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Fatty Acylation Is Important but Not Essential for *Saccharomyces cerevisiae RAS* Function

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Two proteins in the yeast *Saccharomyces cerevisiae* that are encoded by the genes *RAS1* and *RAS2* are structurally and functionally homologous to proteins of the mammalian *ras* oncogene family. We examined the role of fatty acylation in the maturation of yeast *RAS2* protein by creating mutants in the putative palmitate addition site located at the carboxyl terminus of the protein. Two mutations, Cys-318 to an opal termination codon and Cys-319 to Ser-319, were created in vitro and substituted in the chromosome in place of the normal *RAS2* allele. These changes resulted in a failure of *RAS2* protein to be acylated with palmitate and a failure of *RAS2* protein to be localized to a membrane fraction. The mutations yielded a Ras2$^-$ phenotype with respect to the ability of the resultant mutants to grow on nonfermentable carbon sources and to complement *ras1*$^-$ *mutants. However, overexpression of the ras2*$^{Ser\text{-}319}$ product yielded a Ras$^+$ phenotype without a corresponding association of the mutant protein with the membrane fraction. We conclude that the presence of a fatty acyl moiety is important for localizing *RAS2* protein to the membrane where it is active but that the fatty acyl group is not an absolute requirement of *RAS2* protein function.

Genes structurally and functionally homologous to the *ras* family of mammalian oncogenes are found in *Drosophila melanogaster* (38), *Dictyostelium* sp. (32), and both budding and fission yeasts (10, 16, 30). Mammalian *ras* oncogenes were initially identified by the ability of mutationally activated cognates of these genes to induce proliferative transformation of NIH 3T3 cells. They were recognized as relatives of the oncogenic agent carried on Harvey and Kirsten rat sarcoma viruses (13). Proteins encoded by these genes possess GTP-binding activity and weak GTPase activity and are localized to the inner surface of the plasma membrane (27), apparently as a result of the posttranslational addition of a palmitate moiety to a cysteine residue near the carboxyl end (8, 9, 37). The role *ras* proteins normally play in the cell and the means by which activated alleles induce proliferative transformation of mammalian cells is unknown.

In *Saccharomyces cerevisiae* either of two *ras*-homologous genes, *RAS1* and *RAS2*, is required for initiation of the mitotic cell cycle. Spores genotypically *ras1*$^-$ *ras2*$^-$ fail to initiate growth (22), and *ras1* cells carrying a temperature-sensitive allele in *ras2* arrest mitotic growth as unbudded, nongrowing cells at the nonpermissive temperature (S. Garrett, S. Silberberg, and J. R. Broach, unpublished data). Genetic (41) and biochemical (6) results have shown that yeast *RAS* proteins modulate the activity of adenylate cyclase. Mammalian and yeast *ras* protein are cross functional in vivo: a mammalian *ras* protein will complement a yeast *ras1 ras2* mutant strain when expressed in the cell (23), and an activated yeast *RAS1* gene will transform NIH 3T3 cells (11). However, attempts to show that *ras* protein directly regulates adenylate cyclase in eucaryotic systems other than *S. cerevisiae* has failed (2), and a biological role for mammalian *ras* remains to be elucidated.

Fatty acylation has been described in a wide variety of cellular proteins (reviewed in reference 25). At least two nonoverlapping pathways have been described for the addition of fatty acids to proteins (28, 29). In most cases, fatty acylation is associated with membrane attachment (25), although particularly in the case of myristylated proteins, such as pp60$^{v\text{-}src}$, a significant soluble pool of fatty acylated proteins exists (7, 35). Palmitate attachment in H-*ras* protein appears to occur as a thioester linkage to the cysteine residue fourth from the carboxyl terminus (42). This cysteine residue is conserved in all *ras* proteins and occurs as part of the sequence Cys-A-A-X, where A is any aliphatic amino acid and X represents no preference except that it is often the carboxyl-terminal residue. Willumsen et al. (42) have shown that mutations altering the sequence in this region of mammalian H-*ras* protein result in failure of the protein to attach to the cytoplasmic membrane. In addition, the mutation reverses the transformation potential of activated H-*ras* genes.

Fujiyama and Tamanoi (15) have recently shown that yeast cells are capable of adding palmitate to *RAS1* and *RAS2* proteins and that this modification correlates with localization of the protein with the membrane. It is not clear whether palmitate is the only lipid added to the protein, as appears to be the case with mammalian protein, or whether several different lipids can be added. Powers et al. (31) have identified an essential yeast gene, *RAM1*, whose inactivation prevents lipid acylation of H-*ras* expressed in yeasts and is presumed to encode a component of the yeast acylation pathway. In this report, we describe the phenotypic consequences of mutations in the putative palmitate addition site of *RAS2* protein both for the growth of yeasts and for the localization of *RAS2* protein. We found that, although such mutations yield a Ras2$^-$ phenotype, the defect can be abrogated by overexpression of the mutant gene.

MATERIALS AND METHODS

Plasmids and yeast strains. A summary of the yeast strains used in this study can be found in Table 1.

Mutations of the *RAS2* gene carried on plasmid pRAS2 (30) were constructed by site-directed oligonucleotide muta-

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TABLE 1. Yeast strains used in this study

Strain	Genotype
S150-2B[a]	*MATa leu2 ura3 trp1 his3 gal2*
RJ50-2B[b]	*MATa leu2 ura3 trp1 his3 gal2 RAS2::URA3*
RJT-1[c]	*MATa leu2 ura3 trp1 his3 gal2 ras2*$^{opal-318}$
RJS-1[c]	*MATa leu2 ura3 trp1 his3 gal2 ras2*$^{Ser-319}$
SGP-4[c]	*MATα leu2 ura3 trp1 his3 ras1::HIS3 RAS2::URA3*
JR29-2B[d]	*MATa leu2 ura3 trp1 his3 ras2::URA3 can1*
JR28-4C[d]	*MATα leu2 ura3 trp1 his3 gal2 ras1::HIS3 ade8 can1*
RJ824[c]	*MATα leu2 ura3 trp1 his3 gal2 ras2::GAL10-RAS2*$^{Val-19}$
RJ5.1-1[c]	*MATa leu2 ura3 trp1 his3 gal2 ras2::GAL10-RAS2*$^{Val-19, Ser-319}$
RJ9-2B[c]	*MATa leu2 ura3 trp1 his3 gal2 ras2::GAL10-RAS2*$^{Gly-19}$*-URA3*
BJ2169[e]	*MATa leu2 ura3 trp1 prb1-1122, pre1-407 pep4-3*
BJRAS2[c]	*MATa leu2 ura3 trp1 prb1-1122, pre1-407 pep4-3*(YEp51-*RAS2*)
BJRS[c]	*MATa leu2 ura3 trp1 prb1-1122, pre1-407 pep4-3*(YEp51-*ras2*$^{Ser-319}$)
JR270[f]	*MATa/MATα leu2/leu2 trp1/trp1 ura3/ura3 his3/his3 ras1::HIS3/RAS1 RAS2/ras2::URA3*(YEp51-*RAS2*)
JR273[g]	*MATa/MATα leu2/leu2 trp1/trp1 ura3/ura3 his3/his3 ras11::HIS3/RAS1 RAS2/ras2::URA3* (YEp51-*ras2*$^{Ser-319}$)
JR270-3A[h]	*MATa leu2 trip1 ura3 his3 ras1::HIS3 ras2::URA3*(YEp51-*RAS2*)
JR270-4A[h]	*MATa leu2 trp1 ura3 his3 ras1::HIS3 ras2::URA3*(YEp51-*RAS2*)
JR270-8C[h]	*MATα leu2 trp1 ura3 his3 ras1::HIS3 ras2::URA3*(YEp51-*RAS2*)
JR273-2C[h]	*MATa leu2 trp1 ura3 his3 ras1::HIS3 ras2::URA3*(YEp51-*ras2*$^{Ser-319}$)
JR273-5D[h]	*MATa leu2 trp1 ura3 his3 ras1::HIS3 ras2::URA3*(YEp51-*ras2*$^{Ser-319}$)
JR273-6B[h]	*MATα leu2 trp1 ura3 his3 ras1::HIS3 ras2::URA3*(YEp51-*ras2*$^{Ser-319}$)

[a] S150-2b was obtained from D. Botstein and cured of 2μm circle by J. Broach.
[b] This strain was obtained after transformation of S150-2b with a *Kpn*I-*Hin*dIII fragment of pRJ520-6 (pras2-URA3) and selecting for URA$^+$.
[c] This work.
[d] See Kataoka et al. (23) for details of this strain.
[e] Obtained from E. W. Jones (19).
[f] This diploid strain was constructed by crossing JR29-2B with JR28-4C and transforming the resulting diploid with YEp51-*RAS2*.
[g] Same as in footnote *f* except the diploid was transformed with YEp51-*ras2*$^{Ser-319}$.
[h] Individual strains isolated from the sporulation of either JR270 or JR273.

genesis as described by Zoller and Smith (43). Oligonucleotides were synthesized on an Applied Biosystems model 380A DNA synthesizer by the phosphoramidite method. Oligonucleotide 1 (GTGGCTGT[T→A]GTATTATA), containing a single base mismatch substituting serine for cysteine at position 319 of *RAS2*, and oligonucleotide 2 (GGTGGCTG[T→A]TGTATTTAT), converting cysteine 318 to a nonsense codon, were used to generate mutant plasmids pRS-1 and pRTE-1, respectively. The presence of the mutations was confirmed by sequence analysis.

Yeast strains containing mutant *RAS2* alleles were constructed by single-step gene transplacement (34) of strain RJ50-2B, which contains the *URA3* gene inserted 100 base pairs 3′ of the chromosomal *RAS2* gene (from plasmid pRJ520-1) (Table 1). Yeast transformation was done by the lithium acetate procedure described by Ito et al. (21). Linear fragments spanning the *RAS2* gene from either pRS-1 or pRTE-1 were used to transform strain RJ50-2B to Ura$^-$ by selection for resistance to 5-fluoroorotic acid (3) as diagrammed in Fig. 1B. Verification that insertion of the mutant alleles had occurred with attendant loss of the *RAS2-URA3* locus was accomplished by Southern analysis (data not shown).

Yeast strains containing *GAL10-RAS* alleles in place of the normal *RAS2* genes were constructed as follows (refer to Fig. 2 for the structure of the plasmids used to isolate DNA fragments to construct the *RAS2* allele described). Strain RJ824 (*GAL10-RAS2*$^{Val-19}$) was obtained by transforming strain RJ50-2B to 5-fluoroorotic acid resistance with an *Nco*I-*Hin*dIII DNA fragment from plasmid pGRV (S. Cameron and R. Deschenes, unpublished data). Strain RJ9-2B (*GAL10-RAS2::URA3*) was obtained by transforming RJ824 to Ura$^+$ with a *Kpn*I-*Hin*dIII DNA fragment from plasmid pRJ520-1. Strain RJ5.1-1 (*GAL10-RAS2*$^{Val-19, Ser-319}$) was

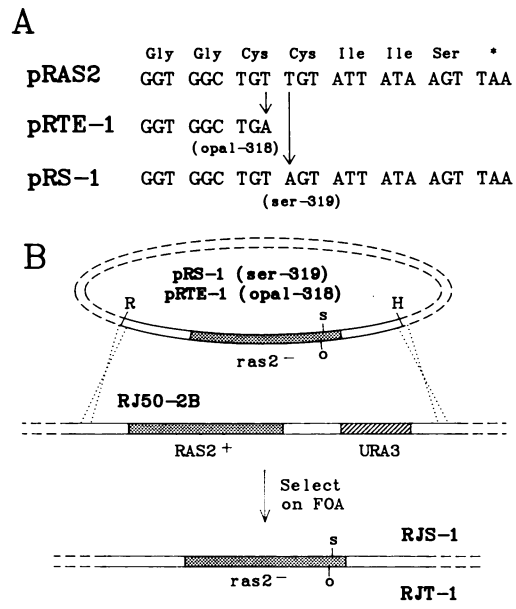

FIG. 1. Carboxyl-terminal mutations of *ras2*. (A) The nucleic acid sequence of the 3′ end of the yeast *RAS2* gene and the predicted amino acid sequence of the carboxyl terminus (residues 316 to 322) of the protein are shown at the top. *, Termination of the *RAS2*, protein. Shown below are the nucleic acid sequence and the predicted amino acid sequence of the two mutant genes created in vitro by oligonucleotide mutagenesis as described in Materials and Methods. (B) Replacement of the chromosomal *RAS2* allele of strain RJ50-2B with the in vitro-generated mutant alleles was acomplished by transforming the strain to resistance to 5-fluoroorotic acid (FOA) with *Eco*RI (R)-*Hin*dIII (H)-digested plasmid pRS-1 or pRTE-1 DNA as diagrammed. S, Serine-319 terminator; O, opal-318, terminator.

obtained by transforming strain RJ824 to Ura$^+$ with plasmid pRSURA linearized with *Bal*I.

For [^{3}H]palmitate labeling and membrane localization experiments, the coding regions of the wild-type *RAS2* gene and the *ras2*$^{Ser-319}$ mutant were cloned into the galactose-inducible expression vector YEp51 (5). The resulting clones, YEp51-*RAS2* and YEp51-*ras2*$^{Ser-319}$, were used to transform the protease-deficient strain BJ2169 (19) in which the plasmids were maintained stably in high copy numbers. Strains JR270-3A, -4A, and -8C were constructed by transforming diploid JR270 (heterozygous for *RAS1* and *RAS2*) with the plasmid YEp51-*RAS2*, sporulating the resulting strain, and dissecting the spores onto rich medium containing 3% galactose. Strains JR272-2C, -6B, and -5D were similarly constructed, except that transformation was done with YEp51-*ras2*$^{Ser-319}$.

Growth medium and genetic techniques. Yeasts were grown in rich medium (1% yeast extract, 2% Bacto-Peptone [both from Difco Laboratories, Detroit, Mich.], with 2% glucose as a carbon source) or synthetic medium (0.67% yeast nitrogen base without amino acids, supplemented with the appropriate amino acids to satisfy auxotrophic requirements and with 2% glucose), except for galactose induction experiments. For galactose induction, cells were pregrown in synthetic medium containing 3% raffinose as the carbohydrate source followed by rich medium containing 4% galactose. Standard procedures for yeast mating, sporulation, and scoring of tetrads were used (36).

DNA isolation and Southern analysis. Total yeast DNA was isolated as described previously (1). DNA was digested with restriction enzymes, electrophoresed in agarose gels, and transferred to nitrocellulose by standard procedures with commercially available enzymes (26). Southern blot analysis was used to confirm all yeast strain constructions (39).

Induction, labeling, and immunoprecipitation. Strains BJRAS2 and BJRS (Table 1) were grown to the stationary phase in synthetic medium without leucine and with 3% raffinose. Cells were diluted into rich medium containing 4% galactose and grown to a density of 2.5×10^7 cells per ml. [^{3}H]palmitate (500 μCi, 65 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added to 10^9 cells in 25 ml of rich medium containing 3% galactose, and the cells were grown for 3 h. Cerulenin (Sigma Chemical Co., St. Louis, Mo.) was added at 1 μg/ml to inhibit fatty acid synthetase and improve incorporation of label into proteins (S. Powers, personal communication). Cell fractionation was done essentially as described by Fujiyama and Tamanoi (15). Cells were harvested by centrifugation at 1,500 × *g* for 5 min and washed once in 0.3 M sorbitol–0.1 M NaCl–5 mM $MgCl_2$–10 mM Tris hydrochloride (pH 7.4) (sorbitol wash). The cells were lysed by vortexing with glass beads (400 to 500 μm) in the same buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 100 U of aprotinin [Sigma] per ml, 1 μM pepstatin [Sigma]). Cellular debris was removed by centrifugation at 1,500 × *g* for 10 min, and a crude membrane fraction was isolated by centrifugation at 100,000 × *g*. The supernatant from this spin was adjusted to 1% Triton and 0.5% deoxycholate and used as the soluble fraction (S100). The pellet was resuspended in sorbitol wash buffer, recentrifuged, and used in subsequent steps as the membrane-enriched fraction (P100).

Immunoprecipitation with the rat anti-Ha-*ras* monoclonal antibody (Y13-259; provided by J. Gibbs, Merck Sharpe & Dohme, West Point, Pa.) was done essentially as described previously (17). Immunoprecipitates were analyzed by electrophoresis in 12.5% acrylamide gels (24).

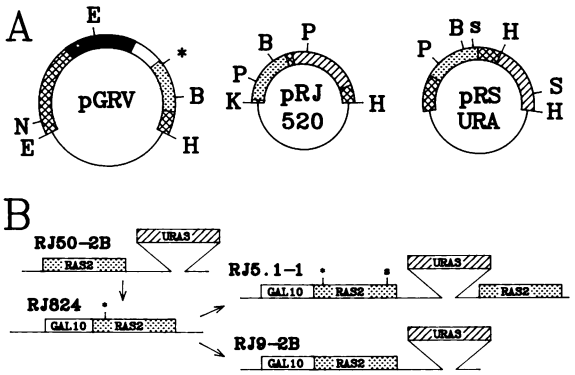

FIG. 2. Construction of yeast strains containing galactose-inducible *RAS2* genes. (A) Shown are diagrams of bacterial plasmid vectors used in the construction of yeast strains with galactose-inducible *RAS2* genes. The *RAS2* coding region is indicated by the stippled segments; 3′ and 5′ noncoding regions of *RAS2* are shown by the cross-hatched segments; the *URA3* gene is indicated by the hatched segments; yeast *LEU2* sequences are indicated by the filled segments; the *GAL10* promoter region is shown by the open segment; and pUC vector sequences are shown by the single line. Restriction enzyme sites include *Eco*RI (R), *Nco*I (N), *Bal*I (B), *Kpn*I (K), *Pst*I (P), *Sma*I (S), and *Hin*dIII (H). The presence of the serine 319 allele is indicated by an s, and the valine 19 allele is shown by an asterisk (*). (B) The structure of the *RAS2* locus in various strains is shown. Strains were derived in the order indicated by transformation with fragments obtained from the vectors shown in panel A as described in Materials and Methods.

RESULTS

Inactivation of *RAS2* by mutations in its conserved carboxyl domain. The carboxyl-terminal sequence of all known *RAS* proteins is Cys-A-A-X, where A is any aliphatic amino acid and X is the last residue. Since mammalian *ras* protein is modified by palmitate addition, apparently through a thioester linkage to the cysteine residue in this sequence, we examined the effects in vivo of modifications of the corresponding region of the yeast *RAS2* gene. Figure 1 shows the sequence of the carboxyl end of yeast *RAS2* protein and the two sequence changes we made in this region by oligonucleotide mutagenesis. The *ras2* gene on plasmid pRTE-1 contains a new termination codon which eliminates all four carboxyl-terminal amino acids, whereas in the protein encoded by the *ras2* gene on plasmid pRS-1, the cysteine residue suspected to be the palmitate acceptor has been changed to a serine. The mutant genes were inserted into the chromosome in place of the normal *RAS2* allele by a one-step transplacement protocol (diagrammed in Fig. 1B) (34). The predicted structure of the *ras2* locus in selected transformants was confirmed by Southern analysis (data not shown).

Both *ras2* mutations were shown to yield a Ras$^-$ phenotype by three criteria. *ras2*$^-$ strains accumulate excess glycogen and stain dark brown in the presence of iodine vapors (14, 41). Figure 3A shows the results of staining patches of a wild-type (wt) *RAS2* strain (RJ50-2B), strain JR29-1D (containing the *ras2*::*URA3* deletion allele), and the two carboxyl-terminal mutants, RJS-1 (*ras2*$^{Ser-319}$) and RJT-1 (*ras2*$^{opal-318}$). As is evident, strains RJS-1 and RJT-1 stain dark, suggesting that they are deficient in *RAS2* function. A second test of *RAS2* protein function is based on the observation that *ras2*$^-$ strains do not grow on nonferment-

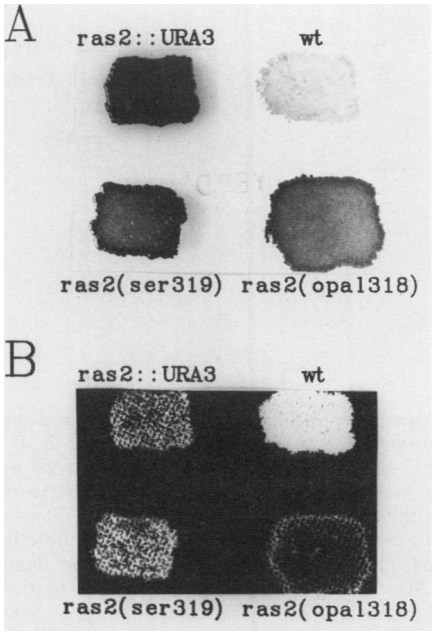

FIG. 3. Phenotypes of *ras2*$^{Ser-319}$ and *ras2*$^{opal-318}$ strains. (A) Patches of JR29-1B (labeled *ras2::URA3*), RJ50-2B (labeled wt), RJS-1 (labeled *ras2*$^{Ser-319}$), and RTE-1 (labeled *ras2*$^{opal-318}$) were grown on rich medium at 30°C for 2 days. Plates were inverted over iodine vapor for approximately 5 min and photographed. (B) A replica of the plate shown in panel A was patched onto rich medium lacking glucose and supplemented with 2% ethanol plus 2% glycerol and incubated at 35°C for 2 days.

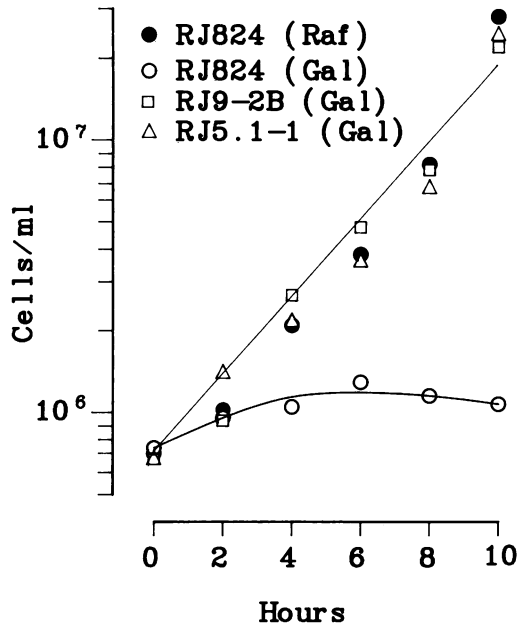

FIG. 4. Growth of strains containing various galactose-inducible *RAS2* genes. Strains RJ824 (*GAL10-RAS2*$^{Val-19}$), RJ9-2B (*GAL10-RAS2*), and RJ5.1-1 (*GAL10-RAS2*$^{Val-19,\ Ser-319}$*-URA3-RAS2*) (see Fig. 2B) were grown to the exponential phase (10^7 cells per ml) in rich medium containing 4% raffinose. Cultures were then diluted to approximately 5×10^5 cells per ml in rich medium containing either raffinose (closed symbols) or 4% galactose (open symbols). At the indicated times, the cell density was determined by counting appropriate samples in a hemacytometer.

able carbon sources at 35°C (14, 40). This is illustrated in Fig. 3B, in which a replica from the same plate as in Fig. 3A was patched to rich medium with 2% ethanol and 2% glycerol as the only carbon sources. Only the wild-type *RAS2* strain grew under these conditions; the mutants behaved in a manner identical to that of the deletion strain. Finally, *ras1*$^-$ *ras2*$^-$ spores failed to grow, since either *RAS1* or *RAS2* is required for initiation of the mitotic cell cycle (22). Thus, as a last test of function of the mutant alleles, we examined whether *ras1 ras2*$^{Ser-319}$ and *ras1 ras2*$^{opal-318}$ strains are viable. Strains RJS-1 and RJT-1 were crossed with strain SGP-4 (*MATα his3 ura3 ras1::HIS3 RAS2::URA3*), and the diploids were sporulated. The *URA3* and *HIS3* genes provided genetic markers for the *ras1* and *RAS2* loci, respectively, so that the *RAS* genotype of every viable spore clone could be unambiguously assigned (barring gene conversion of the *URA3* marker), and assuming Mendelian segregation, the genotype of nonviable spore clones could be inferred. A summary of the viability of spore clones for tetrads from this cross is provided in Table 2. High viability was observed for *RAS1 RAS2*, *RAS1 ras2*, and *ras1 RAS2* spores. However, none of the *ras1 ras2* spores yielded colonies, even after extended incubation. Thus, the carboxyl-terminal mutants

TABLE 2. Complementation of *ras1*$^-$ mutants

Cross	Genotype[a]		No. of spore clones	% Viable
	RAS1	*RAS2*		
SGP-4 (*ras1::HIS3 RAS2::URA3*) × RJS (*RAS1 ras2*$^{Ser-319}$)[b]	+	+	33	100
	−	+	37	95
	+	Ser-319	39	100
	−	Ser-319	33	0
	Unassigned		2	0
SGP-4 (*ras1::HIS3 RAS2::URA3*) × RTE (*RAS1 ras2*$^{opal-318}$)[c]	+	+	33	100
	−	+	46	100
	+	opal-318	45	100
	−	opal-318	33	0
	Unassigned		3	0

[a] The indicated diploid was sporulated and dissected. The genotypes of the resultant spore clones were determined by assessing growth on appropriate prototrophic selection plates. When possible, genotypes of nonviable spores were assigned on the basis of the genotypes of the viable spores in the tetrad, assuming normal Mendelian segregation of *RAS1* and *RAS2*.

[b] Thirty-six tetrads analyzed.

[c] Forty tetrads analyzed.

are completely defective with respect to their ability to complement a *ras1*$^-$ mutant.

Carboxyl-terminal *ras2* mutations are epistatic to *RAS2*-activating alleles. Strains containing *RAS2* mutations analogous to the mammalian transforming alleles (e.g., glycine 19 to valine 19) are sensitive to heat and cold shock and lose viability upon starvation (41). In addition, we found that strains containing such alleles under control of the inducible *GAL10* promoter fail to grow on galactose and with time lose viability under conditions of galactose induction (Fig. 4) (R. J. Deschenes, M. Fedor-Chaiken, and J. R. Broach, unpublished observations). Strains containing a *RAS2* locus with both an activating mutation and a carboxyl-terminal mutation in *cis* were constructed to determine whether failure to add palmitate might suppress the effects of the *RAS2*$^{Val-19}$ allele. Strains RJ824 (carrying *GAL10-ras2*$^{Val-19}$), RJ9-2B (carrying *GAL10-RAS2*), and RJ5.1-1 (carrying *GAL10-ras2*$^{Val-19, Ser-319}$) were constructed by integrating fragments of plasmids constructed in vitro into appropriate recipient strains (see Materials and Methods and Fig. 2B). Resulting strains were propagated in raffinose, a nonrepressing carbon source, and expression of the fusions was induced by transfer to galactose. Under these conditions, strains RJ9-2B and RJ5.1-1 grew equally well on either carbon source, whereas strain RJ824 grew normally on raffinose, but failed to grow after a shift to galactose (Fig. 4). This confirms the results presented above, demonstrating that mutations affecting the extreme carboxyl end of the *RAS2* protein substantially reduce its function.

Carboxyl-terminal mutant *RAS2* proteins are not palmitylated and are not localized to the membrane. Posttranslational modification and subcellular localization of wild-type and mutant *RAS2* proteins were examined in strains in which the proteins were overexpressed. Mutant and wild-type genes were placed under control of the yeast *GAL10* promoter by cloning them into the high-copy expression vector YEp51. Palmitylation of *RAS2* protein was examined directly by [^{3}H]palmitate labeling of the protease-deficient strain BJ2169, harboring either plasmid YEp51-*RAS2* or YEp-*ras2*$^{Ser-319}$, after induction with galactose. *RAS2* protein was immunoprecipitated from extracts of the induced strains, and the extent of its acylation was determined by autoradiography of gel-fractionated immunoprecipitates. The wild-type protein, but not the mutant protein, was labeled by this protocol (Fig. 5). Under these labeling conditions, the label covalently associated with the *RAS2* protein is lipid, a majority of which is palmitate (15). Western blot analysis of the same strains (see below) indicated that the *ras2*$^{Ser-319}$ gene is expressed and that the mutant protein is present at detectable levels. Thus, the absence of labeled *RAS2* protein in strains harboring the mutant gene results from a failure of the mutant protein to be modified rather than from an absence of the mutant protein in the cell. These results are consistent with the hypothesis that the site of palmitylation is the cysteine residue at position 319.

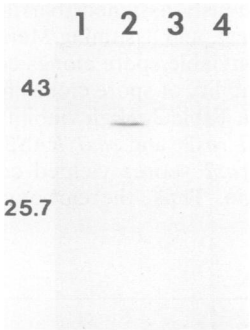

FIG. 5. Palmitylation of wild-type and mutant *RAS2* proteins. Strains BJ2169(YEp51-*RAS2*) (lanes 1 and 2) and BJ2169(YEp51-*ras2*$^{Ser-319}$) (lanes 3 and 4) were grown to the late exponential phase in synthetic medium lacking leucine and containing 3% raffinose. Cells (5×10^8) were removed, washed, transferred to 25 ml of rich medium containing 3% galactose, and incubated for 5 h at 30°C, until the cell density had doubled. Cerelenin (to 3 μg/ml) and 500 μCi of [^{3}H]palmitate (65 Ci/mmol) were added, and incubation continued for 3 h. Extracts were prepared as described in Materials and Methods, and half of each sample was immunoprecipitated with 5 μl of anti-*ras* antibody Y13-259 (lanes 2 and 4). The remaining half was subjected to immunoprecipitation with a nonspecific monoclonal antibody (lanes 1 and 3). The figure shows a 1-week fluorographic exposure of the immunoprecipitates after fractionation on an SDS-polyacrylamide gel treated with PPO-DMSO (2,5-diphenyloxazole–dimethyl sulfoxide) (4). Prestained molecular weight markers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used as size standards (numbers shown are $\times 10^3$).

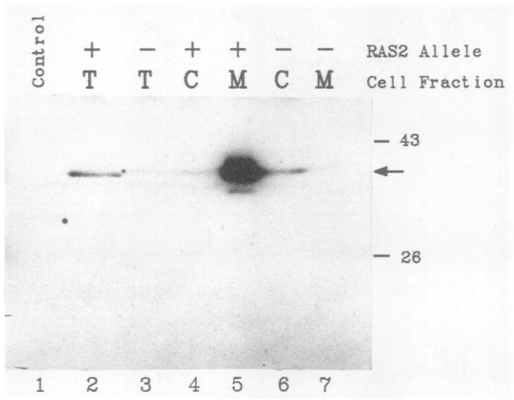

FIG. 6. Membrane localization of wild-type and mutant *RAS2* proteins. Strains BJ2169(YEp51-*RAS2*) (lanes 1, 2, 4, and 5) and BJ2169(YEp51-*ras2*$^{Ser-319}$) (lanes 3, 6, and 7) were pregrown as described in the legend to Fig. 5 and transferred to rich medium containing either 4% galactose (lanes 2 through 7) or 2% glucose (lane 1). Extracts were prepared and either used directly (lanes 1 to 3, designated T) or fractionated into membrane (lanes 5 and 7, designated M) and cytoplasmic (lanes 4 and 6, designated C) fractions. Samples (50 μg) were fractionated by SDS-polyacrylamide, transferred to nitrocellulose, and probed with anti-*ras* antibody, followed by ^{125}I-labeled anti-rat immunoglobulin G (Amersham). Numbers on right are defined in the legend to Fig. 5.

Membrane localization of *RAS2* protein was examined by isolating crude membrane and soluble fractions from strain BJ2169 carrying plasmid YEp-*RAS2* or YEp-*ras2*$^{Ser-319}$ and assessing the partitioning of the *RAS2* protein between the two fractions by Western analysis. Cells were grown in the presence of either glucose (repressing conditions) or galactose (inducing conditions) as described in Materials and Methods. Gel-fractionated extracts of the cultures were transferred to nitrocellulose and probed with anti-*ras* antibody Y13-259. In both strains, galactose induction resulted in production of a protein of 38 to 40 kilodaltons recognized by anti-*ras* antibody (Fig. 6, lanes 2 and 3). The wild-type *RAS2* protein migrated slightly faster than the mutant, consistent with prior observations that suggest that lipid acylation increases the mobility of *ras* protein in sodium dodecyl sulfate (SDS)-polyacrylamide gels (37). When the cells were fractionated into membrane and soluble fractions, we observed that this faster-migrating species was mem-

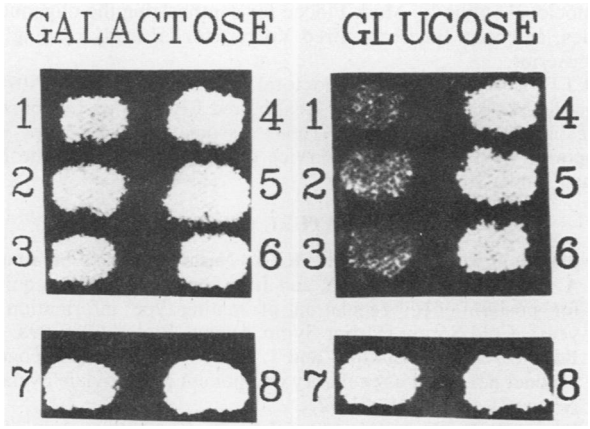

FIG. 7. Overexpression of carboxyl-terminal mutant genes restores function. Patches of *ras1 ras2*(YEp51-*ras2*$^{Ser-319}$) strains 273-2C (1), 273-5D (2), and 273-6B (3) and *ras1 ras2*(YEp51-*RAS2*) strains 270-3A (4), 270-4A (5), and 270-8C (6) were grown on rich medium containing 3% galactose and then replicated to a rich plate containing galactose (left) and to a rich plate containing 2% glucose (right). Plates are shown 2 days after replicating. Also present are the parents, JR29-4C (7) and JR29-2B (8), of the diploid that gave rise to the above strains.

brane specific (Fig. 6, lanes 4 and 5). The membrane fraction from only the wild-type strain contained *RAS2* protein, all of which migrated with the faster mobility. In the strain expressing *ras2*$^{Ser-319}$, only the slower-migrating species was observed, and it appeared only in the soluble fraction (Fig. 6, lane 6).

Using these inducible overexpression strains, we consistently observed higher levels of *RAS2* protein in the wild-type strain than in the mutant strain. This is due to an accumulation of wild-type protein in the membrane fraction after induction and a corresponding absence of accumulation of the mutant protein in either the membrane or the cytosolic fraction (Fig. 6). However, the magnitude of the difference between the levels of the wild-type and mutant proteins as represented in the cell fractionation in Fig. 6 (lanes 4 to 7) is misleading. Since we loaded equal amounts of membrane protein and soluble protein on the gel, the membrane fraction is overrepresented by approximately 10-fold.

Overexpression of *ras2*$^{Ser-319}$ restores *RAS2* function. From the experiments outlined above, we conclude that mutations affecting the acylation of yeast *RAS2* protein abolish its function and prevent its stable membrane association. This could mean that palmitate modification or membrane localization is absolutely required for constituting an active *RAS2* protein. On the other hand, modification or localization could function merely to enhance an already active protein, for example, by bringing it into close proximity to proteins with which it interacts. To distinguish between these two possibilities, we tested whether overexpression of the mutant allele could restore function. YEp-*RAS2* and YEp-*ras2*$^{Ser-319}$ were transformed into a diploid that was heterozygous at both the *RAS1* and *RAS2* loci (Table 1). The resulting strains, JR270 and JR273, were sporulated and dissected onto rich plates containing galactose to allow expression of the plasmid-borne *RAS2* genes. We found that we were able to obtain viable *ras1*$^-$ *ras2*$^-$ spore clones containing YEp-*RAS2* (JR270-3A, -4A, and -8C) or YEp-*ras2*$^{Ser-319}$ (JR273-2C, -6B, and -5D). Thus, *ras2*$^{Ser-319}$ expressed at high levels is able to complement a *ras1 ras2* strain. However, we found that JR273-derived strains could grow only on galactose (Fig. 7). Strains containing YEp-*RAS2* (JR270) were viable on glucose as well as galactose as the sole carbon source. We assume that residual expression from the multiple copies of *GAL10-RAS2* under repressing conditions provides sufficient *RAS2* protein to yield complementation by the wild-type, but not the nonacylated, protein. This result suggests that lipid acylation of *RAS2* protein is not strictly required for function, since a form that is not measurably modified still functions, albeit at a much lower efficiency.

DISCUSSION

Posttranslational acylation of the Harvey viral *ras* protein is essential to its transforming activity. Chen et al. (9) have shown that the protein is acylated through a thioester linkage of palmitate to the cysteine residue at position 187, which is the fourth amino acid from the carboxyl end. Concomitant with acylation, the protein exhibits a greater migration in SDS-polyacrylamide gels and beomes associated with the plasma membrane. Mutational alteration of this cysteine residue abrogates the transforming activity of the viral protein (42). Kirsten viral *ras* protein and cellular *ras* protein are also acylated (8), but the precise structure of these modifications is not known.

The maturation process of the yeast *RAS* proteins is similar to that of Ha-*ras*, although less well characterized. Fujiyama and Tamanoi (15) have shown that *RAS* protein is posttranslationally modified by the addition of lipid. As in Harvey *ras*, lipid attachment is coincident with an increase in mobility in SDS-polyacrylamide gels and association with a membrane fraction. However, the lipid linked to yeast *RAS* protein may be a mixture of several different species, including palmitate, myristate, and laurate (15). The site of attachment has not been identified, but the conservation of the carboxyl-terminal four residues among all known *ras* proteins, including those from yeasts, provides circumstantial evidence that the homologous cysteine residue (position 319) in the yeast protein is the modification site. Consistent with this possibility, we found that mutations that eliminate the carboxyl four residues or that convert cysteine 319 to serine abolish acylation of the protein.

Loss of acylation of the mutant yeast *ras2* proteins can be explained in several ways. The altered protein could fail to assume an appropriate conformation for serving as a substrate for acylation. Fujiyama and Tamanoi (15) have postulated that such a conformational change, apparent as a shift in mobility in polyacrylamide gel electrophoresis, occurs in the cytoplasm before, and independent of, acylation. An alternative explanation for the loss of acylation of the mutant proteins is that they may have a reduced potential to associate with the membrane. If the modification process actually occurs at the membrane, and given the nature of the substrate such a hypothesis is reasonable, then the failure of the mutant proteins to associate with the membrane could well reduce the level of modification of the mutant proteins. A final possibility is that the mutations simply eliminate the site of attachment of the lipid moiety. Given the conservative nature of the cysteine-to-serine substitution and the sequence similarity of the carboxyl end of the yeast *RAS2* protein with that of the Harvey viral *ras* protein, we favor this interpretation. It should be noted that if this interpretation is correct, the failure of the mutant protein to undergo a shift in mobility diminishes the likelihood of the hypothesis of Fujiyama and Tamanoi that *RAS* protein undergoes a major conformational or structural change before acylation.

We found that, although loss of acylation eliminates biological activity of the *RAS2* protein, function can be restored by overexpression of the protein. Restoration of function might result simply from increased levels of the protein compensating either for reduced specific activity of the mutant protein or for an increased turnover rate of the mutant protein. That is, the mutant protein could be inherently less active, or, in the absence of membrane sequestration, the mutant protein could be rapidly degraded. In the former case, increased expression would compensate for the reduced specific activity, and in the latter case, increased rate of synthesis of the mutant protein would yield a steady-state level of the mutant protein equivalent to or higher than that of the wild-type protein under normal conditions. We did, in fact, observe significantly increased accumulation of the wild-type protein over that of the mutant proteins in extended labeling experiments. As an alternative explanation, membrane localization could serve to bring *RAS2* protein into close proximity to other proteins with which it must interact. In this case, overexpression would compensate for loss of the concentrating effect normally achieved by membrane localization by simply increasing the concentration of the protein throughout the cell. The fact that adenylate cyclase is a membrane-localized protein with which *RAS2* protein interacts (41), either directly or indirectly, is consistent with this interpretation. In either event, our results demonstrate that acylation is not absolutely essential for *RAS2* protein function. That is, the absence of acylation does not completely abolish inherent GTP-binding or GTPase activity of the protein, nor does it preclude the protein occupying some topographic site in the cell that might be necessary for interaction with other components in the signal transduction pathway.

Broek et al. (6) and De Vendittis et al. (12) have reported that bacterially produced *RAS2* protein, which undoubtedly lacks fatty acid, will stimulate adenylate cyclase activity when added in vitro to a yeast membrane preparation. The ability of this nonacylated protein to promote activity in vitro could be due to the presence of the appropriate acyl transferase activity in the membrane preparation, which could yield posttranslational activation of the *RAS2* protein during the assay. Alternatively, as suggested by observations presented in this report, *RAS2* protein even lacking acyl modification could well activate adenylate cyclase in vitro, if presented at sufficiently high levels.

A homology between *RAS* and the G proteins of the mammalian hormone response and light transduction systems has been noted (18). However, one distinction between them is in the region important for fatty acylation. No sequence resembling the Cys-A-A-X sequence is present in the bovine adrenal α subunit of G_s, even at its carboxyl terminus (Tyr-Glu-Leu-Leu) (33), and only a weak conservation of the carboxyl sequence is evident in the transducin α subunit (Cys-Gly-Leu-Phe) (20, 24a). Certainly, such proteins may be acylated through a different mechanism or may achieve membrane sequestration through some process other than covalent lipid attachment. An alternative explanation, prompted by our results demonstrating that *RAS2* protein need not be acylated or stably localized to the membrane to be functional, is that the G proteins may use a mechanism independent of lipid-linked membrane attachment to act in signal transduction.

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