Taparowsky et al., 1983; Powers et al., 1984; Reymond et al., 1984; Schejter and Shilo, 1985; Fukui and Kaziro, 1985). Palmitic acid is attached presumably via a thioester bond to the cysteine residue in this conserved region (Willumsen et al., 1984a; Buss and Sefton, 1986), and it is believed that palmitylation is responsible at least in part for membrane localization. Mutant *ras* genes that have substitutions or deletions in this region fail to become acylated, fail to localize to the membrane, and fail to transform cells (Willumsen et al., 1984a, 1984b). In the case of *src* proteins, myristic acid is attached to the N-terminal alanine residue via an amide linkage (Buss and Sefton, 1985; Schultz et al., 1985). Mutants of *src* that have substitutions at this residue are defective in function, acylation, and membrane localization (Kamps et al., 1986). Thus for *src*, as well as for *ras*, acylation appears to be a critical event in membrane targeting.

Another protein that may depend upon acylation for membrane targeting is the secreted peptide **a**-factor. **a**-factor is the mating pheromone produced specifically by mating type **a** (*MATa*) haploids of S. cerevisiae (Wilkinson and Pringle, 1974; Betz et al., 1981). Two genes, *MFa1* and *MFa2*, that encode the polypeptide precursors of **a**-factor have been characterized (Brake et al., 1985). These precursor coding sequences do not have a hydrophobic signal sequence. However, the predicted C-terminal sequence of both the **a**-factor precursors is Cys-Val-Ile-Ala, in consensus with the C-termini of eukaryotic *ras* proteins (Table 1).

Here we report the identification and cloning of a gene in yeast required for a step in the production of both *RAS* proteins and **a**-factor. We show that this gene is required for the acylation and membrane localization of *RAS* proteins. We suggest that this gene encodes the acyltransferase responsible for acylation of *RAS* proteins and **a**-factor.

## Results

### Identification of an Extragenic Suppressor of *RAS2*$^{val19}$ that Alters *RAS* Activity

Certain missense mutations profoundly alter the activity of eukaryotic *RAS* genes. In particular, mutated H-*ras* genes that encode valine instead of glycine at the twelfth codon are capable of inducing tumorigenic transformation in NIH 3T3 cells (Taparowsky et al., 1983; Reddy et al., 1982; Tabin et al., 1982). Yeast cells with a corresponding mutation at codon 19 of *RAS2* have an altered response to nutritional stress. These *RAS2*$^{val19}$ cells are relatively inefficient at arresting in G1 and maintaining viability when starved for an essential nutrient such as nitrogen or sulfur (Toda et al., 1985). Related to this phenotype is the inability of stationary phase *RAS2*$^{val19}$ cells to tolerate heat shock (Sass et al., submitted; unpublished data). Unlike wild-type cells, which become resistant to a short heat shock treatment when they enter the resting state (Iida and Yahara, 1984), saturated cultures of *RAS2*$^{val19}$ cells yield few survivors (0.01%) after heat shock (see Experimental Procedures). Of the surviving cells, 1% are stably heat shock resistant (Hsr$^{+}$). Thus, we have been able to obtain spontaneous phenotypic revertants of *RAS2*$^{val19}$ cells at a frequency of $10^{-6}$. Using the *ras1*$^{-}$ *RAS2*$^{val19}$ strain PT1-6, approximately 1% of the stable phenotypic revertants isolated at 25°C had the additional property of temperature-sensitive growth (ts). Presumably, these mutants contain a lesion that suppresses *RAS2*$^{val19}$-induced sensitivity to heat shock at 25°C and that prevents cells from sustained growth at 35°C. We restricted our analysis to this set of ts mutants.

Table 1. Common C-Terminal Sequences in *RAS* Proteins, a-Factor, and Other Proteins

| Gene | Species | C-Terminal Sequence | Reference |
|---|---|---|---|
| *RAS1* | S. cerevisiae | gly cys *cys* ile ile cys | Powers et al., 1984 |
| *RAS2* | S. cerevisiae | gly cys *cys* ile ile ser | Powers et al., 1984 |
| *ras1* | S. pombe | cys lys *cys* val leu ser | Fukui and Kaziro, 1985 |
| Dd-*ras* | Dictyostelium | lys gln *cys* leu ile leu | Reymond et al., 1984 |
| *Dras3* | Drosophila | val pro *cys* val leu leu | Schejter and Shilo, 1985 |
| H-*ras* | Human | cys lys *cys* val leu ser | Taparowsky et al., 1983 |
| K-*ras* | Human | lys lys *cys* ile ile met | Taparowsky et al., 1983 |
| N-*ras* | Human | leu pro *cys* val val met | Taparowsky et al., 1983 |
| Transducin-γ | Bovine | gly gly *cys* val ile ser | Hurley et al., 1984 |
| *rho* | Aplysia | gly gly *cys* val val leu | Madaule and Axel, 1985 |
| *RHO1* | S. cerevisiae | lys lys *cys* val leu leu | Maduale et al., 1986 |
| *RHO2* | S. cerevisiae | asn cys *cys* ile ile leu | Maduale et al., 1986 |
| *MFa1* | S. cerevisiae | pro ala *cys* val ile ala | Brake et al., 1985 |
| *MFa2* | S. cerevisiae | pro ala *cys* val ile ala | Brake et al., 1985 |
| Consensus | | cys A A X | |

In the consensus sequence Cys-A-A-X, A is an aliphatic amino acid (either valine, leucine, or isoleucine), and X is the C-terminal amino acid. Transducin-γ represents the γ subunit of bovine retinal transducin.

Forty-three independent ts revertants were analyzed in detail. Thirty-nine were shown to contain recessive mutations as judged by the growth at 35°C of diploids formed by mating to wild-type strains. We expected two classes of revertants that would not be informative for the purposes of this study: ts *ras2* mutants and ts *cyr1* mutants. The latter class is expected because we have established that the major biological effects of *RAS* in yeast are mediated through adenylate cyclase (Toda et al., 1985; Broek et al., 1985), encoded by the *CYR1* gene (Kataoka et al., 1985a). Indeed, complementation analysis with known *ras2* and *cyr1* mutants revealed that 25 of the 39 revertants fell into one of these complementation groups, and these revertants have not been studied further.

One ts recessive revertant, STS11, that did not fall into either the *cyr1* or *ras2* complementation groups was mated to the wild-type strain RS16-4C for tetrad analysis. The segregation pattern of heat shock resistance indicated the presence of an extragenic suppressor of $RAS2^{val19}$. This suppressor, which we call *supH*, also suppressed other phenotypes induced by $RAS2^{val19}$ including the carbohydrate storage defect and the nitrogen-starvation viability defect (Toda et al., 1985). By complementation analysis, 8 of the remaining 14 recessive ts revertants fell into the *supH* class. Combined tetrad analysis of three of these (STS11, STS17, and STS18) showed that the ts and the $Hsr^+$ phenotypes cosegregated; all 27 of the $RAS2^{val19}$ progeny that were $Hsr^+$ (and thereby contained *supH*) were ts, and all 31 of the $RAS2^{val19}$ progeny that were $Hsr^-$ (and therefore lacking *supH*) were not temperature sensitive (non-ts). These data indicate that the same mutation is responsible for both suppression of $RAS2^{val19}$ and for temperature-sensitive growth in *supH* strains.

Since *supH* suppresses $RAS2^{val19}$, one possibility is that *RAS* proteins are no longer functional in *supH* mutants. To test whether the *RAS* protein present in *supH* strains was still capable of stimulating adenylate cyclase, we prepared membranes from both wild-type and *supH* strains and assayed their adenylate cyclase activity (see Experimental Procedures). In the presence of $Mn^{2+}$, which activates adenylate cyclase independently of *RAS* and guanine nucleotides (Broek et al., 1985), adenylate cyclase activities were comparable in both wild-type and *supH* strains (Table 2). In contrast, the *RAS*-dependent adenylate cyclase activity, assayed in the presence of $Mg^{2+}$ or $Mg^{2+}$ plus guanine nucleotides, was very low in the *supH* strain compared with wild-type (Table 2). This effect was observed regardless of whether *supH* strains harbored either the wild-type *RAS2* or activated $RAS2^{val19}$ allele (data not shown). This biochemical profile is similar to that found in membranes from $ras1^-$ $ras2^-$ strains (Toda et al., 1985; Broek et al., 1985) and demonstrates that *supH* affects the function of *RAS*. Confirmation that *supH* membranes behaved as if they were $ras^-$ was shown by the addition of *RAS2* protein purified from E. coli, which fully restored $Mg^{2+}$-guanine nucleotide-dependent activity (Table 2). Apparently, whatever cellular defect results from the deficiency of *SUPH*, it does not affect the ability of adenylate cyclase to respond to *RAS2* proteins purified from E. coli.

### The Role of *SUPH* in Essential Processes Other than the *RAS*-Adenylate Cyclase Pathway

If the sole essential role of the *SUPH* gene product were to allow *RAS* proteins to function, we could expect to suppress the ts defect of *supH* strains by the same set of genetic manipulations that suppress $ras1^-$ $ras2^-$ lethality, for example, overexpression of the adenylate cyclase gene *CYR1* or overexpression of the cAMP-dependent protein kinase gene *TPK1* (Kataoka et al., 1985a; T. Toda et al., unpublished results). We transformed 3 mutant strains each containing a different ts *supH* allele (STS11, STS17, and STS18) with the high copy plasmid YEp*TPK1* which contains a gene encoding a cAMP-dependent protein kinase (T. Toda et al., unpublished results). This plasmid suppresses $ras1^-$ $ras2^-$ and relieved the ts phenotype of STS11, but not of STS17 or STS18. Similarly, the ts phenotype of STS11, but not STS17 or STS18, could also

Table 2. Adenylate Cyclase Activity in Membranes

| Genotype[b] | Assay Conditions[a] Mn2+ (1) | Mg2+ (2) | Mg2+, Gpp(NH)p (3) | Mg2+, Gpp(NH)p, *RAS2* (4) |
|---|---|---|---|---|
| *SUPH+ RAS2* | 24 | 3.3 | 8.5 | 11 |
| *supH- RAS2* | 21 | 0.4 | 1.1 | 16 |

[a] Membranes from the strains SP1 (*SUPH+*) and RS40-4C (*supH-*) were prepared, and adenylate cyclase activity was assayed as described in Experimental Procedures. Thirty micrograms of membranes was assayed either in the presence of (1) 2.5 mM $Mn^{2+}$ or (2) 2.5 mM $Mg^{2+}$, or (3) 2.5 mM $Mg^{2+}$ and 50 μM Gpp(NH)p or (4) 2.5 mM $Mg^{2+}$ and 50 μM Gpp(NH)p and 70 units of *RAS2* protein as indicated (see Experimental Procedures). Gpp(NH)p (guanosine-5'(β,γ-imino) trisphosphate) is a nonhydrolyzable analog of GTP. Adenylate cyclase activity is expressed in units of pmol of cAMP generated per mg of membrane protein per min. The values indicated are the averages of duplicate samples that deviated <10% from the average. Essentially identical results were obtained when the assay was performed at 25°C or 35°C.

[b] Full genotypes are given in Experimental Procedures.

be suppressed by the overexpression of adenylate cyclase activity (Kataoka et al., 1985a). Thus, two out of three alleles of *supH* must inactivate other essential functions in addition to *RAS*. The *supH* allele of STS11 appears to be somewhat leaky in that the only essential function inactivated at 35°C appears to be *RAS*. All three of the *supH* mutants exhibited the same terminal arrest phenotype: after 4 hr at 35°C, they accumulated as unbudded cells (75%–90%).

### *supH* Causes a Mating Defect in *MATa* Cells Resulting from a Defect in a-Factor Production

During the genetic analysis of *supH* mutants, we noted that *supH* caused a mating defect in **a** cells, but not in α cells. To examine this **a**-specific sterile phenotype more carefully, we back-crossed the strains RS40-4C and RS40-5A, both of which contained the *supH* allele from STS11, to the wild-type strains EG123 and 246.1.1, respectively, to form diploids that were *MATa/MATα supH-/SUPH+*. Twenty-nine tetrads were analyzed. Whereas all 58 *MATα* segregants (28 of which were ts) were mating-proficient, only 28 of the *MATa* segregants could mate; the remaining 30 *MATa* segregants were sterile. Each member of this latter class of *MATa* nonmaters was also ts. Thus, *supH* causes **a**-specific sterility, in addition to a ts phenotype in both **a** and α cells.

The observation that a mutation influencing *RAS* also influenced mating ability was surprising, since there was no previously known connection between *RAS* and mating. Two main aspects of the mating phenotype of **a** cells are production of the pheromone **a**-factor and response to the pheromone α-factor. We determined whether either of these processes was defective in *supH* mutants. Response to α-factor for several *MATa supH* segregants from the cross described above was assessed by microscopic observation of single cells after exposure to α-factor (see Experimental Procedures). Using the criteria of arrest of bud formation and alteration in cell morphology, the **a** *supH* mutants behaved in a manner identical with wild-type **a** cells, indicating that the mutants are responsive to α-factor. In contrast, the *supH* mutants exhibited a greatly reduced level of **a**-factor (Figure 1). Quantitation of this defect indicated a >200-fold deficiency of pheromone activity in the culture medium of mutant strains (S. Michaelis, unpublished results). Thus, the **a**-specific mating defect caused by the *supH* mutation can be attributed to a defect in production of **a**-factor, which is known to be essential for mating (S. Michaelis, unpublished results).

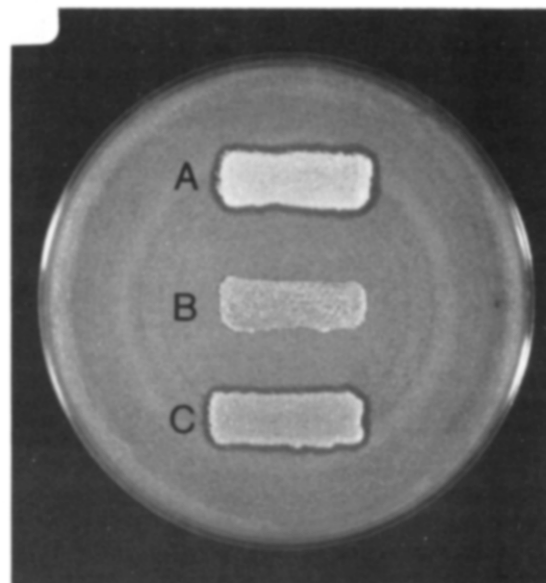


Figure 1. Halo Assay for Production of **a**-Factor by a *supH* Mutant
The *MATa* strains shown are (A) EG123 (*SUPH+*), (B) RS40-5A (*supH-*), (C) RS40-5A carrying pYPG1, which contains the *SUPH-STE16* gene. Secretion of **a**-factor by the patched cells prevents growth of the surrounding lawn, resulting in a halo of growth inhibition.

### *SUPH* Is Allelic to *STE16*, a Gene Required for the Biosynthesis of a-Factor

The phenotype of *supH* mutants is similar to three **a**-specific sterile mutants, *ste6* (J. D. Rine, Ph.D. thesis, University of Oregon, Eugene, Oregon, 1979; Wilson and Herskowitz, 1984), *ste14* (L. C. Blair, Ph.D. thesis, University of Oregon, Eugene, Oregon, 1979), and *ste16* (K. L. Wilson, Ph.D. thesis, University of California, San Francisco, San Francisco, K. Wilson and I. Herskowitz, submitted). These three mutants are all defective in the production of **a**-factor, but still exhibit normal levels of transcription of the **a**-factor precursor genes *MFa1* and *MFa2* (Brake et al., 1985; S. Michaelis et al., unpublished results). We observed that *ste16* mutants, unlike *ste6* and *ste14* mutants, grew slowly, although this was not a ts defect. We tested whether the *ste16* mutation is responsible for slow growth by analyzing tetrads from a *MATa/MATα STE16+/ste16-* diploid formed by crossing strains EG123 and H1192. All 23 sterile *MATa* progeny were slow growing, whereas the 25 *MATa* progeny that mated efficiently grew at wild-type rates. Thus, the *ste16* mutation causes a growth defect.

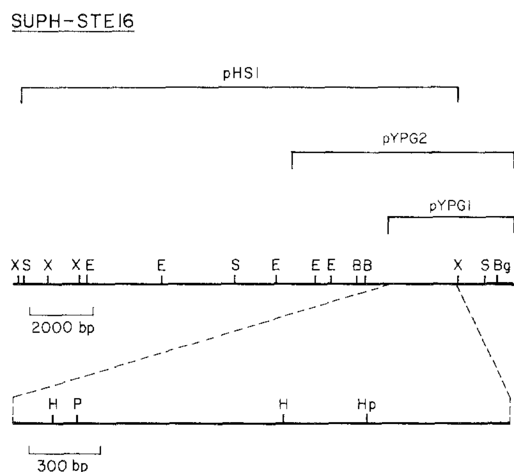


Figure 2. Composite Restriction Endonuclease Map of *SUPH-STE16* Locus

Above the map is shown the Sau3A inserts of plasmids that contain the *SUPH-STE16* gene. Within the map, all sites for a given restriction endonuclease are indicated except for BglII, for which only one of several sites is shown. Restriction endonucleases used were: E, EcoRI; H, HindIII; P, PstI; X, XbaI; S, SphI; Hp, HpaI; Bg, BglII; B, BamHI.

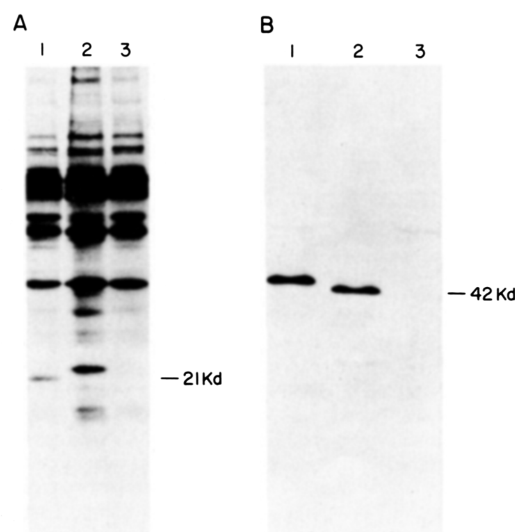


Figure 3. Immunoprecipitation of H-*ras* and *RAS2* Proteins

(A) Strains carrying p*ADH-H-ras* were labeled for 3 hr with [$^{35}$S]methionine as described in Experimental Procedures. *RAS* proteins were immunoprecipitated with monoclonal antibody 259 (Furth et al., 1982) and the samples analyzed by SDS-PAGE and autoradiography. Lane 1 shows the H-*ras* protein immunoprecipitated from the *SUPH*$^+$ strain SP1 carrying p*ADH-H-ras*. Lane 2 shows the H-*ras* protein from the *supH*$^-$ strain RS41-5A carrying p*ADH-H-ras*. Lane 3 shows a mock immunoprecipitation of the SP1 extract without monoclonal antibody 259. The position of the 21 kd form of mature H-*ras* protein is indicated.
(B) Strains carrying the *RAS2* overexpression plasmid YEp*RAS2*-1 were analyzed as described in (A). Lane 1 shows the *RAS2* protein immunoprecipitated from the *supH*$^-$ strain RS40-17C carrying YEp*RAS2*-1. Lane 2 shows the *RAS2* protein immunoprecipitated from the *SUPH*$^+$ strain SP1 carrying YEp*RAS2*-1. Lane 3 shows a mock immunoprecipitation of the SP1 extract without antibody. The position of the 42 kd for the mature *RAS2* protein is indicated.

Given the close phenotypic similarities between *supH* mutants and *ste16* mutants, we tested whether the two mutations can complement each other and whether they are allelic. We mated the *MATα supH*$^-$ strain RS40-4C to the *MATa ste16*$^-$ strain H1171. To allow for mating, strain H1171 contained the plasmid pYPG2, which suppresses the *ste16* mating defect (see below). After selecting the diploid, we screened for diploid segregants that were Ura$^-$ and had lost the plasmid. Diploids that lost the plasmid were very slow growing at 37°C, whereas diploids that retained the plasmid grew normally at 37°C. Thus, *ste16* and *supH* are in the same complementation group. Furthermore, we examined tetrads from a diploid that had lost the plasmid. The 48 *MATα* haploid progeny mated efficiently, whereas none of the 50 *MATa* segregants were mating-proficient. Thus, *ste16* and *supH* are allelic and represent the same gene, which we temporarily refer to as *SUPH-STE16*.

## Cloning the *SUPH-STE16* Gene

The *STE16* and *SUPH* genes were cloned by two independent methods based on the two different aspects of their mutant phenotypes. For *STE16*, two plasmids that reverse the mating defect of *ste16*$^-$ mutants were obtained from a YEp24-based yeast genomic library (K. L. Wilson, Ph.D. thesis, 1985). These two plasmids, pYPG1 and pYPG2, also restore **a**-factor production to *ste16*$^-$ strains. For *SUPH*, three plasmids that could complement the ts phenotype of *supH* mutant strain RS40-4C were obtained from a yeast genomic library constructed in the shuttle vector YCp50 (see Experimental Procedures). Two of these plasmids contained the *RAS2* gene, and we confirmed that some but not all *supH*$^-$ strains could be suppressed by overexpression of *RAS2* (S. Powers, unpublished). The other plasmid, pHS1, was not related to any of the cloned genes of the *RAS*-adenylate cyclase pathway. Comparison of the restriction maps of the inserts of pYPG1, pYPG2, and pHS1 revealed that they contained a common region of 2.1 kb (Figure 2). pYPG1 and pHS1 restored **a**-factor production to *supH* mutants (see Figure 1), and also complemented their growth defect, showing that the common 2.1 kb region encodes the complementing gene.

We showed by genetic analysis that the gene we had cloned truly corresponded to the *SUPH-STE16* gene rather than, for example, to a gene such as *RAS2* that can complement *supH-ste16* mutations when overexpressed. By integrative transformation we constructed *SUPH-STE16*$^+$ strains whose only copy of *URA3*$^+$ information was tightly linked to the locus corresponding to the cloned DNA (see Experimental Procedures). In crosses to a *supH*$^-$ *ura3*$^-$ strain (RS40-4C), the segregation pattern of *URA3*$^+$ and *supH*$^-$ indicated complete linkage (no recombinants in 33 tetrads), and we therefore conclude that the cloned gene is indeed *SUPH-STE16*.

## *SUPH-STE16* Is Required for the Maturation of *RAS* Proteins

The processing of eukaryotic *RAS* proteins results in at least three observable changes: fatty acid acylation, a

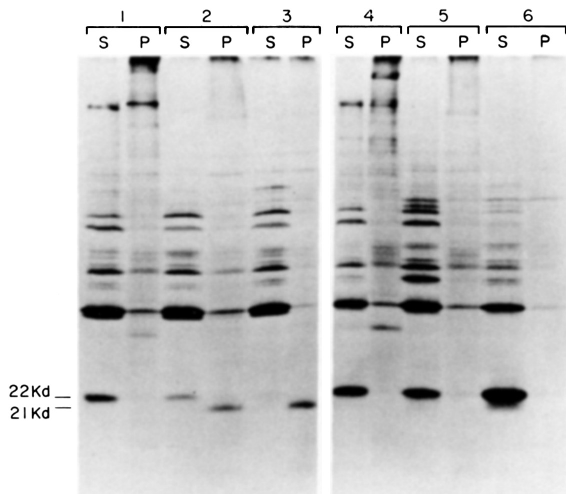


Figure 4. Pulse-Chase and Cell Fractionation Analysis of H-*ras* Protein

Strains carrying p*ADH-H-ras* were labeled for 5 min with [$^{35}$S]methionine. Portions of the cultures were incubated for either an additional 20 min or 2 hr with an excess of cold methionine. Extracts were separated into soluble and insoluble fractions as described in Experimental Procedures. Lanes 1S and 1P show the H-*ras* protein immunoprecipitated from the soluble (S) and the membrane pellet (P) fractions of the strain RS41-5A carrying both p*ADH-H-ras* and the *SUPH*$^+$ plasmid pHS1 (5 min pulse). Lanes 2S and 2P, 20 min chase of the above; lanes 3S and 3P, 2 hr chase of the above; lanes 4S and 4P, the H-*ras* protein immunoprecipitated from the soluble and membrane pellet fractions of the *supH*$^-$ strain RS41-5A carrying p*ADH-H-ras* (5 min pulse). Lanes 5S and 5P, 20 min chase of above; lanes 6S and 6P, 2 hr chase of above. The positions of the 22 kd precursor of H-*ras* protein and the 21 kd mature H-*ras* protein are indicated.

shift in mobility seen by SDS-PAGE analysis, and membrane localization (Shih et al., 1982; Papageorge et al., 1982; Sefton et al., 1982). It is not known whether these three changes are separable, although it has been suggested that the shift in mobility is not caused by acylation (Buss and Sefton, 1986). However, all three of these changes are thought to involve the C-terminus of *RAS* (Willumsen et al., 1984a; 1984b). Eukaryotic *RAS* proteins and the **a**-factor precursors terminate with the sequence Cys-A-A-X (Table 1). This led us to consider the possibility that **a**-factor production might involve some if not all of these changes, and that *SUPH-STE16* effects the C-terminal processing of both *RAS* proteins and **a**-factor.

In mammalian cells, the H-*ras* protein is initially synthesized as a soluble cytoplasmic 22 kd precursor that matures into a membrane-bound 21 kd protein (Shih et al., 1982). When H-*ras* is expressed in yeast, this same maturation process occurs (Kataoka et al., 1985b; Clark et al., 1985), and an analogous situation has been reported for the *RAS2* protein in yeast (Fujiyama and Tamanoi, 1986). We tested whether *SUPH-STE16* affects this maturation of *RAS* proteins. For this purpose, we transformed *SUPH*$^+$ and *supH*$^-$ strains with high copy plasmids containing *RAS2*. Since the mobility shift is easier to follow with H-*ras*, we also transformed wild-type and *supH* strains with the high copy plasmid p*ADH-H-ras* (which contains a human H-*ras* cDNA transcribed from the yeast alcohol dehydrogenase [*ADH1*] promoter). We labeled wild-type and *supH*$^-$ cells for 3 hr with [$^{35}$S]methionine to ensure that the predominant *RAS* species in wild-type cells was mature *RAS* protein, and analyzed the immunoprecipitated *RAS* proteins by SDS-PAGE. As seen in Figure 3A, all the H-*ras* protein observed in the *SUPH*$^+$ strain has a relative mobility of 21 kd, whereas all the H-*ras* in the *supH*$^-$ mutant has a relative mobility of 22 kd. Likewise, the *RAS2* protein migrates more slowly in the *supH*$^-$ mutant than the *RAS2* protein observed in the wild-type *SUPH*$^+$ strain (Figure 3B). This observation suggests that *supH*$^-$ strains are synthesizing *RAS* precursors but that maturation does not occur. The *supH*$^-$ strains were equally defective in this regard at both 25°C and 35°C (data not shown). It is not surprising to observe this biochemical defect at the permissive temperature, since other phenotypes, such as the **a**-factor defect, are also exhibited at 25°C. To confirm that this mobility defect results from the *supH* mutation, we analyzed four isogenic strains that had either lost or retained an extrachromosomal plasmid containing the *SUPH*$^+$ gene (see Experimental Procedures). The two *SUPH*$^+$ strains produced a faster migrating form of *RAS*, whereas the two *supH*$^-$ strains did not (data not shown).

## *SUPH-STE16* Is Required for the Membrane Localization of *RAS* Proteins

In both mammalian cells and in yeast the H-*ras* precursor is found primarily in the cytoplasmic fraction, while the H-*ras* mature form is found in the membrane fraction (Shih et al., 1982; Papageorge et al., 1982; Clark et al., 1985). We performed pulse-chase and localization analyses of the H-*ras* protein in both a *supH*$^-$ strain and its isogenic *SUPH*$^+$ counterpart and analyzed the immunoprecipitated [$^{35}$S]methionine-labeled H-*ras* proteins by SDS-PAGE. In Figure 4, it can be seen that the H-*ras* protein of wild-type cells is initially synthesized as a soluble, slower migrating form that over time matures into a faster migrating protein that is insoluble and apparently membrane-bound. In *supH*$^-$ cells, the soluble precursor does not mature into a faster migrating form, and little if any membrane localization in achieved over time (Figure 4). Essentially identical results were obtained with *RAS2* (data not shown). Thus, *SUPH-STE16* is required for both the maturation of *RAS* and its membrane localization.

## *SUPH-STE16* Is Required for the Acylation of *RAS* and Other Membrane Proteins

We tested whether *SUPH-STE16* is required for *RAS* protein acylation by examining the presence of fatty acid on *RAS2* protein in both *SUPH*$^+$ and *supH*$^-$ strains. To facilitate analysis, both strains contained the high copy plasmid p*GAL10-RAS2*, which uses the galactose-inducible *GAL10* promoter for high levels of *RAS2* expression. Strains were induced and then labeled with [$^3$H]palmitic acid and in parallel with [$^{35}$S]methionine. Figure 5 shows that equal amounts of [$^{35}$S]methionine-labeled *RAS2* protein were immunoprecipitated in both *SUPH*$^+$ and *supH*$^-$ strains. However, essentially no detectable fatty acid-labeled *RAS2* protein was found in the *supH*$^-$ strain, in contrast to a significant amount of fatty acid-labeled *RAS2* protein in *SUPH*$^+$ cells. Subsequent treatment of the

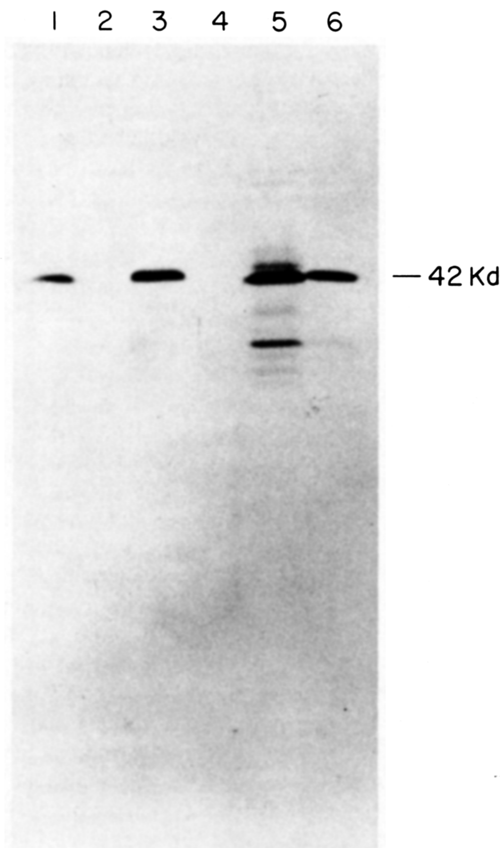


Figure 5. Analysis of Fatty Acid Acylation of *RAS2* Protein

Strains carrying p*GAL10-RAS2* were grown to 2 × $10^7$ cells/ml in synthetic medium with 2% raffinose and then induced with 5% galactose. [$^3$H]palmitic acid or [$^{35}$S]methionine was added immediately after induction. The cells were labeled for 2 hr, and extracts were prepared, immunoprecipitated, and analyzed as described in Experimental Procedures. Lane 1 shows the *RAS2* protein immunoprecipitated from the *SUPH*$^+$ strain SP1 carrying p*GAL10-RAS2* and labeled with [$^3$H]palmitic acid. Lane 2 shows the *RAS2* protein immunoprecipitated from the *supH*$^-$ strain RS41-5A carrying p*GAL10-RAS2* with labeling conditions identical with those in lane 1. Strains and conditions for lanes 3 and 4 were identical with lanes 1 and 2, respectively, except that labeling with [$^3$H]palmitic acid was in rich medium (YP + 5% galactose). Lanes 5 and 6 show the [$^{35}$S]methionine-labeled *RAS2* protein immunoprecipitated from p*GAL10-RAS2* carrying strains, SP1 and RS41-5A, respectively. The position of the 42 kd *RAS2* protein is indicated by the arrow.

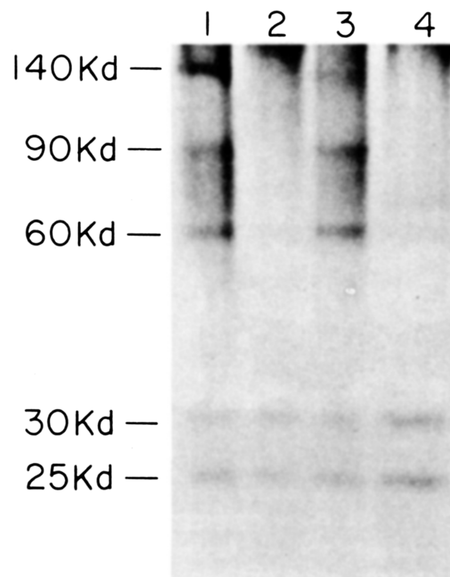


Figure 6. Analysis of Acylated Membrane Proteins

Twenty-five milliliter cultures of four isogenic *SUPH*$^+$ and *supH*$^-$ strains, described in the Results and in Figure 4, were grown to a density of 2 × $10^7$ cells/ml, spun down, and resuspended in 25 ml YPD containing 2 mCi of [$^3$H]palmitic acid. After a 2 hr labeling, membrane extracts were prepared and analyzed by SDS-PAGE (see Experimental Procedures). Lanes 1 and 3, acylated membrane proteins from *SUPH*$^+$ strains; lanes 2 and 4, acylated membrane proteins from *supH*$^-$ strains.

SDS-polyacrylamide gel shown in Figure 5 with 0.1 M KOH in 20% methanol, which cleaves the thioester bond linking the fatty acid to *RAS* (Sefton et al., 1982), followed by autoradiography, confirmed that the tritium label seen in Figure 5 was due to fatty acid attachment and not to conversion of the label into the general amino acid pool (data not shown). Western blot analysis of the [$^3$H]palmitic acid-labeled extracts showed that equal amounts of *RAS2* had been synthesized in both the *supH*$^-$ and the *SUPH*$^+$ strain (data not shown). Thus, the *SUPH-STE16* gene product is required for *RAS* protein acylation.

We also tested whether *SUPH-STE16* alters the acylation of any other membrane proteins. Membranes were prepared from [$^3$H]palmitic acid-labeled *supH*$^-$ and *SUPH*$^+$ strains and analyzed by SDS-PAGE (see Experimental Procedures). In *SUPH*$^+$ strains, we observed major acylated proteins of approximate molecular weights 140 kd, 90 kd, 60 kd, 30 kd, and 25 kd (Figure 6). The $^3$H-label could be removed from all five of these acylated proteins by treatment with 0.1 M KOH in 20% methanol, suggesting that the linkage of the fatty acid to the proteins was via ester or thioester bonds (data not shown; Schlesinger et al., 1983). The 30 kd and 25 kd acylated proteins are present in the *supH*$^-$ mutants. However, we detected little if any 140 kd, 90 kd, and 60 kd acylated proteins in *supH*$^-$ strains (Figure 6). Thus, in *supH*$^-$ strains, several other membrane proteins aside from *RAS* fail to become acylated.

## Discussion

We report the identification of mutations in a gene, *SUPH*, which result in defective *RAS* activity and defective mating by **a** cells. *SUPH* is shown to be identical with *STE16*, a gene required for the production of the mating pheromone **a**-factor. We redesignate *SUPH-STE16* as *RAM* (RAS and **a**-factor maturation function). We demonstrate that *ram*$^-$ mutants are defective in maturation, membrane localization, and acylation of *RAS* proteins. As discussed in the following section, our findings lead to the proposal that the *RAM* gene product is responsible for acylation. Because both *RAS* and **a**-factor require *RAM* and share the proposed acylation site Cys-A-A-X, we suggest that **a**-factor (or its precursor) contains a fatty acid whose addition is also mediated by *RAM*.

We have demonstrated that in *ram*$^-$ strains the *RAS*-dependent adenylate cyclase activity, but not adenylate cyclase itself, is attenuated. This shows that the *RAM*-dependent modification is important for *RAS* function, in accordance with the finding that mutant H-*ras* genes that lack acylation sites are biologically inactive (Willumsen et al., 1984a, 1984b). Since yeast adenylate cyclase can be stimulated in vitro by the addition of *RAS2* protein purified from E. coli, it is possible that the *RAM*-dependent modification is not absolutely required for *RAS* function, but that it merely facilitates *RAS* function because of its effect on localization. It should be noted that the concentration of *RAS2* protein purified from E. coli, which is required for stimulation of adenylate cyclase in vitro, is at least 30-fold higher than the concentration of *RAS2* protein found in wild-type yeast cells (Broek et al., 1985; D. Broek, unpublished). Additional work is required to resolve this question.

### Role of *RAM* in Posttranslational Modification of *RAS* Proteins

The processing of eukaryotic *RAS* proteins results in at least three observable changes: fatty acid acylation, a shift in mobility seen by SDS-PAGE analysis, and membrane localization. It is not known whether the mobility shift is due to acylation or some other modification. Indeed, the precise sequence of events that comprise these changes is unknown. However, because mutant *RAS* proteins lacking acylation sites undergo none of these changes (Willumsen et al., 1984a, 1984b), it is reasonable to presume that fatty acid acylation is a required preliminary event. Since *RAS* proteins in *ram*$^-$ strains fail to undergo any of these changes, it is our expectation that *RAM* encodes the fatty acid acyltransferase. We cannot exclude the possibility that *RAM* encodes a protein required to "prepare" *RAS* and other proteins for acylation. The resolution of this problem may be possible by the biochemical analysis of the *RAM* protein and by structural analysis of *RAS* proteins found in *ram*$^-$ strains.

### Role of *RAM* in a-Factor Biosynthesis

The two peptide mating pheromones, **a**-factor and α-factor, are likely to differ substantially in their manner of secretion. It has been demonstrated that the biosynthesis of α-factor employs the classical secretory pathway involving the endoplasmic reticulum, Golgi, and secretory vesicles (Julius et al., 1984). Structural considerations suggest that **a**-factor employs a novel mode of secretion. Although the structure of active secreted **a**-factor has not been precisely determined, it is likely that **a**-factor is a oligopeptide of 11–15 residues and that it is processed from a precursor either 36 residues in length (encoded by *MFa1*) or 38 residues in length (encoded by *MFa2*) (Betz et al., 1981; Brake et al., 1985). These precursors lack an amino-terminal signal sequence and sites for Asn-linked glycosylation, which are hallmarks of proteins destined for the secretory pathway. However, the presence of the C-terminal sequence Cys-A-A-X suggests that the **a**-factor precursor is acylated. The observation that *ram*$^-$ mutants are defective both in *RAS* acylation and **a**-factor production is consistent with this notion. Acylation might play a role in **a**-factor secretion; for example, fatty acid addition might occur in the cytoplasm to facilitate membrane targeting, or it might occur at the membrane as a prerequisite for a later step in secretion. Another possibility is that active **a**-factor is a lipopeptide, as is known to be the case for mating pheromones in two species of basidiomycetous yeasts (Sakagami et al., 1981; Kamiya et al., 1979). Determination of the structure of **a**-factor and the mutant **a**-factor molecules that accumulate in *ram*$^-$ strains should aid in understanding the pathway of biosynthesis of this pheromone.

### Role of *RAM* in Other Cellular Processes

We have shown that other proteins besides **a**-factor and *RAS* are altered by *ram*$^-$ mutations. For example, three of the major acylated membrane proteins observed in wild-type cells fail to become acylated in *ram*$^-$ mutants. It is likely that these proteins share with *RAS* and **a**-factor the C-terminal consensus sequence. Other proteins that terminate with the Cys-A-A-X sequence include the γ-subunit of the GTP-binding protein transducin (Hurley et al., 1984) and the *RAS*-related *RHO* gene products, which are highly conserved in eukaryotes and are present in yeast (Madaule and Axel, 1985; Madaule et al., 1986; see also Table 1). Interestingly, the G1 arrest phenotype of the more stringent *ram*$^-$ mutants is not altered by high copy plasmids expressing the cAMP-dependent protein kinase gene *TPK1*, although overexpression of *TPK1* alleviates the G1 arrest of *ras*$^-$ mutants. This argues that attenuation of some distinct *RAM*-dependent pathway(s) is capable of inducing G1 arrest even when the cAMP effector pathway is activated. *ram*$^-$ mutants provide an access to identification of such pathways.

## Experimental Procedures

### Yeast Strains

H1171 (*MATa ste16 leu2 ura3 his3 his4 trp1 tyr1 lys2 cry*$^R$) and H1192 (*MATα ste16 leu2 his3 his4 trp1 lys2*) were isolated by K. Wilson (unpublished). RC757 (*MATα sst2 his6 met1 can1 cyh2 rme1*) was the strain used for the lawn to test for **a**-factor halo (Chan and Otte, 1982). Strains EG123 and 246.1.1 are two isogenic (*leu2 ura3 his4 trp1*) *MATa* and *MATα* strains, respectively (Siliciano and Tatchell, 1984). PT1-6 (*MATa RAS2*$^{val19}$ *ras1::URA3 leu2 ura3 his3 trp1 ade8 can1*) was derived by transformation from the previously described *RAS1*$^+$ strain TK161R2V (Toda et al., 1985). STS11, STS18, and STS17 are ts Hsr$^+$ revertants isolated from PT1-6 (see below). RS16-4C (*MATα ura3 his3 trp1 ade8 ade2 can1 SUP*$^{84L}$) was derived from the Esposito strain K382-19D after three backcrosses to SP1 (*MATa leu2 ura3 his3 trp1 ade8 can1*) (Toda et al., 1985). *SUP*$^{84L}$ is a dominant suppressor of the *RAS2*$^{val19}$-induced sporulation defect; it does not effect any other phenotypes induced by *RAS2*$^{val19}$ (Toda et al., 1985). RS40-4C (*MATα supH ura3 his3 trp1 ade8 can1*), RS40-5A (*MATa supH ura3 his3 trp1 ade8 can1*) and RS40-17C (*MATa supH leu2 ura3 his3 trp1 ade8 can1*) were derived from a cross between STS11 and RS16-4C. RS41-5A (*MATα supH leu2 ura3 his3 trp1 ade8 can1*) and RS41-8D (*MATa supH RAS2*$^{val19}$ *leu2 ura3 his3 trp1 ade8 can1*) were derived from a cross between STS18 and RS16-4C. T50-31 (*MATα cyr1 leu2 ura3 his3 trp1*) was constructed as described (Toda et al., 1985). The four isogenic strains (see Results) were derived from RS41-5A that was first transformed with the *SUPH*$^+$ *URA3*$^+$ plasmid pHS1, then transformed with the *ADH-H-ras LEU2*$^+$ plasmid pLD95. Two Ura$^+$ SupH$^+$ and two Ura$^-$ SupH$^-$ segregants were derived from the double transformant.

### Plasmids

YEp*RAS2*-1 is a 2μ-based *LEU2*$^+$ plasmid that overexpresses the *RAS2* gene (Powers et al., 1984). YEp*TPK1* is a 2μ-based *LEU2*$^+$ plasmid that overexpresses the cAMP-dependent protein kinase gene *TPK1* (T. Toda et al., unpublished). p*GAL10-RAS2*, which contains *GAL10-RAS2*, was constructed by T. Kataoka (Kataoka et al., 1985b) and has the *RAS2* coding region inserted into the correct orientation of the *GAL10* expression plasmid YEp51 (Broach et al., 1983). pLD95, (called p*ADH-H-ras* in the text) is a 2μ-based *LEU2*$^+$ plasmid in which human H-*ras* cDNA is transcribed from the yeast alcohol dehydrogenase (*ADH1*) promoter (constructed by L. Davidow and M. Gollahera, Pfizer Central Research, personal communication). YIpSM98 contains the 4.2 kb BamHI–BglII fragment of pYPG2 (see Results) inserted into the BamHI site of the integration vector YIp5 (Struhl et al., 1979).

### Media, Genetic Manipulations, and Physiological Assays

Yeast transformations, complementation assays, and tetrad analyses were performed as previously described (Kataoka et al., 1985a; Wilson and Herskowitz, 1984). Yeast were grown in YPD (2% peptone, 1% yeast extract, and 2% glucose) or, to maintain selective pressure for plasmids, in synthetic medium (0.67 g/l Yeast Nitrogen Base, 2% glucose, plus appropriate auxotrophic supplements). Matings were performed by selection for prototrophy. For mating-deficient strains (*MATa supH-ste16*) rare mating events occurred at a sufficient frequency to be obtained using this selection. Mating-type determinations for *supH-ste16* strains were performed by replica plating cells onto separate synthetic medium plates containing lawns of tester strains DC14 (*MATa his1*) or H227 (*MATa lys1*) and DC17 (*MATα his1*) or H1793 (*MATα lys1*), and assessing growth of prototrophic diploids 2 days later. All *supH-ste16* nonmaters could be unambiguously assigned as *MATa* by allowing them to incubate with the tester strains for 2 days on YPD before scoring for growth on synthetic plates.

To isolate ts Hsr$^+$ revertants of PT1-6, 50 subclones were kept at saturation at 25°C in YPD for 3 days and then heat shocked at 50°C for 30 min before spreading aliquots on YPD plates. Six days later, surviving colonies were replica-plated to assay for Hsr and ts phenotypes. Heat shock on plates was performed with preheated plates at 55°C with incubation periods of 15 to 45 min.

Responsiveness to α-factor was determined by a confrontation assay essentially as described (MacKay and Manney, 1974) using the strain 246.1.1 as a source for α-factor. The halo assay for **a**-factor production was performed as described previously (Wilson and Herskowitz, 1984). The *supH* strains used in this study can grow well at 30°C, so that most experiments were performed at this temperature unless otherwise indicated. ts growth was generally assayed at 35°C.

### Cloning and Integrative Mapping

For the cloning of *SUPH*, plasmid-dependent non-ts Ura$^+$ transformants of the *supH*$^-$ *ura3*$^-$ strain RS40-4C were obtained after transformation with DNA from a yeast genomic library of Sau3A partial digestion fragments contained in the *URA3*$^+$ shuttle vector YCp50. DNA was isolated from these transformants (Struhl et al., 1979), transformed into E. coli, and the rescued plasmids were tested for suppression of *supH* and examined for their relationship to known genes of the *RAS*-adenylate cyclase pathway by restriction mapping and Southern analysis. The *SUPH* candidate pHS1 contained a 13.9 kb insert; subcloning experiments showed that the 3.1 kb BamHI junction fragment complemented the *supH* ts growth defect (see Figure 2). Plasmids containing the *STE16* gene were isolated from a YEp24 clone bank (K. L. Wilson, Ph.D. thesis, 1985). The common region of DNA shared by the *STE16* plasmid pYPG1 and the *SUPH* plasmid pHS1 was established by analysis with the restriction enzymes HindIII, PstI, and BamHI. For integrative mapping, we constructed the plasmid YIpSM98 (see above). Integration of YIpSM98 was targeted to sequences homologous to the 4.2 kb BamHI–BglII insert of pYPG2 by cleavage with XbaI (Orr-Weaver et al., 1981). Targeted integration of YIpSM98 into the chromosome at the *SUPH-STE16* locus was accomplished by digesting the plasmid DNA with XbaI, followed by transformation into EG123 (*SUPH-STE16*$^+$) and selection for Ura$^+$ transformants (Orr-Weaver et al., 1981).

### Adenylate Cyclase Assays

Yeast membranes and *RAS2* protein from E. coli were prepared as described (Broek et al., 1985). Assays were performed as described (Broek et al., 1985). One unit of *RAS2* protein is defined as that which will bind 1 pmol of [$^3$H]GDP at 37°C after 45 min in the presence of 50 mM Tris-HCl buffer (pH 7.5) and 3 mM $MgCl_2$.

### Metabolic Labeling with [$^3$H]Palmitic Acid and [$^{35}$S]Methionine

All labeling was performed at 30°C, unless otherwise indicated, with exponentially growing yeast cultures seeded at a density of 1–2 × $10^7$ cells/ml. Synthetic medium was adjusted to pH 6.8; this allowed for more efficient uptake of palmitic acid. For the experiment shown in lanes 3 and 4 of Figure 5, labeling was performed in rich medium (YP + 5% galactose; pH 6.8). For labeling with [$^3$H]palmitic acid (New England Nuclear; 5 mCi/mmol in ethanol) the label was concentrated 10-fold to 50 mCi/ml. Two mCi of [$^3$H]palmitic acid was added directly to 25 ml cultures, and incubated for 2 hr. For labeling with [$^{35}$S]methionine, 50–500 μCi was added to 5 ml cultures in synthetic medium adjusted to pH 6.8.

Labeled cells were washed once in ice-cold extraction buffer A (50 mM potassium phosphate, pH 7.4; 150 mM NaCl; 1% Triton X-100; 1% sodium deoxycholate; 1 mM β-mercaptoethanol; and 1 mM PMSF). The pellet was frozen at −80°C and lysed in 200 μl of ice-cold buffer A with an equal volume of glass beads. Cells were disrupted by vortexing 6 times in 1 min bursts followed by chilling on ice. The extract was clarified by centrifugation at 15,000 × g for 30 min and stored at −80°C.

### Cell Fractionation

Labeled cells were washed once and lysed in ice-cold extraction buffer B (50 mM potassium phosphate, pH 7.4; 150 mM NaCl; 1 mM β-mercaptoethanol; and 1 mM PMSF) as described above. Cellular debris was removed by centrifugation at 1000 × g for 10 min, and the cellular extract was then further centrifuged at 45,000 rpm in a Ti50 rotor (Beckman) for 30 min. The supernatant was decanted (soluble fraction). The pellet was washed with 5 ml of buffer B and recentrifuged. The washed pellet was resuspended in 200 μl of buffer A, sonicated for 10 sec, and placed on ice for 30 min to extract membrane proteins. Remaining insoluble material was removed by centrifugation at 15,000 × g for 30 min.

### Immunoprecipitations

Two microliters of the rat monoclonal antibody 259 (10 mg/ml; Furth et al., 1982) was added to 200 μl of extract plus buffer A. After 1 hr rotation at 4°C, was added 15 μl of packed Protein A-Agarose (Boehringer Mannheim) that had been precoated with 150 μg of rabbit antibody to rat IgG (Cappel) (Furth et al., 1982). After 1 hr of rotation at 4°C, the immunoprecipitates were washed 2–4 times with buffer A containing 0.1% SDS. Following a suggestion of Bart Sefton (Salk Institute), for all of the experiments involving [$^3$H]palmitic acid labeling, the *RAS* proteins were liberated from the washed precipitates by addition of sample buffer containing 1 mM β-mercaptoethanol and 2% SDS at room temperature and loaded onto the gel without boiling.

### Other Methods

SDS-PAGE analysis was carried out as previously described (Broek et al., 1985) except that the gels were soaked in DMSO for 30 min immediately following electrophoresis. After another 30 min wash with DMSO, the gels were soaked in DMSO-PPO (New England Nuclear) for 1 hr, washed in water for 30 min, and then processed for autoradiography as described (Broek et al., 1985). In situ treatment of the gel displayed in Figure 5 with 0.1 M KOH in 20% methanol was performed as described (Sefton et al., 1982). Membrane extracts for the experiment shown in Figure 6 were isolated as described above. The extracts were exhaustively delipidated by sequential extraction with 40-fold volumes of 2:1 chloroform:methanol, 1:2 chloroform:methanol, 10:10:3 chloroform:methanol:water, and finally acetone (Schlesinger, 1983). After each extraction, the protein pellet was resuspended in buffer B containing 2% SDS and was sonicated briefly. Before loading onto the gel, the samples were adjusted to 10% glycerol.

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