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Evidence for an *S*-Farnesylcysteine Methyl Ester at the Carboxyl Terminus of the *Saccharomyces cerevisiae RAS2* Protein†

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ABSTRACT: The protein products of yeast and mammalian *ras* genes are posttranslationally modified to give mature forms that are localized to the inner surface of the plasma membrane. We have previously demonstrated that the mature form of the *Saccharomyces cerevisiae RAS2* gene product is methyl esterified at a modified C-terminal cysteine residue. Here we provide evidence that this residue is an *S*-farnesylcysteine α-carboxyl methyl ester. This result establishes common posttranslational modifications for *RAS* proteins and fungal sex factors. These polypeptides exhibit sequence similarities at their C-termini that appear to be the critical recognition elements for a common set of modification enzymes. In mammalian cells, proteins with analogous C-terminal sequences appear to be prenylated and carboxyl methylated by a similar mechanism.

Peptides and proteins translated with C-terminal sequences including a cysteine residue in the fourth position from the terminus are candidates for a series of modification reactions including lipidation, proteolysis, and methyl esterification (Clarke et al., 1988). For example, the genes for the **a** mating factors from *Saccharomyces cerevisiae* encode C-terminal sequences of Cys-Val-Ile-Ala (Brake et al., 1985). The mature form of this factor, however, is lacking the terminal three amino acids, and the newly exposed cysteine residue is farnesylated at the sulfhydryl group and methylated at the carboxyl group (Betz et al., 1987; Anderegg et al., 1988). A similar sequence is present in the gene for the mating pheromone of the basidiomycetous yeast *Rhodosporidium toruloides* (Akada et al., 1989), and a similar C-terminal structure is present, although no evidence has been presented for its methyl esterification (Kamiya et al., 1978). In other proteins where terminal Cys-Xaa-Xaa-Xaa sequences are encoded by the gene, evidence has been obtained for at least some of these modification reactions. In the retinal cGMP phosphodiesterase, a C-terminal cysteine methyl ester has been demonstrated in a membrane binding domain (Ong et al., 1989). Members of the nuclear lamin family have been reported to be methyl esterified at unidentified sites (Chelsky et al., 1987), and evidence has been presented consistent with the farnesylation of the terminal cysteine residues (Beck et al., 1988; Wolda & Glomset, 1988; Farnsworth et al., 1989; Vorburger et al., 1989a). Additionally, several other peptides and proteins have been shown to be either isoprenylated at cysteine residues and/or methyl esterified at C-terminal cysteine residues where no information is available on the original coding region. These include the peptidyl sex factors from the jelly fungi *Tremella mesenterica* (Sakagami et al., 1981) and *Tremella brasiliensis* (Ishibashi et al., 1984), as well as mammalian small molecular weight G-proteins (Yamane & Fung, 1989) and potentially similar species (Backlund & Aksamit, 1988; Ota & Clarke, 1989).

We have been interested in this type of posttranslational processing in the mammalian and yeast ras protooncogene products, all of which contain Cys-Xaa-Xaa-Xaa C-terminal sequences (Barbacid, 1987; Santos & Nebreda, 1989). Evidence has been presented for the methyl esterification of the mammalian H-ras (Clarke et al., 1988) and N-ras (Gutierrez et al., 1989), as well as yeast *RAS2* protein (Deschenes et al., 1989), and for the isoprenylation of mammalian H-ras, K-ras,

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and N-ras proteins (Hancock et al., 1989; Casey et al., 1989; Schafer et al., 1989). The specific nature of these modifications and their relationship to each other, however, have not yet been determined. In this study, we present chemical evidence that the conserved cysteine at the carboxyl terminus of *RAS2* protein in yeast is in fact modified by attachment of an isoprenylated farnesyl (C-15) lipid through a thioether linkage and by attachment of a methyl ester on its α-carboxyl group.

Experimental Procedures

Srains and Plasmids. A yeast strain that overexpresses *RAS2* protein [JR830, *MATα leu2 ura3 trp1 his3 pep4*::*URA3*(YEp-*RAS2*)] was constructed by transformation of JR345-6A with a 2-μm based plasmid (YEp51) containing the *RAS2* gene behind a galactose-inducible promoter (Broach et al., 1983). Two *RAS2* alleles, wild type and SER^{318} mutant, were expressed in this fashion. The SER^{318} allele was constructed by oligonucleotide mutagenesis (Zoller & Smith, 1982). Sequence analysis was performed, confirming that the predicted C-terminal sequence has changed from -*Cys*-Cys-Ile-Ile-Ser to -*Ser*-Cys-Ile-Ile-Ser. The parent strain was made protease deficient by genetically disrupting *PEP4*, a gene required for the activation of lysosomal proteases (Hemmings et al., 1981). A more detailed description of this strain and the plasmid used to overproduce *RAS2* can be found elsewhere (Deschenes & Broach, 1987; Deschenes et al., 1989). All yeast manipulations were performed as described by Sherman et al. (1986).

Sulfate Labeling and Immunoprecipitation of RAS2 Protein. Cells were grown to a density of 4×10^7 cells/mL in 25 mL of synthetic medium (2% raffinose) lacking leucine to select for retention of the plasmid. Total protein was labeled by growth in [^{35}S]sulfate essentially as described by Deshaies and Schekman (1987). Intracellular sulfate pools were depleted by growing the strain in 25 mL of synthetic medium lacking sulfate until arrest occurs (generally 24–36 h). The medium was then supplemented to a final concentration of 0.05 mM sulfate, and expression of the plasmid-borne *RAS2* gene was induced by the addition of 4% galactose for 3 h. Cells were then washed to remove free sulfate and resuspended in 5 mL of synthetic medium containing 4% galactose, 1 mCi of $^{35}SO_4$ (2.5–4.0 Ci/mg, Amersham) was added, and the cells were labeled for 8–12 h. Cells were harvested by centrifugation, washed once in sorbitol buffer (0.3 M sorbitol, 0.1 M sodium chloride, 5 mM magnesium chloride, 10 mM Tris-HCl, pH 7.4), and lysed by vortexing with glass beads (425–600 μm, Sigma) in 1–2 mL of sorbitol buffer containing the following protease inhibitors: 100 units/mL aprotinin (Sigma), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1 μM pepstatin (Sigma). Unbroken cells and cellular debris were removed by centrifugation at 1500*g*. Crude membrane (P_{100}) and soluble (S_{100}) fractions were prepared by centrifugation at 100000*g* for 60 min in a Beckman SW50 Ti rotor. Immunoprecipitation of *RAS2* protein from the extracts was performed with anti-ras monoclonal antibody Y13-259 (Oncogene Science) as described elsewhere (Deschenes & Broach, 1987; Furth et al., 1982). The purity of the immunoprecipitated *RAS2* protein was documented previously (Deschenes et al., 1989).

Preparation of Standards of S-trans,trans-Farnesylcysteine and S-trans,trans-Farnesylcysteine Methyl Ester. S-trans,trans-Farnesylcysteine methyl ester was prepared from farnesyl bromide and cysteine methyl ester. *S-trans,trans*-Farnesyl bromide was prepared as described by Corey et al. (1972) and used immediately without purification. Cysteine methyl ester hydrochloride (0.4 mmol) and potassium bicarbonate (2.0 mmol) were dissolved in a minimal amount of water (3 mL) in a borosilicate test tube (18 × 150 mm). This solution was added to a 100-mL round-bottomed flask containing farnesyl bromide (1.2 mmol) at room temperature. *N,N*-Dimethylformamide (6 mL) was added until the bromide dissolved. At 30 min, thin-layer chromatography with silica gel plates revealed that cysteine methyl ester with an $R_f = 0.44$ in 1-butanol–acetic acid–water (8:1:1, v/v/v) had completely reacted to form a new ninhydrin-positive spot with an $R_f = 0.71$ in the same solvent system. The solution was concentrated in vacuo and dried overnight to remove residual *N,N*-dimethylformamide. The oily resiue was taken up into ethyl acetate (25 mL) and extracted with water (25 mL). The organic fraction was concentrated in vacuo and purified further on a silica gel column (silica gel 60, Merck 9385, 1.7 × 20.5 cm) eluted with acetonitrile. Appropriate fractions containing farnesylcysteine methyl ester were pooled, and final purification was accomplished by preparative HPLC with an Ecnosphere C18 reverse-phase column (Alltech/Applied Science, 10-mm inside diameter by 190-mm length, 5-μm spherical particles). The column was eluted at 3.0 mL/min at room temperature for 20 min in 40% solvent B followed by a linear gradient of 40% solvent B to 100% solvent B over 15 min (solvent A is 0.1% trifluoroacetic acid in water, and solvent B is 0.1% trifluoroacetic acid–90% acetonitrile–9.9% water). *S-trans,trans*-Farnesylcysteine methyl ester eluted from 13 to 20 min. This peak was pooled and concentrated on a Speedvac lyophilizer for 15 min to remove the organic fraction, and the remainder was lyophilized overnight. *S-trans,trans*-Farnesylcysteine methyl ester elutes at 25.5 min (one peak) under the analytical high-performance liquid chromatography conditions described in Figure 1. Thin-layer chromatography of this ester on silica gel plates (Merck 5735) revealed a ninhydrin-positive spot with an $R_f = 0.51$ in acetonitrile and $R_f = 0.81$ in 1-butanol–acetic acid–water (8:1:1, v/v/v). The R_f of the purified material in the latter solvent system is slightly larger than that measured for the crude reaction product due to the absence of the other components. Mass spectral analysis of farnesylcysteine methyl ester, performed at the University of California, Riverside, Mass Spectral Facility, revealed an experimental exact mass of 339.2234, calculated ($C_{19}H_{33}NO_2S$) 339.2232 ($\Delta = 0.6$ ppm). *S-trans,trans*-Farnesylcysteine was prepared from farnesylcysteine methyl ester by base hydrolysis. *S-trans,trans*-Farnesylcysteine methyl ester (250 nmol) was dissolved in 0.100 mL of 1 M sodium hydroxide and incubated for 4.0 h at 45 °C. After neutralization with dilute hydrochloric acid, farnesyl cysteine was purified by preparative HPLC on an Econosphere C18 reverse-phase column (Alltech/Applied Science, 10-mm inside diameter by 190-mm length, 5-μm spherical particles). The column was eluted at 3.0 mL/min at room temperature by a linear elution gradient of 0% solvent B to 100% solvent B over 50 min (solvent A is 0.1% trifluoroacetic acid in water, and solvent B is 0.1% trifluoroacetic acid–90% acetonitrile–9.9% water). *S-trans,trans*-Farnesylcysteine eluted at 27 min, and this peak was collected and concentrated in vacuo.

Enzymatic Digestion of RAS2 Protein. Thiolester-linked fatty acids were removed from wild-type *RAS2* protein by treatment with neutral hydroxylamine after the method of Wainfan and Bruggen (1957). Sepharose beads containing immunoprecipitated ^{35}S-labeled *RAS2* protein (approximately 60 μL) were added to 0.5 mL of 0.2 M hydroxylamine hydrochloride, pH 7.0, in a 1.5-mL polypropylene microcentrifuge tube and allowed to mix for 30 min at room temperature

on a rotating shaker. This sample was centrifuged for 5 min at 13600*g* and the supernatant removed. The beads were then washed with 0.5 mL of 20 mM sodium phosphate, pH 6.8, and recentrifuged as above, and the supernatant was removed. The radioactivity of each supernatant was determined by liquid scintillation spectrophotometry in ACS II scintillation fluid (Amersham). Enzymatic digestion of Ser^{318} *RAS2* mutant was performed without hydroxylamine incubation.

The *RAS2* protein containing hydroxylamine-treated beads were then treated with proteolytic enzymes to digest the protein to free amino acids. Two protocols were used. The first was modified from that of Van der Werf and Koshland (1977). The beads were resuspended in 0.5 mL of 20 mM sodium phosphate, pH 6.8, 7.5 units of Pronase E (*Streptomyces griseus*, Sigma type XIV, 5.8 units/mg of protein; 1 unit of hydrolyzes casein to produce Folin-Ciocalteu color equivalent to 1 μmol of tyrosine per minute at pH 7.5 at 37 °C) was added, and the mixture was incubated at 32 °C for 19 h. The beads were then centrifuged (13600*g*) for 5 min, and a small aliquot of the supernatant was taken for determination of total radioactivity. The remainder of the supernatant was then treated with 17.5 units of leucine aminopeptidase M (porcine kidney microsomes, Sigma type IV-S, 20 units/mg of protein; 1 unit hydrolyzes 1.0 μmol of L-leucine-*p*-nitroanilide per minute at pH 7.2 at 37 °C) and 38 units of prolidase (porcine kidney, Sigma, 205 units/mg of protein; 1 unit hydrolyzes 1.0 μmol of Gly-Pro per minute at pH 8.0 at 37 °C), and the digestion was continued for an additional 4 h at 32 °C. These latter proteases were obtained as ammonium sulfate suspensions at pH 7.7 and 8.0, respectively, and were used after centrifugation and resuspension in 20 mM sodium phosphate, pH 6.8. Following completion of the digestion, the sample was centrifuged for 5 min (13600*g*), and the supernatant was removed. A small aliquot of the supernatant was analyzed for radioactivity, and the remainder was lyophilized.

The second protocol was developed to avoid loss of the methyl ester linkage during the digestion. Sepharose beads containing immunoprecipitated ^{35}S-labeled *RAS2* protein were mixed with 0.37 unit of *Staphylococcus aureus* V8 protease (Sigma type XVII, 4.5 units/mg of solid; 1 unit will hydrolyze casein to Folin-Ciocalteu color equivalent to 1.0 μmol of tyrosine per minute at pH 7.5 at 37 °C) in 0.5 mL of 0.1 M 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane 1,3-diacetate, pH 7.0, containing 30 mM calcium chloride and 30 mM magnesium chloride. The mixture was incubated at 37 °C for 30 min, and then 2.6 units of leucine aminopeptidase M was added. The incubation was continued at 37 °C for an additional 90 min. At this point, an equivalent amount of leucine aminopeptidase M (2.6 units) was added, and the incubation was continued at 37 °C for a total digestion time of 3 h. Following completion of the digestion, the sample was centrifuged for 5 min (13600*g*), and the supernatant was removed. A small portion of the supernatant was analyzed for radioactivity. The beads were washed with 0.2 mL of acetonitrile. The acetonitrile wash was combined with the initial supernatant and analyzed immediately by HPLC.

Results

Modification of RAS2 Protein from S. cerevisiae by Isoprenylation of a Cysteine Residue. We identified modified cysteine residues in immunoprecipitates of ^{35}S-labeled yeast *RAS2* protein after enzymatic digestion of the protein into free amino acid residues. Samples were initially treated with hydroxylamine under conditions similar to those previously shown to remove more than 95% of the protein-bound palmitoyl groups of this protein (Fujiyama & Tamanoi, 1990). Controls

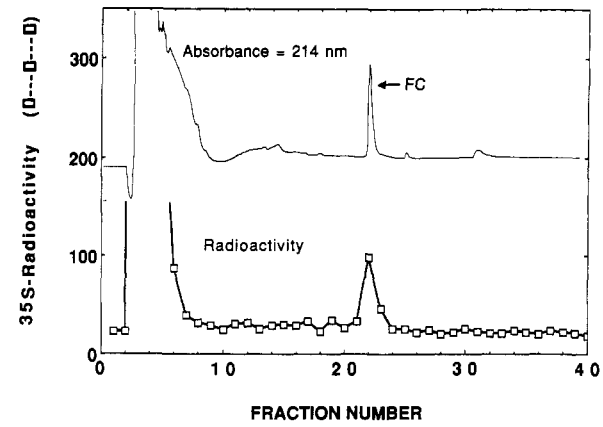

FIGURE 1: Isolation of [^{35}S]farnesylcysteine by HPLC from hydroxylamine-treated and proteolytically digested ^{35}S-labeled *RAS2* protein from *S. cerevisiae*. Immunoprecipitated ^{35}S-labeled *RAS2* protein was treated initially with neutral hydroxylamine and was then digested with Pronase E followed by a mixture of leucine aminopeptidase M and prolidase as described under Experimental Procedures. The lyophilized residue was resuspended in 1.0 mL of methanol–water (1:3 v/v). A sample (0.750 mL) was combined with 25 nmol of farnesylcysteine standard prepared as described under Experimental Procedures and fractionated by HPLC on a C18 reverse-phase column (Econosphere, Alltech/Applied Science, 4.6-mm inside diameter by 250-mm length) with a linear elution gradient of 0–100% solvent B (solvent A is 0.1% trifluoroacetic acid in water, and solvent B is 0.1% trifluoroacetic acid–90% acetonitrile–9.9% water) at 1.0 mL/min over 50 min at room temperature. Farnesylcysteine was detected by its UV absorption at 214 nm (solid line) on a Waters Model 441 detector; the peak at 22.6 min corresponds to 0.8 absorbance unit. One-minute fractions were collected and assayed for total radioactivity (□) by counting 50 μL of each fraction in 10 mL of scintillation fluid (Amersham, ACS II). The farnesylcysteine (FC) standard eluted in fractions 22 and 23 with 3500 cpm of the ^{35}S radioactivity.

performed with synthetic farnesylcysteine methyl ester demonstrated that this neutral hydroxylamine treatment did not cleave the methyl ester or isoprenoid modification. The hydroxylamine-treated *RAS2* protein was subsequently fragmented with Pronase, leucine aminopeptidase, and prolidase. Under these conditions, it would be expected that C-terminal α-methyl esters would be hydrolyzed by the nonspecific esterase activity of the Pronase preparation (Narahashi et al., 1970). Controls experiments showed that farnesylcysteine methyl ester was in fact hydrolyzed under these digestion conditions.

We tested whether radiolabeled species were present in this digest that would comigrate with specific S-isoprenylated cysteine derivatives on a C18 reverse-phase HPLC system designed to resolve these compounds. Such fractionation of the enzymatic digest revealed that the bulk of the radioactivity was eluted in early fractions at the position expected for free [^{35}S]methionine and [^{35}S]cysteine (Figure 1). However, a significant amount of radioactivity eluted later in the column at 22 min in the same fraction as a standard of the C-15 isoprenoid *S*-farnesylcysteine that was mixed with the sample before application to the column. Under the same conditions, the corresponding C-5 [*S*-(dimethylallyl)], C-10 (*S-trans*-geranyl), and C-20 [*S*-(*all-trans*-geranylgeranyl)] cysteine derivatives eluted at 11, 16, and 27 min, respectively. The recovery of total radioactivity injected from this chromatography step was 85%.

Although this result suggested that a *RAS2* cysteine residue was modified by an *S*-farnesyl group, we wanted to confirm this assignment by using additional high-resolution chromatography steps. First, we prepared fluorescent *o*-phthalaldehyde derivatives because HPLC methodology for sepa-

Table I: Recovery of Farnesylcysteine from Immunoprecipitated ^{35}S-Labeled *RAS2* following Enzymatic Digestion

sample	^{35}S radioactivity (cpm)	
	wild type	Ser^{318} mutant
supernatant of hydroxylamine-treated beads	72000	
supernatant of Pronase/leucine aminopeptidase/prolidase digest of hydroxylamine-treated beads	214825 (26853)[a]	95211 (13602)[a]
farnesylcysteine	3500 (13%)[b]	4420 (33%)[b]

[a] The expected radioactivity in a single cysteine or methionine residue is calculated on the basis of a content of six methionine and two cysteine residues in the mature protein with the same specific radioactivity. In the Ser^{318} mutant, there is only one cysteine residue. [b] Yield calculated on the basis of a single modified cysteine residue.

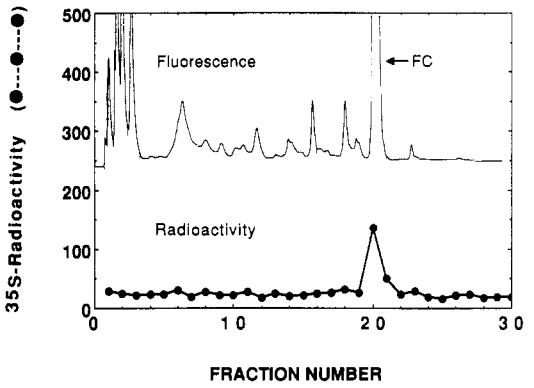

FIGURE 2: Confirmation of the identity of [^{35}S]farnesylcysteine by HPLC fractionation after *o*-phthalaldehyde derivatization. The fractions containing the farnesylcysteine standard from reverse-phase HPLC (Figure 1; fractions 22 and 23) were pooled and concentrated in vacuo. The residue was resuspended in 0.200 mL of methanol–water (1:3 v/v). A sample (60 μL, 300 cpm) was mixed with additional farnesylcysteine (500 pmol), and the mixture was derived with *o*-phthalaldehyde as described by Jones (1981). The solution was injected on a Waters C18 Resolve column (4.6-mm inside diameter by 150-mm length) with a linear elution gradient of 40–100% solvent B [solvent A is 50 mM sodium acetate–50 mM sodium phosphate, pH 7.4–tetrahydrofuran–methanol (96:2:2 v/v/v), and solvent B is methanol–tetrahydrofuran (90:10 v/v)] at 1.0 mL/min over 15 min at room temperature. Fluorescence was detected by a Gilson Model 121 fluorometer (RFU = 1.0) with a 305–394-nm bandwidth excitation filter and a 430–470-nm emission filter (solid trace). One-minute fractions were collected and assayed for total radioactivity (●) by counting the entire fraction (1.0 mL) in 10 mL of scintillation fluid (Amersham, ACS II).

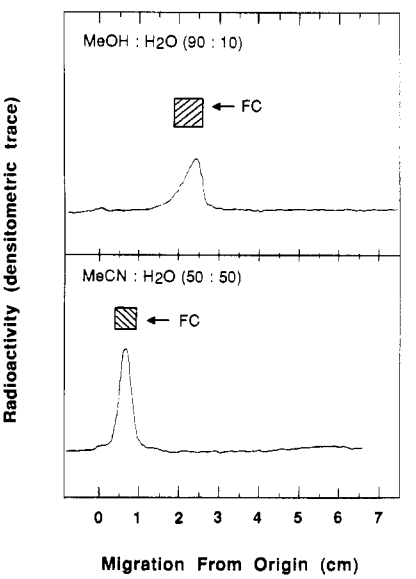

FIGURE 3: Confirmation of the identity of the proteolytic product as farnesylcysteine by reverse-phase thin-layer chromatography. A sample (60 μL, 300 cpm) of pooled and concentrated fractions 22 and 23 in methanol–water (1:3 v/v) from the reverse-phase HPLC shown in Figure 1 was mixed with additional farnesylcysteine standard (10 nmol) as described in the legend to Figure 2. This material was applied to a spot on each of the 5 × 10 cm long reverse-phase C18 glass plates (Whatman). The plates were developed in 600-mL covered chambers containing either a methanol–water (90:10 v/v) (top panel) or an acetonitrile–water (1:1 v/v) (bottom panel) solvent system. The farnesylcysteine standard was visualized by ninhydrin spray (cross-hatched area). Total radioactivity was determined by densitometric scan of an autoradiograph (Kodak XAR-5 film) after a 2-week exposure.

rating closely related amino acid derivatives, such as leucine and isoleucine, had already been well established (Jones, 1981). The radiolabeled fractions coeluting with the farnesylcysteine standard (Figure 1; fractions 22 and 23) were pooled, derivatized with *o*-phthalaldehyde, and analyzed by HPLC. The major peak of radiolabel was found to coelute exactly with the *o*-phthalaldehyde farnesylcysteine derivative (Figure 2). The recovery of total radioactivity injected from this chromatography step was 62%. Second, we analyzed similar samples by reverse-phase thin-layer chromatography. In two solvent systems the ^{35}S radioactivity cochromatographed exactly by autoradiography with the farnesylcysteine standard visualized with ninhydrin spray (Figure 3). To illustrate the resolving power of this latter separation system, we note that the R_f of the farnesylcysteine synthetic standard in methanol–water (9:1) was 0.35 while the farnesylcysteine methyl ester standard and an undigested peptide fragment containing farnesylcysteine methyl ester did not migrate at all under these conditions (R_f = 0).

The recovery of radioactivity in the farnesylcysteine peak in Figure 1 represents about 13% of that expected for the labeling of a single cysteine residue in the *RAS2* protein (Table I). A somewhat better yield was obtained with the variant of the *RAS2* protein in which the cysteine residue immediately preceding the conserved cysteine residue was converted to a serine residue (Table I). We performed a digestion protocol identical with that employed with the wild-type *RAS2* protein on an ^{35}S-labeled Ser^{318} mutant *RAS2* protein. The supernatant was analyzed as described in Figure 1 and resulted in a 33% recovery of farnesylcysteine (Table I). The ^{35}S-labeled farnesylcysteine isolated cochromatographed with synthetic farnesylcysteine when derivatized with *o*-phthalaldehyde on reverse-phase HPLC and on reverse-phase thin-layer chromatography plates. The reason for the improved yield with the serine mutant is not clear. It is possible that Cys^{318} is modified in some way that renders the Cys^{318}–Cys^{319} bond resistant to proteolytic cleavage.

Isolation of [^{35}S]*Farnesylcysteine Methyl Ester from a* Ser^{318} *RAS2 Mutant.* The enzymatic digestion protocol detailed earlier for the isolation of [^{35}S]farnesylcysteine results in the cleavage of the methyl ester linkage (see above). Therefore, we developed a new digestion protocol using enzymes that would spare the α-methyl ester linkage. This was accomplished by a short digestion protocol with *S. aureus* V8 protease followed by leucine aminopeptidase M as detailed under Experimental Procedures. The supernatant from the digestion was fractionated immediately by reverse-phase HPLC, and the results are shown in Figure 4A. The majority of the radioactivity elutes in fractions 3 and 4 in positions consistent for the elution of radiolabeled free methionine and

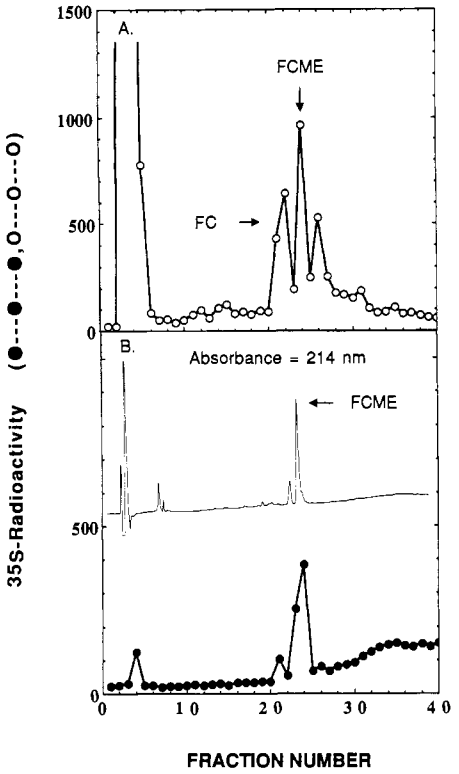

FIGURE 4: Isolation of [^{35}S]farnesylcysteine methyl ester by HPLC from proteolytically digested ^{35}S-labeled *RAS2* protein from *S. cerevisiae*. Immunoprecipitated ^{35}S-labeled *RAS2* protein was digested with *S. aureus* V8 protease followed by leucine aminopeptidase M as described under Experimental Procedures. (A) The supernatant was fractionated by HPLC on a C18 reverse-phase column as described in Figure 1. One-minute fractions were collected and assayed for total radioactivity (O) by counting 100 μL of each fraction in 10 mL of scintillation fluid (Amersham, ACS II). (B) Fraction 24 (100 μL, 964 cpm) was mixed with farnesylcysteine methyl ester standard prepared as described under Experimental Procedures and fractionated by HPLC as described in Figure 1. Farnesylcysteine methyl ester (FCME) was detected by its UV absorption at 214 nm (solid line) on a Waters Model 441 detector; the peak at 23.9 min corresponds to 0.5 absorbance unit. One-minute fractions were collected and assayed for total radioactivity (●) by counting the entire fraction in 10 mL of scintillation fluid (Amersham, ACS II). Farnesylcysteine methyl ester (FCME) standard eluted in fractions 23 and 24 with 600 cpm of ^{35}S radioactivity.

cysteine. However, a significant amount of radioactivity elutes at fraction 24 and migrates in a position identical with that of the synthetic *S*-farnesylcysteine methyl ester standard. Under these conditions, we find that standards of *S*-(dimethylallyl)- (C-5), *S*-geranyl- (C-10), and *S*-(geranylgeranyl)cysteine methyl ester elute at 15, 20, and 29 min, respectively, and are well separated from the radioactive peak seen here. Reverse-phase thin-layer chromatography (data not shown) and subsequent HPLC analysis confirm the identification of the radioactive peak as *S*-farnesylcysteine methyl ester (Figure 4B). The peak eluting in fractions 21 and 22 (Figure 4A) corresponds to farnesylcysteine as confirmed by reverse-phase thin-layer chromatography (data not shown). Finally, a significant amount of radioactivity elutes in fraction 26. Reverse-phase thin-layer chromatography of this material demonstrates that it does not correspond to either the farnesylcysteine or the farnesylcysteine methyl ester standard (data not shown). However, further digestion of fraction 26 with leucine aminopeptidase M generated farnesylcysteine methyl ester. Therefore, this peak is apparently a fragment containing farnesylcysteine methyl ester that was digested incompletely. The yields of farnesylcysteine methyl ester and farnesylcysteine were 30% and 33%, respectively

Table II: Recovery of Farnesylcysteine Methyl Ester from Immunoprecipitated ^{35}S-Labeled *RAS2* following Enzymatic Digestion

sample	^{35}S radioactivity (cpm) of Ser^{318} mutant
supernatant of *S. aureus* V8 protease/leucine aminopeptidase digest	223 622 (31 946)[a]
farnesylcysteine methyl ester	9 640 (30%)[b]
farnesylcysteine	10 715 (33%)[b]
incompletely digested fragment containing farnesylcysteine methyl ester	5 260 (16%)[b]

[a] The expected radioactivity in a single cysteine or methionine residue is calculated on the basis of a content of six methionine and one cysteine residue in the mature protein with the same specific radioactivity. [b] Yield calculated on the basis of a single modified cysteine residue.

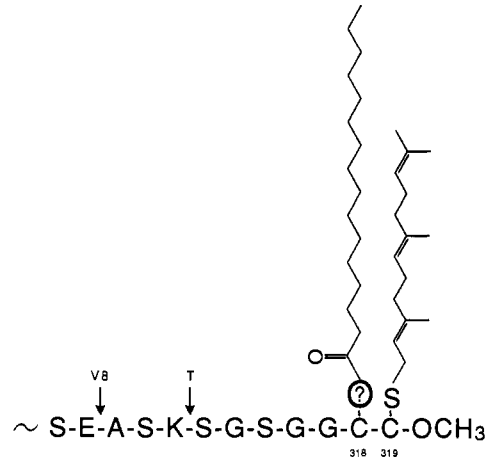

FIGURE 5: Structure of the C-terminus of the mature *S. cerevisiae* *RAS2* protein. The last three residues of the primary translation product are cleaved, and Cys^{319}, the resultant C-terminal residue, is modified by *S*-farnesylation and α-carboxyl methylation. Covalent linkage of palmitate has been localized to Cys^{318} (Fujiyama & Tamanoi, 1990).

(Table II). The recovery of total radioactivity injected from the initial chromatography fractionation was 80%. The isolation of farnesylcysteine from enzymatic digestion of the Ser^{318} mutant was apparently the result of methyl ester hydrolysis rather than a partially methylated mutant *RAS2* protein. A similar enzymatic digestion performed at pH 6.0 produced only the methyl esterified farnesylcysteine residue.

Discussion

Several lines of evidence have suggested that ras proteins undergo a series of posttranslational modifications at their C-termini including proteolysis, prenylation, and carboxyl methylation (Clarke et al., 1988; Casey et al., 1989; Deschenes et al., 1989; Hancock et al., 1989; Schafer et al., 1989). Until now, analogies with related modifications at the C-termini of peptidyl fungal mating factors (Kamiya et al., 1978, 1979a,b; Sakagami et al., 1981; Ishibashi et al., 1984; Miyakawa et al., 1985; Anderegg et al., 1988) have provided the best indication as to the details of the chemistry involved. In this paper we provide chemical evidence for the presence of an *S*-farnesylcysteine methyl ester (Figure 5) at the C-terminus of the *S. cerevisiae* *RAS2* protein. Previous work, using both mass spectral and NMR techniques, had shown that mammalian proteins could be modified by either *S-trans,trans*-farnesyl (Farnsworth et al., 1989) or *S*-(*all-trans*-geranylgeranyl) (Farnsworth et al., 1990; Rilling et al., 1990; Yamane et al., 1990) groups. Our work strongly suggests that the isoprenoid

Table III: Eucaryotic Proteins Containing -Cys-Xaa-Xaa-Xaa C-Terminal Tails[a]

protein	carboxyl-terminal sequence	lipidation on conserved Cys	methylation	proteolysis of terminal three amino acids
fungal mating pheromones				
S. cerevisiae **a** factor	-Asp-Pro-Ala -Cys-Val-Ile-Ala[b]	farnesyl[c]	yes[c]	yes[c]
T. brasiliensis (A-9291-I)	-Ser-Gly-Gly -Cys[d,e]	oxidized farnesyl[d]	yes[d]	
T. mesenterica (A-10)	-Asn-Gly-Tyr -Cys[e,f]	oxidized farnesyl[f]	yes[f]	
R. toruloides rhodotorucine A	-Arg-Asn-Gly -Cys-Thr-Val-Ala[g]	farnesyl[h]	no[h]	yes[h]
ras proteins				
human/mouse Ha-*ras*	-Ser-Cys-Lys -Cys-Val-Leu-Ser[i]	polyisoprene[i]	yes[j]	yes[k]
human Ha-*ras*-1 variant	-Ser-Ser-Lys -Cys-Val-Leu-Ser[i]	polyisoprene[i]		
rat Ha-*ras*-1	-Ser-Cys-Lys -Cys-Val-Leu-Ser[l]			
chicken Ha-*ras*-1	-Asn-Cys-Lys -Cys-Val-Ile-Ser[m]			
human Ki-*ras*-2A	-Ile-Lys-Lys -Cys-Ile-Ile-Met[n]			
mouse Ki-*ras*-2A	-Ile-Lys-Lys -Cys-Val-Ile-Met[o]			
human Ki-*ras*-2B	-Lys-Thr-Lys -Cys-Val-Ile-Met[i]	polyisoprene[i,p]		
rat Ki-*ras*-2B	-Arg-Thr-Arg -Cys-Ile-Val-Met[q]			
mouse Ki-*ras*-2B	-Arg-Thr-Arg -Cys-Thr-Val-Met[n]			
human N-*ras*	-Gly-Leu-Pro -Cys-Val-Val-Met[i]	polyisoprene[i]	yes[k]	yes[k]
mouse N-*ras*	-Gly-Ser-Pro -Cys-Val-Leu-Met[r]			
Drosophila Dras1	-Arg-Phe-Lys -Cys-Lys-Met-Leu[s]			
Drosophila Dras2/64B	-Lys-Arg-Lys -Cys-Cys-Leu-Met[s]			
Dictyostellium discoideum Dd*ras*	-Lys-Lys-Gln -Cys-Leu-Ile-Leu[t]			
Saccharomyces pombe SPRAS	-Thr-Lys-Cys -Cys-Val-Ile-Cys[u]			
S. cerevisiae RAS1	-Gly-Gly-Cys -Cys-Ile-Ile-Cys[v]			
S. cerevisiae RAS2	-Gly-Gly-Cys -Cys-Ile-Ile-Ser[v]	farnesyl[w]	yes[w]	yes[w]
ras-related small G-proteins				
Drosophila Dras3	-Lys-Val-Pro -Cys-Val-Leu-Leu[x]			
human/mouse R-*ras*	-Gly-Cys-Pro -Cys-Val-Leu-Leu[y]			
human *rap*1A/K*rev*-1	-Lys-Lys-Ser -Cys-Leu-Leu-Leu[z]			
human *rap*1B	-Lys-Ser-Ser -Cys-Gln-Leu-Leu[aa]			
human *rap*2	-Lys-Ser-Pro -Cys-Val-Leu-Met[z]			
Aplysia rho	-Lys-Gly-Gly -Cys-Val-Val-Leu[ab]			
human *rho*A	-Lys-Ser-Gly -Cys-Leu-Val-Leu[ac]			
human *rho*B	-Ile-Asn-Cys -Cys-Lys-Val-Leu[ac]			
human *rho*C	-Arg-Arg-Gly -Cys-Pro-Ile-Leu[ac]			
human *rac*1	-Lys-Arg-Lys -Cys-Leu-Leu-Leu[ad]			
human *rac*2	-Lys-Arg-Ala -Cys-Ser-Leu-Leu[ad]			
human *ral*A	-Arg-Glu-Arg -Cys-Cys-Ile-Leu[ae]			
human *ral*B	-Lys-Glu-Arg -Cys-Cys-Leu-Leu[ae]			
Saguinus oedipus ral	-Arg-Glu-Arg -Cys-Cys-Ile-Leu[af]			
S. cerevisiae RHO1	-Lys-Lys-Lys -Cys-Val-Leu-Leu[ag]			
S. cerevisiae RHO2	-Ala-Asn-Cys -Cys-Ile-Ile-Leu[ag]			
S. cerevisiae RSR1	-Ala-Ser-Thr -Cys-Thr-Ile-Leu[ah]			
heterotrimeric (large) G-proteins				
bovine brain G-protein (γ-subunit)	-Lys-Phe-Phe -Cys-Ala-Ile-Leu[ai]	geranylgeranyl[bk]	yes[aj]	yes[aj]
bovine transducin (γ)-subunit)	-Lys-Gly-Gly -Cys-Val-Ile-Ser[ak]	farnesyl[bl]	yes[bl]	yes[bl]
bovine transducin (α-subunit)	-Leu-Lys-Asp -Cys-Gly-Leu-Phe[al]	no[am]	no[am]	no[am]
bovine/rat Go (α-subunit)	-Leu-Arg-Gly -Cys-Gly-Leu-Tyr[an]			
human/bovine/rat/mouse $G_i(1)$, $G_i(2)$ (α-subunit)	-Leu-Lys-Asp -Cys-Gly-Leu-Phe[ao]	no[an]	no[an]	no[an]
human/bovine/rat/mouse $G_i(3)$ (α-subunit)	-Leu-Arg-Glu -Cys-Gly-Leu-Tyr[ao]			
S. cerevisiae STE18 (γ-subunit)	-Ser-Val-Cys -Cys-Thr-Leu-Met[ap]	polyisoprene[bm]		
nuclear lamin proteins				
human lamin A	-Pro-Gln-Asn -Cys-Ser-Ile-Met[aq]	polyisoprene[ar]		
Xenopus laevis lamin A	-Pro-Gln-Asn -Cys-Ser-Ile-Met[as]			
chicken lamin A	-Pro-Gln-Gly -Cys-Ser-Ile-Met[at]	polyisoprene[au]		
murine lamin B	-Glu-Arg-Ser -Cys-Val-Val-Met[av]	farnesyl[aw]	yes[ax]	
chicken lamin B1	-Glu-Arg-Ser -Cys-Val-Val-Met[at]	polyisoprene[au]		
chicken lamin B2	-Ser-Arg-Gly -Cys-Leu-Val-Met[ay]	polyisoprene[au]		yes[au]
X. laevis L_1	-Asn-Lys-Asn -Cys-Ala-Ile-Met[az]			
X. laevis L-III	-Asp-Pro-Ser -Cys-Ser-Ile-Met[ba]			
Drosophila lamin B	-Asn-Glu-Lys -Cys-Ala-Ile-Met[bb]			
additional proteins				
bovine cGMP phosphodiesterase (α-subunit)	-Ser-Lys-Ser -Cys-Cys-Val-Gln[bc]		yes[bc]	yes[bc]
human cAMP phosphodiesterase	-Leu-Gln-Ser -Cys-Thr-Ile-Ile[bd]			
human extracellular superoxide dismutase	-Glu-Ser-Glu -Cys-Lys-Ala-Ala[be]			
human(2′–5′)oligo(A) synthetase E18	-Asp-Trp-Thr -Cys-Thr-Ile-Leu[bf]			
mouse(2′–5′)oligo(A) synthetase	-Asp-Trp-Thr -Cys-Ile-Leu-Leu[bg]			
human/rat gap junction protein	-Ser-Asp-Arg -Cys-Ser-Ala-Cys[bh]			
leukemia antigen	-Glu-Lys-Lys -Cys-Arg-Val-Trp[bi]			
rabbit phosphorylase kinase (α-subunit)	-His-Ser-Ile -Cys-Ala-Met-Gln[bj]			

[a] Proteins listed contain an aliphatic residue (Ala, Ile, Val, Leu, Met) in the pentultimate position and a cysteine residue in the fourth position from the end. [b] Brake et al., 1985. [c] Anderegg et al., 1988. [d] Ishibashi et al., 1984. [e] The primary transcript sequence has not been determined. The sequence of the mature polypeptide is given. [f] Sakagami et al., 1981. [g] Akada et al., 1987. [h] Kamiya et al., 1978, 1979a,b. [i] Hancock et al., 1989. [j] Clarke et al., 1988. [k] Gutierrez et al., 1989. [l] Ruta et al., 1986. [m] Westaway et al., 1986. [n] McGrath et al., 1983. [o] George et al., 1985. [p] Casey et al., 1989. [q] Shimizu et al., 1983. [r] Guerrero et al., 1985. [s] Brock, 1987. [t] Reymond et al., 1984. [u] Fukui & Kaziro, 1985. [v] Powers et al., 1984.

Table III (Continued)

[w] This study. [x] Schejter & Shilo, 1985. [y] Lowe et al., 1987. [z] Pizon et al., 1988a. [aa] Pizon et al., 1988b. [ab] Madaule & Axel, 1985. [ac] Chardin et al., 1989. [ad] Didsbury et al., 1989. [ae] Chardin & Tavitian, 1989. [af] Chardin & Tavitian, 1986. [ag] Madaule et al., 1987. [ah] Bender & Pringle, 1989. [ai] Gautam et al., 1989. [aj] Fung et al., 1990. [ak] Hurley et al., 1989 [al] Lochrie et al., 1985. [am] West et al., 1985. [an] Itoh et al., 1986. [ao] Beals et al., 1987. [ap] Whiteway et al., 1989. [aq] Fisher et al., 1989. [ar] Beck et al., 1988. [as] Wolin et al., 1987. [at] Peter et al., 1989. [au] Vorburger et al., 1989a. [av] Hoger et al., 1988. [aw] Modification determined for human species; Farnsworth et al., 1989. [ax] Chelsky et al., 1987. [ay] Vorburger et al., 1989b. [az] Krohne et al., 1987. [ba] Stick, 1988. [bb] Gruenbaum et al., 1988. [bc] Ong et al., 1989. [bd] Kurihara et al., 1988. [be] Hjalmarsson et al., 1987. [bf] Benech et al., 1985. [bg] Ichii et al., 1986. [bh] Kumar & Gilula, 1986. [bi] Shipp et al., 1988. [bj] Zander et al., 1988. [bk] Yamane et al., 1990. [bl] Fukada et al., 1990. [bm] Finegold et al., 1990.

group on yeast *RAS2* is an example of the first type of modification. However, because we have not been able to isolate sufficient quantities of the proteolytic products for mass spectral or NMR analyses, we cannot eliminate the possibility that the modification of the yeast protein is a group closely related to *trans,trans*-farnesyl.

The results reported here provide additional data linking the posttranslational modifications of ras proteins and peptide mating factors in yeast. In *S. cerevisiae*, the *RAS2* gene, as well as those for **a** mating factor, encodes a cysteine residue three amino acids from the C-terminus (Powers et al., 1984; Brake et al., 1985). Both primary gene products are then processed similarly. A farnesyl moiety is added in a thioether linkage of this cysteine residue. The three amino acids to the carboxyl-terminal side of the last cysteine are cleaved; the cysteine is carboxyl methylated. A common posttranslational processing pathway would explain how mutations at a single locus, designated *DPR1* or *RAM1*, can cause a deficiency in both **a** factor expression and *RAS2* activity (Powers et al., 1986; Fujiyama et al., 1987). From sequence similarities between *DPR1* and thiol proteases it has been suggested that this protein catalyzes the proteolytic cleavage event (Goodman et al., 1988). Consistent with this possibility is the observation that *RAS2* is not proteolytically cleaved in a *dpr1* mutant (Fujiyama & Tamanoi, 1990). However, the same result would be obtained if *RAM1*/*DPR1* encoded a step earlier in the pathway that is required for subsequent cleavage. In fact, it has recently been found that *ram1* mutant strains fail to carry out the farnesylation of yeast *RAS2* proteins (L. Fahr and R. J. Deschenes, unpublished observations).

In mammalian cells, nuclear lamin B has been shown to be farnesylated and carboxyl methylated (Chelsky et al., 1987; Farnsworth et al., 1989), and preliminary results indicate that several other proteins may be similarly modified. These include the α-subunit of cGMP phosphodiesterase from retinal rods (Ong et al., 1989), the γ-subunit of G-proteins in brain (Fung et al., 1990), and several different ras-related GTP-binding proteins from a variety of different mammalian tissues (Backlund & Aksamit, 1988; Yamane & Fung, 1989; Ota & Clarke, 1989; Maltese et al., 1990). DNA sequence data indicate that many of these proteins are translated with a cysteine three residues from the C-terminus within a conserved Cys-Xaa-Xaa-Xaa-COOH motif termed a CXXX tail (Table III), where the residue following the cysteine is generally aliphatic and the penultimate residue is always aliphatic. Besides the CXXX tail, nuclear lamin B, the fungal sex factors, ras and ras-related GTP-binding proteins, the α-subunit of cGMP phosphodiesterase, and the γ-subunit of G-proteins do not appear to be structurally or functionally related. Moreover, when a CXXX tail was genetically linked to an unrelated carrier sequence (protein A), the product was prenylated (Hancock et al., 1989). Thus, it appears that a CXXX tail is the essential recognition element for this type of posttranslational processing, although it is not clear why some proteins are farnesylated and some are geranylgeranylated (Farnsworth et al., 1990; Rilling et al., 1990; Yamane et al., 1990). In Table III, we have listed several additional candidate proteins for these modifications that have CXXX tail sequences encoded by cDNA sequences with penultimate aliphatic residues.

An interesting example of a CXXX-like C-terminal sequence that is probably not modified occurs in the α-subunits of some members of the large G family of GTP-binding proteins (e.g., the G_i's, G_t, and G_o; Table III). In all of these polypeptides, the cysteine is followed by a glycine residue, and in at least two studies it has been directly shown that the tail remains intact in the mature protein (West et al., 1985; Itoh et al., 1986). The conserved cysteine residue corresponds to the site of ADP-ribosylation of these proteins by pertussis toxin (West et al., 1985), a reaction that would be expected to be blocked by S-isoprenylation. It is tempting to speculate that it is the presence of the adjacent glycine residue that prevents modification in these proteins.

CXXX-tailed proteins often contain additional cysteines upstream of the conserved cysteine. These are candidates for another type of modification, palmitoylation (Hancock et al., 1989; Fujiyama & Tamanoi, 1990). In *S. cerevisiae RAS2*, it has been suggested that Cys^{318} is the site of palmitoylation (Fujiyama & Tamanoi, 1990; Figure 5). Prevention of palmitoylation has only a minimal effect on biological activity and membrane localization of yeast *RAS2* (Deschenes & Broach, 1987). In mammalian ras proteins, the palmitoylation reaction is associated with membrane-association (Gutierrez et al., 1989). Since pulse–chase and subcellular fractionation studies indicate that palmitoylation occurs late in the posttranslational processing of *S. cerevisiae RAS2* (Tamanoi et al., 1988; Fujiyama et al., 1987), it seems reasonable that *RAS2* is not palmitoylated until it becomes membrane associated. Cysteines localized near the plasma membrane are generally subject to palmitoylation [for reviews, see Olson (1988), Towler et al. (1988), and Schultz et al. (1988)].

In *S. cerevisiae*, both **a** factor and *RAS* regulate the activities of membrane-associated proteins. **a** factor binds to a membrane receptor of the β-adrenergic receptor/rhodopsin superfamily to control the activity of a heterotrimeric G-protein (Whiteway et al., 1989), and *RAS2* regulates the activity of a membrane-bound adenylate cyclase (Toda et al., 1985). The data are consistent with farnesylation/methylation acting to increase the effective concentration at target sites within the membrane, although the possibility that these modifications induce an improved conformation for substrate/target interaction has not been excluded. Buss et al. (1989) have demonstrated that attachment of a myristoyl group in amide linkage to the N-terminus of Ha-ras can functionally replace the C-terminal farnesyl for membrane localization. This modification leads to transformation presumably because the protein is locked in an active conformation. Thus, in addition to its involvement in membrane targeting, farnesylation and/or methylation may play a crucial role in the regulation of *RAS* activity.

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