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Maturation of Isoprenylated Proteins in *Saccharomyces cerevisiae*

MULTIPLE ACTIVITIES CATALYZE THE CLEAVAGE OF THE THREE CARBOXYL-TERMINAL AMINO ACIDS FROM FARNESYLATED SUBSTRATES *IN VITRO**

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Eukaryotic polypeptides containing COOH-terminal -CXXX sequences can be posttranslationally modified by isoprenylation of the cysteine residue via a thioether linkage, proteolytic removal of the three terminal amino acids, and α-carboxyl methylation of the cysteine residue. Through the development of an indirect coupled assay, we have identified three *in vitro* activities in the yeast *Saccharomyces cerevisiae* that can catalyze the proteolytic cleavage of the three COOH-terminal amino acids of the synthetic peptide substrate *N*-acetyl-KSKTK[*S*-farnesyl-Cys]VIM. One of these is the vacuolar protease carboxypeptidase Y. Using a mutant strain deficient in this enzyme, we find evidence for an additional soluble activity as well as for a membrane-associated activity. These latter activities are candidates for roles in the physiological processing of isoprenylated protein precursors. They are both insensitive to inhibitors of serine and aspartyl proteinases but are sensitive to sulfhydryl reagents and 0.5 mM $ZnCl_2$. The soluble activity appears to be a metalloenzyme, inhibitable by 2 mM *o*-phenanthroline but not by 1 mM *N*-ethylmaleimide, whereas the membrane-associated enzyme is inhibitable by 1 mM *N*-ethylmaleimide but not 2 mM *o*-phenanthroline. We show that the membrane-bound protease is not an activity of the membrane-bound methyltransferase, because protease activity is observed in membrane preparations that lack the *STE14*-encoded methyltransferase. The soluble activity appears to be a novel carboxypeptidase of approximately 110 kDa that catalyzes a processive removal of amino acids from the COOH terminus from both the farnesylated and nonfarnesylated substrate, but not from three other unrelated peptides. Finally, we find no evidence for nonvacuolar membrane or soluble activities that catalyze the ester hydrolysis of *N*-acetyl-*S*-farnesyl-L-cysteine methyl ester.

In eukaryotic cells, there are a number of proteins and polypeptides whose initial translation product contains a -Cys-Xaa-Xaa-Xaa sequence at the COOH terminus, where Xaa represents a variety of amino acid residues. This sequence can target these polypeptides for a series of posttranslational modifications that include sequential lipidation with either a farnesyl or geranylgeranyl isoprene group on the cysteine residue, proteolysis of the three terminal amino acids, and methyl esterification of the newly exposed α-carboxyl group of the cysteine residue (Clarke *et al.*, 1988; Hancock *et al.*, 1989; Glomset *et al.*, 1990; Maltese, 1990; Rine and Kim, 1990, Stimmel *et al.*, 1990, Der and Cox, 1991; Hancock *et al.*, 1991; Clarke, 1992). The COOH-terminal amino acid of this motif appears to specify the nature of the isoprene group; polypeptides ending with a leucine residue are geranylgeranylated, and those ending with methionine, serine, alanine or glutamine residues are targeted for farnesylation (Kawata *et al.*, 1990; Kinsella *et al.*, 1991, Yamane *et al.*, 1991).

We have been interested in defining this processing pathway in the yeast *Saccharomyces cerevisiae*. Much has already been done in characterizing both the lipidation and methylation reactions, the first and last steps of this pathway. Two distinct isoprenyltransferases have been identified which can catalyze the isoprenylation reaction in yeast. A farnesyltransferase comprised of two subunits, the RAM1 (Powers *et al.*, 1986; Fujiyama *et al.*, 1987; Goodman *et al.*, 1988) and RAM2 gene products (Goodman *et al.*, 1990; Moores *et al.*, 1991; He *et al.*, 1991), appears to be responsible for the lipidation of -CXXX-containing polypeptides such as the RAS proteins (Goodman *et al.*, 1990), the *STE18* gene product (Finegold *et al.*, 1990), and the **a**-factor mating pheromone (Anderegg *et al.*, 1988; Marcus *et al.*, 1991), by a C_{15} isoprenoid group. The second enzyme is a geranylgeranyltransferase that is comprised of two subunits encoded by the *CDC43/CAL1* gene (Johnson *et al.*, 1991a; Johnson *et al.*, 1991b; Ohya *et al.*, 1991) and the *RAM2* gene by the addition of a C_{20} geranylgeranyl moiety (Finegold *et al.*, 1991; Moores *et al.*, 1991). Similar enzymes have been characterized in mammalian cells (Reiss *et al.*, 1990; Chen *et al.*, 1991; Joly *et al.*, 1991; Moores *et al.*, 1991; Reiss *et al.*, 1991; Seabra *et al.*, 1991; Yoshida *et al.*, 1991; Yokayama *et al.*, 1991).

The enzymes involved in the final methylation step have also been well characterized. We have shown that a membrane-bound COOH-terminal methyltransferase, the product of the *STE14* gene in *S. cerevisiae*, can catalyze the methylation of both farnesylated and geranylgeranylated substrates (Hrycyna and Clarke, 1990; Hrycyna *et al.*, 1991). This methyltransferase is responsible for the *in vivo* methylation of several cellular polypeptides including RAS1, RAS2, and the mating pheromone **a**-factor (Hrycyna *et al.*, 1991) and is essential for the secretion and biological activity of **a**-factor (Hrycyna *et al.*, 1991; Marcus *et al.*, 1991). A mammalian counterpart possessing similar activity to the STE14 methyltransferase has also been described (Stephenson and Clarke, 1990, 1992; Volker *et al.*, 1991).

In contrast, not much is known to date about the proteolytic processing enzymology either in yeast or other cell types. This

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processing event was shown to occur indirectly by the isolation of α-carboxyl-methylated cysteine residues from proteins whose cDNA codes for 3 more COOH-terminal amino acids (Ong *et al.*, 1989; Stimmel *et al.* 1990; Yamane *et al.*, 1990; Yamane *et al.*, 1991). Direct evidence for proteolysis of the 3 COOH-terminal amino acids of the $p21^{ras}$ protein in mammalian cells was provided by Gutierrez *et al.*. (1989), and for the yeast RAS2 protein, by Fujiyama and Tamanoi (1990). More recently, a membrane-associated protease activity in canine pancreatic microsomes has been reported which increases the membrane binding of farnesylated $p21^{H\text{-}ras}$ 2-fold over the non-proteolyzed species (Hancock *et al.*, 1991). In the present study, we characterize enzyme activities responsible for the proteolytic cleavage reaction which removes the 3 carboxyl-terminal amino acids from farnesylated -*CXXX* precursors in *S. cerevisiae*. Taking a biochemical approach using synthetic peptide substrates, we have identified multiple activities which can catalyze the reaction in both soluble and membrane subcellular fractions of *S. cerevisiae*. We also find that the yeast vacuolar enzyme carboxypeptidase Y can catalyze the reaction *in vitro*. Similar results have recently been obtained by Ashby *et al.* (1992).

MATERIALS AND METHODS

Yeast Strains, Media, and Growth Conditions—Yeast strains used in this study are listed in Table I. SM1058 was formerly designated EG123 (Michaelis and Herskowitz, 1988). Strain SM1188 was derived from SM1058 by a single-step gene replacement in which a significant portion of the *STE14* coding region is deleted and substituted by the selectable marker *TRP1* (Hrycyna *et al.*, 1991). Unless otherwise indicated, strains were propagated on YEPD medium containing 1% (w/v) yeast extract (Difco), 2% (w/v) Bacto-Peptone (Difco), and 2% (w/v) D-glucose.

Preparation of Crude Membrane and Cytosolic Fractions—Cells were grown to an OD_{600} of 0.9–1.2. Membranes and soluble fractions were prepared essentially as described previously (Hrycyna and Clarke, 1990) except that the membrane fraction was not further purified by sucrose density gradient centrifugation. The protein concentrations were approximately 35 mg/ml for the membrane fraction and 6.5–8.25 mg/ml for the soluble fraction.

Synthetic Substrates—*N*-[^{14}C]Acetyl-L-Lys-L-Ser-L-Lys-L-Thr-L-Lys-L-Cys-L-Val-L-Ile-L-Met was synthesized by Dr. Janis Young at the UCLA Peptide Synthesis Facility and purified by HPLC.[1] The crude peptide (1300 nmol) was dissolved in 500 μl of nanopure water and separated on a preparative scale Econosphere C_{18} reverse-phase column (Alltech/Applied Scientific, 10-mm inner diameter × 190-mm length) equilibrated in 100% solvent A at room temperature (solvent A is 0.1% trifluoroacetic acid in water and solvent B is 0.1% trifluoroacetic acid, 90% acetonitrile, 9.9% water). The column was eluted with a linear 2% solvent B/min gradient at a flow rate of 3 ml/min. The peptide eluted at 17–18 min. The amino acid composition (Gilbert *et al.*, 1988) was determined to be consistent with the proposed structure and the specific activity was found to be 162.8 cpm/nmol. This HPLC-purified peptide was farnesylated by a modification of the method of Stephenson and Clarke (1990) yielding *N*-[^{14}C]acetyl-L-Lys-L-Ser-L-Lys-L-Thr-L-Lys-*S-trans,trans*-farnesyl-L-Cys-L-Val-L-Ile-L-Met (*N*-Ac-KSKTK[*S*-farnesyl-Cys]VIM). The peptide (500 nmol) was dissolved in 0.2 ml of dimethyl sulfoxide and 0.050 ml of 50 mM guanidine carbonate (2500 nmol) followed by the addition of 0.050 ml of a 1:20 (v/v) solution of farnesyl bromide (9200 nmol) (Aldrich) in dichloromethane. The reaction was allowed to continue for 10 min at room temperature and then the mixture was dried under vacuum in a Savant SpeedVac apparatus. The products were resuspended in 0.4 ml of 1:1 (v/v) acetonitrile:water and fractionated by HPLC using the same reverse-phase column described above but equilibrated in 20% solvent B at room temperature. The column was eluted using a linear gradient of 1.5% solvent B/min over 40 min followed by a 2%/min linear gradient to 100% solvent B over an additional 10 min at a flow rate of 3 ml/min. The *N*-Ac-KSKTK[*S*-farnesyl-Cys]VIM product eluted at 29.6–29.9 min; the peak of UV absorbance at 214 nm was coincident with the radioactivity. The concentration of peptide in each assay was quantified by liquid scintillation counting using the specific radioactivity determined above. *N*-Acetyl-*S*-farnesyl-L-cysteine and *N*-acetyl-*S*-farnesyl-L-cysteine [^{3}H]methyl ester were prepared as described previously by Hrycyna *et al.* (1991). The peptide DRVYIHPF (angiotensin II) was purchased from Chemical Dynamics (South Plainfield, NJ), whereas GGDA was a Vega product (Tucson, AZ). The synthesis and characterization of the peptide VYPNSA was described previously (Stephenson and Clarke, 1989).

TABLE I
Saccharomyces cerevisiae strains

Name	Genotype	Source
SM1058	*MAT***a** *trp1 leu2 ura3 his4 can1 STE14*$^+$	S. Michaelis[a]
SM1188	*ste14*Δ*::TRP1*, isogenic to SM1058	S. Michaelis[a]
ABYS1	*MAT***a** *pra1 prb1 prc1 cps1 ade*	D. Meyer[b]

[a] Johns Hopkins University (Hrycyna *et al.*, 1991).
[b] UCLA (Achstetter *et al.*, 1984).

In Vitro Coupled Protease Assay—Protease enzyme activity was measured in yeast membrane and soluble fractions both in the presence and absence of the synthetic peptide substrate. Membrane preparations of the *Escherichia coli* strain BSG24 expressing the *STE14-Trp*E fusion protein were used as the source of COOH-terminal methyltransferase activity. The fusion protein was induced in *E. coli* and extracts were prepared as described previously by Hrycyna *et al.* (1991) at a specific activity of 3.2 pmol of methyl groups transferred per min/mg of protein at a protein concentration of approximately 15 mg/ml. Peptide (1.5 nmol) was dried in a 1.5-ml polypropylene microcentrifuge tube and resuspended in 20–27 μl of 100 mM Tris·HCl buffer (pH 7.52). Potential sources of protease activity (see below) were generally incubated with 3–5 μl of the *E. coli* membrane fraction, 20 μl (1 nmol) of *S*-adenosyl-L-[^{14}C-*methyl*] methionine (ICN, 50 mCi/mmol; final concentration of 16.7 μM) in 100 mM Tris·HCl (pH 7.52), and the Tris buffer to a final volume of 50 μl. After incubation at 37 °C, protease activity was quantified by measuring the picomoles of methyl groups transferred to each newly exposed cysteine residue per min/mg of protease source protein. The reaction was stopped by the addition of 55 μl of 1 M NaOH, 1% sodium dodecyl sulfate (w/v). The solution was vortexed, and 85 μl was immediately spotted onto a piece of folded thick filter paper (2 × 8 cm; Bio-Rad, catalog No. 165–0962). The paper was placed in the neck of a 20-ml scintillation vial containing 10 ml of scintillation fluid (Research Products, Safety Solve), and the vial was capped. After 2 h, the filter paper was removed and radioactivity counted in a liquid scintillation counter. Volatile [^{14}C]methanol released by the cleavage of the methyl esters by base diffuses into the scintillation fluor, whereas other nonvolatile material remains on the filter paper (Ota and Clarke, 1989).

Protease Inhibition Assays—Assays were performed as described above except that the protease was preincubated for 10–15 min at 37 °C with the potential inhibitor prior to the addition of the other reaction components.

Esterase Activity Assays—Enzyme activity was measured in yeast soluble and membrane fractions utilizing *N*-acetyl-*S*-farnesylcysteine [^{3}H]methyl ester as the substrate. Each reaction mixture contained substrate (6600–6800 cpm dried in 1.5-ml polypropylene microcentrifuge tubes), between 2 and 10 μl of yeast membranes, cytosol, or carboxypeptidase Y as the esterase source, and 100 mM Tris·HCl (pH 7.52) to a total volume of 50 μl. After a 40-min incubation at 37 °C, the reaction was quenched with the addition of 55 μl of 0.1 M sodium phosphate (pH 2.0), 1% (w/v) sodium dodecyl sulfate, and the [^{3}H]methanol released was quantified by the vapor diffusion assay described above.

RESULTS

Multiple Activities in S. cerevisiae Catalyze the Removal of the 3 Terminal Amino Acids from Farnesylated -CXXX-containing Peptides in Vitro—The 3 terminal amino acids found on farnesylated or geranylgeranylated proteins containing the -*CXXX* consensus sequence at the COOH terminus must be cleaved before α-carboxyl methyl esterification of the cysteine residue can occur. We were interested in identifying an activ-

[1] The abbreviations used are: HPLC, high performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; pHMB, *p*-hydroxymercuribenzoate.

ity in *S. cerevisiae* responsible for this proteolytic cleavage event. We first tested the possibility that the methyltransferase itself could catalyze both the cleavage of these 3 residues and the subsequent methylation reaction. The yeast *STE14* methyltransferase gene can be expressed as a *Trp*E-fusion membrane protein in *E. coli*, an organism that appears to lack the enzymes in the protein isoprenylation pathway (Hrycyna *et al.*, 1991; He *et al.*, 1991). Membrane preparations from these cells readily catalyze the methylation of short synthetic peptides containing COOH-terminal farnesyl or geranylgeranyl cysteine residues (Hrycyna *et al.*, 1991). We then asked whether this *E. coli* membrane fraction could catalyze the methylation of a peptide containing the three additional amino acids at the COOH terminus. We found, however, that the peptide *N*-Ac-KSKTK[*S*-farnesyl-Cys]VIM, based on the sequence of the COOH terminus of the human Ki-*ras*-2B protein, was not a methyl acceptor (Table II). This result indicates that the methyltransferase cannot catalyze the removal of the 3 terminal amino acids and implies that the methyltransferase, at least as it is expressed in *E. coli*, and the protease represent distinct enzymes.

This system was then utilized as an indirect assay to detect proteolytic cleavage activity. We reasoned that any enzyme that was capable of removing the terminal 3 amino acids would render the farnesylated peptide an effective methyl acceptor. Clear evidence has been obtained that both the mammalian (Stephenson and Clarke, 1990) and the yeast (Hrycyna and Clarke, 1990) methyltransferase activity is dependent upon the presence of an isoprenylated COOH-terminal cysteine residue. No methylation has been observed in unmodified peptides. Therefore, we mixed the membrane fraction from *E. coli* expressing the yeast STE14 methyltransferase described above with membrane and soluble extracts from *ste14*-deficient *S. cerevisiae* cells in the presence of the farnesylated peptide containing the 3 additional amino acids. The success of this assay is dependent upon an excess of methyltransferase in the reaction mixture so that the protease reaction is rate-limiting. Controls showed that no protease activity was present in the soluble fraction from *E. coli* that would generate a methylatable substrate from the *N*-Ac-KSKTK[*S*-farnesyl-Cys]VIM. On the other hand, both yeast membrane and soluble fractions demonstrated good activity (Table II).

Using this novel assay, we have identified at least three distinct activities in yeast responsible for the cleavage reaction (Fig. 1, Table II). Using the membrane fraction from the *S. cerevisiae* strain SM1188 (Table I) and the soluble fraction from the *S. cerevisiae* strain ABYS1 (Table I), we were able to show significant incorporation of methyl groups into the peptide substrate in each fraction which was linearly dependent upon amount of enzyme added (Fig. 1, Table II). The ABYS1 strain was used because it lacks four major vacuolar proteases, including proteinase A, proteinase B, carboxypeptidase Y, and carboxypeptidase S (Table I). This is important because we find that purified carboxypeptidase Y alone can catalyze the production of the methyltransferase substrate (Table II). The presence of protease in the ABYS1 soluble fraction indicates that this activity is distinct from these vacuolar proteases. Similarly, the presence of protease activity in the membrane fraction of the ABYS1 strain (at a specific activity of about 1.5-fold greater than that of the SM1188 strain) indicates that the membrane activity is also distinct from that of the four vacuolar proteases. From the specific activities presented in Table II as well as the observed distribution of protein between the soluble and membrane fractions (35:65), we calculate that the total soluble activity in the ABYS1 strain is about 1.2 times that of the total membrane activity in the SM1188 strain.

The Membrane-associated and Soluble Activities Are Distinct Enzymes as Determined by Protease Inhibition Studies— The four major classes of proteases are the metalloproteases, the serine proteases, the sulfhydryl proteases, and the aspartyl proteases (Jones, 1991, Wolf, 1986). We were interested in determining what types of proteases the soluble and membrane-bound activities described here represented. Because carboxypeptidase Y could catalyze the reaction, we chose to perform all further characterizations of the soluble enzyme using extracts from the ABYS1 strain to avoid contributions to the activity from this vacuolar enzyme. Neither the membrane-associated nor the soluble activity was sensitive to serine protease inhibitors such as PMSF, dichloroisocoumarin, or leupeptin, nor to the aspartyl protease inhibitor pepstatin (Table III). On the other hand, both activities were sensitive to sulfhydryl modifying reagents to varying degrees. $ZnCl_2$ inhibited both forms of the protease at a concentration of 0.5 mM. *p*-Hydroxymercuribenzoate (0.5 mM) completely inhibited the membrane-associated enzyme but had only a partial effect on the soluble form (Table III). Similarly, 1.0 mM *N*-ethylmaleimide completely abolished the activity of the membrane-associated enzyme but had little to no effect on the soluble species (Table III). Another striking difference between these two enzymes is their distinct sensitivities to

TABLE II

Distinct protease and methyltransferase activities are responsible for the COOH-terminal modification of farnesylated -CXXX containing peptides

Methyltransferase source	Protease source	Substrate	Protease specific activity[a]	
			No added protease	Added protease
			pmol/min/mg membrane protein	*pmol/min/mg protease protein*
E. coli membranes[b]		None	0.78	
E. coli membranes		*S*-Farnesyl-Ac-KSKTKCVIM	1.1	
E. coli membranes + *E. coli* cytosol[b]		*S*-Farnesyl-Ac-KSKTKCVIM		2.2
E. coli membranes + yeast membranes[c]		*S*-Farnesyl-Ac-KSKTKCVIM		69.8
E. coli membranes + yeast cytosol[d]		*S*-Farnesyl-Ac-KSKTKCVIM		158.3
E. coli membranes + purified CPY[e]		*S*-Farnesyl-Ac-KSKTKCVIM		1385.4

[a] Each value represents averages from four to five separate experiments. For values with added protease, the background of *E. coli* membranes plus peptide substrate was subtracted.

[b] Cytosol and membranes prepared from *E. coli* strain BSG24 (p*TrpE-STE14*$_{(1-239)}$) expressing the full-length *STE14* gene product as a *Trp*E fusion protein.

[c] *S. cerevisiae* strain SM1188.

[d] *S. cerevisiae* strain ABYS1.

[e] Purified carboxypeptidase Y from bakers' yeast (Sigma).

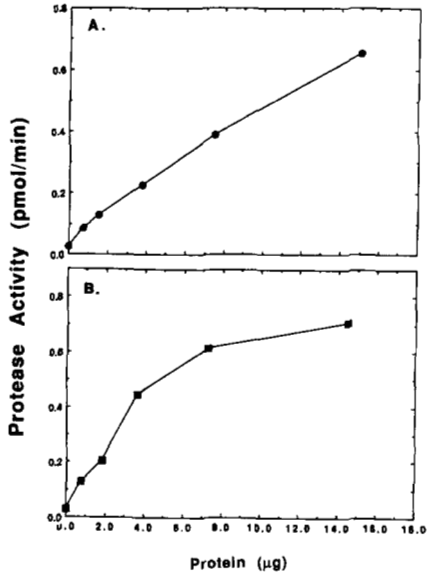

FIG. 1. **Dependence of soluble and membrane-associated protease activity on amount of enzyme.** The indicated amount of a crude membrane fraction from *S. cerevisiae* strain SM1188 (*A*) or the soluble fraction from strain ABYS1 (*B*) was incubated in reaction mixtures containing *N*-Ac-KSKTK[*S*-farnesyl-Cys]VIM (1.5 nmol), the membrane fraction from an *E. coli* strain expressing the STE14 methyltransferase (45 μg protein), 1 nmol *S*-adenosyl-L-[^{14}C-*methyl*]methionine (50 mCi/mmol) in 100 mM Tris·HCl (pH 7.52), and the Tris buffer to a final volume of 50 μl and final *S*-adenosyl-L-[^{14}C-*methyl*]methionine concentration of 16.7 μM. The reaction mixtures were incubated for 30 min at 37 °C and assayed as described under "Materials and Methods." A minimal background value from control incubations without peptide was subtracted from each point.

TABLE III

Inhibition of the soluble and membrane-associated proteases responsible for the cleavage of the 3 terminal amino acids from the farnesylated peptide N-Ac-KSKTKCVIM

Inhibitor	% of control	
	Membranes	Soluble
None	100[a]	100[b]
0.5 mM $ZnCl_2$	20.1	3.0
4 mM PMSF	78.2	
5 mM PMSF		86.7
20 μg/ml leupeptin	93.0	102.0
20 μg/ml pepstatin	97.6	94.1
0.5 mM *p*-hydroxymercuribenzoate	7.0	32.3
1.0 mM *N*-ethylmaleimide	9.1	112.5[c]
20 μg/ml dichloroisocoumarin	89.1[d]	91.0[d]
0.5 mg/ml E-64	61.3[e]	
0.75 mg/ml E-64		56.8[e]
2.0 mM *o*-phenanthroline	78.5[f]	11.2[g]
20 μg/ml aprotinin		100.4

[a] Represents 0.482 pmol/min activity.
[b] Represents 0.793 pmol/min activity.
[c] 100% control represents 0.953 pmol/min activity.
[d] Control incubations with solvent alone (*N*, *N*-dimethylformamide) gave comparable results.
[e] Control incubations with solvent alone (1:1, ethanol:water) gave comparable results.
[f] 100% control represents 0.376 pmol/min activity.
[g] 100% control represents 0.263 pmol/min activity.

the metal chelator *o*-phenanthroline. We found that the membrane-associated enzyme is not affected by a concentration of 2.0 mM *o*-phenanthroline, whereas the soluble activity is abolished at this concentration (Table III). From these data, it is evident that these two forms represent distinct enzymes which both function to cleave the terminal 3 amino acids of a synthetic peptide in our assay system. We propose that the membrane-associated protein may be a sulfhydryl-containing protease due to its sensitivity to sulfhydryl modifying reagents. Although this enzyme is insensitive to the thiol protease inhibitor E-64 (Table III), at least one other protease has been identified that is inhibited by sulfhydryl modifying reagents but shows no sensitivity to this compound (Fagan and Waxman, 1991). Since the soluble form is sensitive to *o*-phenanthroline, but not to EDTA, this enzyme could represent a metal-containing carboxypeptidase (Folk *et al.*, 1960).

The Soluble Enzyme Is a Novel Processive Carboxypeptidase of Molecular Weight 110,000—Because the major part of the protease activity detected with our assay was in the ABYS1 soluble fraction, we decided to further characterize this fraction. We fractionated this material by Sephacryl S-200 gel filtration chromatography. Protease activity eluted in a single sharp peak that corresponded to a molecular weight for the native enzyme of approximately 110 kDa (Fig. 2). Since carboxypeptidase Y has a native molecular mass of 62 kDa, and the extract was from the ABYS1 mutant, this activity cannot be attributed to carboxypeptidase Y contamination.

We were interested next in determining the pathway by which the soluble enzyme cleaves the peptide substrate. We developed an assay which allowed us to distinguish between an endoproteolytic cleavage event and a carboxypeptidase activity. The assay involves monitoring the cleavage reaction by amino acid analysis and is based on the fact that only free amino acids will form a fluorescent compound when derivatized with *o*-phthalaldehyde (Jones *et al.*, 1981, Pfeifer *et al.*,

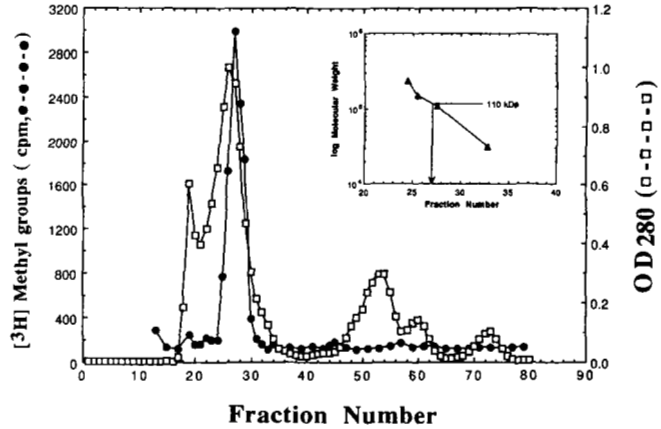

FIG. 2. **Molecular weight determination of the soluble protease from *S. cerevisiae* by gel filtration chromatography on Sephacryl S-200.** 1.3 ml (9.75 mg of protein) of the 100,000 × *g* soluble fraction prepared from *S. cerevisiae* strain ABYS1 was loaded onto a Sephacryl S-200 column (Pharmacia LKB Biotechnology Inc., 1.5 cm diameter × 70 cm) equilibrated with 100 mM Tris·HCl (pH 7.52 at 4 °C). The enzyme was eluted with the same buffer at a flow rate of 0.13 ml/min and 1.9-ml fractions were collected. The protein profile at 280 nm of the fractions was obtained (□—□), and 15 μl of each fraction was assayed for protease activity (●—●) by the coupled methyltransferase assay as described under "Materials and Methods." Fractions were assayed as described in the Worthington Enzyme Manual (Worthington, 1988) for the endogenous marker proteins glucose-6-phosphate dehydrogenase, (M_r 230,000, tetramer; Nogae and Johnston, 1990; Yue *et al.*, 1969), alcohol dehydrogenase (M_r 148,000; Russell *et al.*, 1983), and hexokinase (M_r 110,000; Stachelek *et al.*, 1986) except that the reactions were scaled down to 1 ml. NADP was used for the hexokinase and glucose 6-phosphate assays, and NAD was used for assaying alcohol dehydrogenase. Superoxide dismutase (M_r 31,000; Bermingham-McDonogh *et al.*, 1988) was assayed by a modification of the method of Heikkila and Cabbat (1976). The buffer used was 100 mM Tris·HCl (pH 7.52), and the final reaction mixture volume was 1 ml. The *inset* shows the position of the peak activity *versus* their native molecular weights.

1983). If the 3 amino acids were released as a tripeptide, no fluorescence would be observed. This assay also allows for a more direct determination of specific activity of the protease because it does not depend on the methyltransferase assay. Both the farnesylated and non-farnesylated peptide *N*-Ac-KSKTKCVIM were initially used as substrates for the reaction. Carboxypeptidase Y was used as a control enzyme and the partially purified gel filtration preparation of the ABYS1 soluble fraction (*fraction 27*; Fig. 3) was used as the source for the soluble protease activity.

Carboxypeptidase Y cleaved both substrates as expected, first releasing methionine, then isoleucine, then valine in a time-dependent fashion (Fig. 3, *top panels*). We found that this enzyme was capable of additional cleavage reactions from the COOH terminus of the non-farnesylated peptide but we observed no evidence for its ability to cleave past the farnesyl-cysteine residue. For example, in the experiment shown in Fig. 3, free lysine (but not threonine or serine) was released from the non-farnesylated peptide, whereas neither lysine, threonine, or serine release was detected from the farnesylated peptide. This latter observation explains why we observed activity in the coupled assay; the carboxypeptidase Y was slowed or stopped by the presence of the farnesyl-cysteine residue generating the farnesylated peptide lacking the 3 amino acids that was the substrate for the methyltransferase.

The soluble 110-kDa enzyme partially purified on gel filtration also released free amino acids from the carboxyl terminus of the peptide substrates but gave a significantly different profile under similar assay conditions. At each time point, all 3 of the COOH-terminal amino acids were found to be released in equivalent amounts for both the farnesylated and non-farnesylated peptides (Fig. 3, *bottom panels*). These data indicate that the soluble enzyme is a carboxypeptidase active on both farnesylated and non-farnesylated peptides. We confirmed this result by showing that the soluble activity is inhibited 87.5% by the addition of 0.8 mM unmodified peptide in the coupled assay (data not shown). This enzyme is characterized by the apparent simultaneous release of at least the 3 terminal amino acid residues, suggesting a processive type of mechanism. To our knowledge, this type of carboxypeptidase activity has not been previously identified in yeast (Wolf, 1986; Jones, 1991) or in other tissues.

Unlike carboxypeptidase Y, we observed that this carboxypeptidase is capable of cleaving past the farnesylcysteine residue, although at a rate slower than that seen for the COOH-terminal 3 residues described above. For example, we detected about 0.4 mol of threonine released per mol of farnesylated *N*-Ac-KSKTKCVIM at the 35-min time point (see Fig. 3). Free lysine was also detected. The latter amino acid was difficult to quantitate due to the low fluorescent

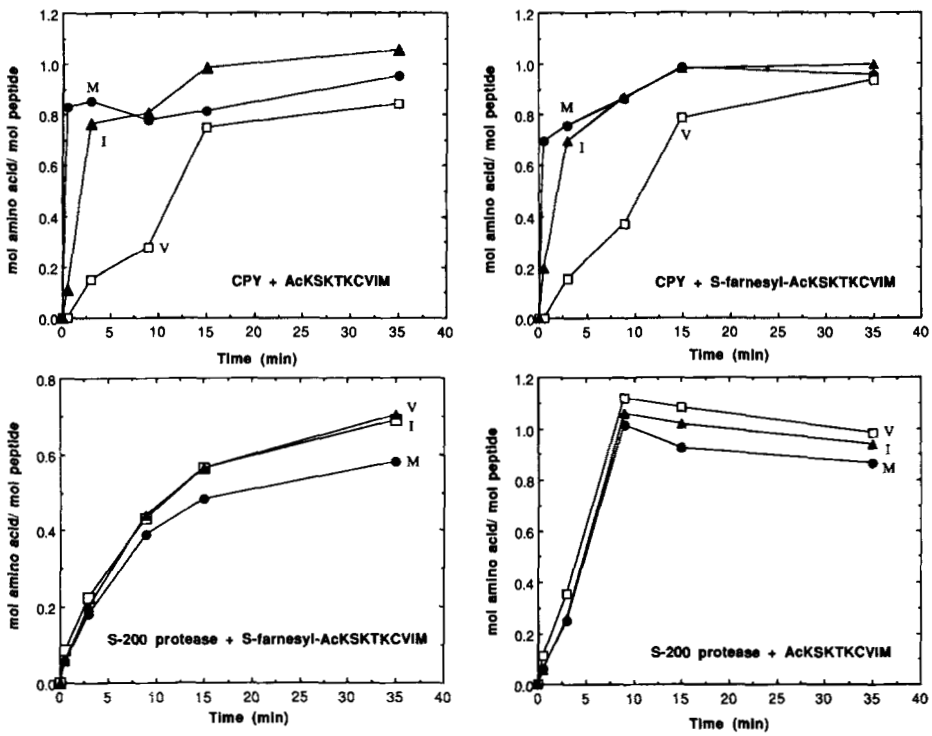

FIG. 3. **Analysis of the cleavage reaction of the soluble protease and carboxypeptidase Y by amino acid analysis.** Incubations contained either the *S*-farnesylated (1.1 nmol) or non-farnesylated (1.4 nmol) peptide *N*-Ac-KSKTKCVIM and either fraction 27 (17.5 μg of protein) from the S-200 column preparation of the soluble *S. cerevisiae* protease (*bottom panels*) or carboxypeptidase Y (*CPY*) (0.5 μg of protein from *S. cerevisiae*, Sigma) (*top panels*) and 100 mM Tris·HCl (pH 7.52) to a final volume of 50 μl. After incubation at 37 °C for various times, the reaction was quenched with 75 μl of *o*-phthalaldehyde solution (0.4% (w/v) *o*-phthalaldehyde (Fluka), 10% (v/v) methanol, and 0.4% β-mercaptoethanol (Sigma) in 0.45 M potassium borate (pH 10.2)) and spun at 13,600 × *g* for 3 min. *o*-Phthalaldehyde-derivatized amino acids were separated from the reaction mixture (10 μl) on a Waters Resolve C_{18} reverse-phase column (3.9 × 150 mm, 5-μm spherical silica) equilibrated in 90% Buffer A and 10% Buffer B (Buffer A is 50 mM sodium acetate and 50 mM sodium phosphate (pH 7.4), 2% (v/v) methanol, 2% (v/v) tetrahydrofuran; Buffer B is 65% (v/v) methanol, 35% (v/v) water). Fluorescence was monitored by a Gilson model 121 fluorometer. The column was eluted using a linear gradient of 2% Buffer B/min over 45 min at a flow rate of 1 ml/min, washed for 5 min in 100% Buffer B, and re-equilibrated for 15 min in 90% Buffer A prior to the next injection. Quantification of the derivatized amino acids was based on the fluorescence of amino acid standards (Pierce Chemical Co., Standard H; 75 pmol).

yield of the *o*-phthalaldehyde derivative but appeared to be at least 1.0 mol/mol of peptide at this time point. Similar results were obtained with the non-farnesylated peptide. In neither case was free serine released.

The specificity of the soluble 110-kDa carboxypeptidase activity was tested using peptides with carboxyl-terminal sequences unrelated to those of the precursors of the isoprenylated proteins as potential substrates. We could detect no release of amino acids from the peptide DRVYIHPF (angiotensin II) when incubated with the soluble enzyme for either 10 or 30 min under the conditions shown in Fig. 3 that result in the cleavage of the COOH-terminal 3 amino acids of *N*-Ac-KSKTKCVIM. Control experiments with the DRVYIHPF peptide with carboxypeptidase Y under the conditions described in Fig. 3 resulted in the full release of phenylalanine, histidine, and isoleucine and the partial release of tyrosine. Similarly, we could detect no release of carboxyl-terminal amino acids from the peptides GGDA or VYPNSA with the soluble 110-kDa carboxypeptidase under the conditions shown in Fig. 3.

Using this assay for free amino acid release, we measure specific activities for farnesylated *N*-Ac-KSKTKCVIM cleavage by the soluble 110-kDa activity and carboxypeptidase Y that are approximately 20–90 times as great as that observed in the coupled methyltransferase assay for either enzyme (Fig. 3; Table II). This result indicates that only a subfraction of peptides containing a COOH-terminal farnesylated cysteine are generated at any one time.

We have also assayed the membrane-bound protease activity in this assay. We could detect no release of free amino acids from either the farnesylated or non-farnesylated *N*-Ac-KSKTKCVIM peptide under conditions that resulted in the methylation of the farnesylated peptide (data not shown). These results suggest that the membrane-bound activity is not a carboxypeptidase.

Esterase Activity in S. cerevisiae—Because many proteases are also esterases, we were interested in exploring the possibility that the same enzyme(s) that cleave the peptide bond on the carboxyl side of the farnesylcysteine could also cleave a methyl ester at this point. Therefore, we sought to biochemically identify an esterase activity in *S. cerevisiae* specific for farnesylated substrates that could possibly serve to modulate the level of methylation of these substrates *in vivo*. We thus incubated *N*-acetyl-*S*-farnesylcysteine-[^{3}H]methyl ester with the soluble fraction of the SM1188 yeast strain and found evidence for an activity which could catalyze the cleavage of the methyl ester. No activity was observed, however, in the membrane fraction (Table IV). Parallel experiments using the soluble fraction from ABYS1 and purified carboxypeptidase Y as a control enzyme confirmed that this activity was due to contamination of the cytosolic fraction by vacuolar carboxypeptidase Y as a result of cell disruption and was not due to a specific esterase. Therefore, at least under the conditions of our assay, no specific activity capable of cleaving this methyl ester linkage was identified in the cytosol or membrane fractions of *S. cerevisiae* strains.

TABLE IV

Methyl esterase activity in membrane and soluble fractions of S. cerevisiae using N-acetyl-[S-farnesyl-Cys]-methyl ester as a substrate

Enzyme source	Specific activity
	pmol/min/mg protein[a]
SM1188 Membrane	0.05
SM1188 Soluble	5.3
ABYS1 Soluble (−PMSF)[b]	0.2
ABYS1 Soluble (+PMSF)[b]	0.1
Carboxypeptidase Y[c]	605
ABYS1 gel filtration fraction 27[d]	0.5

[a] Represents picomoles of [^{3}H]methyl groups released as methanol per min/mg of protein. A small background value from control incubations in the absence of esterase source was subtracted. Each value represents the average of duplicate incubations.

[b] Soluble fraction was prepared either in the presence or absence of PMSF (1 mM).

[c] Purified from bakers' yeast (Sigma).

[d] Peak protease activity from S-200 gel filtration chromatography of the ABYS1 soluble fraction (see Fig. 3).

DISCUSSION

We have developed a novel assay to detect protease activities that cleave the three terminal amino acids from farnesylated peptide substrates containing the -C*XXX* motif. This assay, which represents the first successful separation of the methylation and proteolytic components, allowed us to identify three distinct proteolytic activities in the yeast *S. cerevisiae*, a membrane-associated enzyme and two soluble enzymes, one of which we showed to be carboxypeptidase Y. Although carboxypeptidase Y digestion is presumably not physiologically relevant for processing outside of the vacuole, the other two distinct proteases are potential candidates for the *in vivo* proteolysis of farnesylated polypeptides containing the -C*XXX* COOH-terminal structure. The membrane-associated activity is similar to an enzyme recently described in both rat liver and *S. cerevisiae* cells that catalyzes the removal of the 3 terminal amino acids as a tripeptide from a farnesylated -C*XXX*-containing substrate (Ashby *et al.*, 1992). We show that the soluble enzyme is a carboxypeptidase that operates in a processive manner to remove the COOH-terminal 3 amino acids. However, if the resulting -*S*-farnesylcysteine COOH-terminal residue is not "capped" by the addition of a methyl group, further cleavage can occur.

The potential competition between the methyltransferase and the carboxypeptidase for COOH-terminal isoprenylated cysteine residues suggests a mechanism for the regulation of the levels of the modified proteins. Previous studies on the processing of RAS2 protein in yeast strains lacking STE14 methyltransferase activity (Hrycyna *et al.*, 1991) showed the accumulation of a soluble form migrating on sodium dodecyl sulfate-gel electrophoresis at the position of the non-isoprenylated precursor p41 (Fujiyama *et al.*, 1987). Since these cells contain active farnesyltransferase activity, it was not clear why the loss of the methylation reaction should dramatically slow the isoprenylation reaction. Our characterization of the soluble protease activity in this work, however, suggests that isoprenylated proteins may form normally but may be subject to further cleavage by this carboxypeptidase in the absence of methyltransferase. This reaction would remove the farnesylated cysteine residue, resulting in the reversion of the polypeptide to a form which comigrates with the completely unmodified protein. Presumably, the loss of several amino acids would have little effect on the migration of the protein in comparison to cleavage of the isoprene group. This explanation may account for the apparent persistence of the precursor species. The feasibility of this mechanism depends on the localization of the soluble protease in the cell. Preliminary results suggest that the soluble protease is not enriched in a vacuolar preparation of ABYS1 and thus may be localized to the cytosol *in vivo* (data not shown).

Further work will be required to demonstrate the physiological roles of both the membrane-associated and soluble enzymes. We cannot at this time, therefore, rule out other possible functions for either enzyme. Since the carboxypeptidase is active on the non-farnesylated substrate, it could represent a nonspecific protease whose actual cellular func-

tion *in vivo* is to rapidly degrade polypeptide precursors that are not properly modified. Significantly, we do not detect cleavage of three peptides with COOH-terminal sequences unrelated to the -CVIM peptide. This result suggests that this enzyme may be specific for substrates such as isoprenylated protein precursors with hydrophobic COOH termini; additional studies will be needed to map out the specificity of this carboxypeptidase more exactly and test this hypothesis. Similarly, it will be important to localize the membrane-associated protease to help define its cellular function.

The possibility that COOH-terminal methylation may be a reversible regulatory reaction in the cell suggests the existence of enzymes that catalyze methyl ester cleavage. The bacterial chemotactic response is regulated by reversible methylation reactions, and a similar pathway may occur in eukaryotic cells (Clarke, 1985; Volker *et al.*, 1991; Clarke, 1992). The observation that mammalian formylpeptide chemoreceptor action is mediated by a large G-protein (Polakis *et al.*, 1988) along with the finding that the γ-subunit of this G-protein is α-carboxyl-methylated at its COOH terminus (Yamane *et al.*, 1990) provides support for the earlier hypothesis that transient methylation can regulate eukaryotic receptor function (Venkatasubramanian *et al.*, 1980). Recently, it has been shown that the treatment of mouse peritoneal macrophages with saturating amounts of *N*-acetyl-*S*-farnesylcysteine, a competitive inhibitor of the COOH-terminal protein methyltransferase, inhibits their chemotactic response (Volker *et al.*, 1991).

On the other hand, there is little evidence to support the removal of COOH-terminal methyl groups in cells. For example, no evidence for methyl group turnover in mammalian $p21^{H\text{-}ras}$ over a 2-h period has been observed (Gutierrez *et al.*, 1989). However, *N*-acetyl-*S*-farnesylcysteine-[^{3}H]methyl ester has been shown to be hydrolyzed by a retinal rod outer segment preparation (Perez-Sala *et al.*, 1991). We sought to identify a similar esterase activity in *S. cerevisiae* specific for COOH-terminal farnesylcysteine methyl ester structures. Our initial studies, which involved incubating the same substrate [^{3}H]methyl ester with membrane and soluble fractions from a yeast strain lacking COOH-terminal methyltransferase activity, pointed to the existence of such an activity (Table IV). However, our determination that purified carboxypeptidase Y from bakers' yeast readily catalyzed the cleavage of the methyl ester suggested that vacuolar contaminants might contribute to such an activity. Upon assaying the soluble fraction from ABYS1, we observed that the activity was missing (Table IV). Thus, our results indicate that the activity we first observed was probably due to carboxypeptidase Y and not to a specific esterase.

Our failure to demonstrate non-vacuolar methyl esterase activity in yeast cells, as well as our finding that the potential proteases themselves cannot cleave the methyl ester linkage (Table IV), adds support to the hypothesis that the cellular function of the methylation reaction is to protect these modified proteins from further protease digestion. It is possible, however, that an esterase activity does exist for specific protein or peptide substrates that is dependent upon upstream protein sequences or conformations that would not be present on the *N*-acetyl-*S*-farnesylcysteine methyl ester model substrate used in this and other studies.

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REFERENCES

Achstetter, T., Emter, O., Ehmann, C., and Wolf, D. H. (1984) **259,** 13334–13343
Anderegg, R. J., Betz, R., Carr, S. A., Crabb, J. W., and Duntze, W. (1988) *J. Biol. Chem.* **263,** 18236–18240
Ashby, M. N., King, D. S., and Rine, J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** in press
Bermingham-McDonogh, O., Gralla, E. B., and Valentine, J. S. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 4789–4793
Chen, W.-J., Andres, D. A., Goldstein, J. L., Russell, D. W., and Brown, M. S. (1991) *Cell* **66,** 327–334
Clarke, S. (1985) *Annu. Rev. Biochem.* **54,** 479–506
Clarke, S. (1992) *Annu. Rev. Biochem.* **61,** 355–386
Clarke, S., Vogel, J. P., Deschenes, R. J., and Stock, J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 4643–4647
Der, C. J., and Cox, A. D. (1991) *Cancer Cells* **3,** 1–11
Fagan, J. M., and Waxman, L. (1991) *Biochem. J.* **277,** 779–786
Finegold, A. A., Schafer, W. R., Rine, J., Whiteway, M., and Tamanoi, F. (1990) *Science* **249,** 165–169
Finegold, A. A., Johnson, D. I., Farnsworth, C. C., Gelb, M. H., Judd, S. R., Glomset, J. A., and Tamanoi, F. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4448–4452
Folk, J. E., Piez, K. A., Carroll, W. R., and Gladner, J. A. (1960) *J. Biol. Chem.* **235,** 2272–2277
Fujiyama, A. K., and Tamanoi, F. (1990) *J. Biol. Chem.* **265,** 3362–3368
Fujiyama, A. K., Matsumoto, K., and Tamanoi, F. (1987) *EMBO J.* **6,** 223–228
Gilbert, J. M., Fowler, A., Bleibaum, J., and Clarke, S. (1988) *Biochemistry* **27,** 5227–5233
Glomset, J. A., Gelb, M. H., and Farnsworth, C. C. (1990) *Trends Biochem. Sci.* **15,** 139–142
Goodman, L. E., Perou, C. M., Fujiyama, A. K., and Tamanoi, F. (1988) *Yeast* **4,** 271–281
Goodman, L. E., Judd, S. R., Farnsworth, C. C., Powers, S., Gelb, M. H., Glomset, J. A., and Tamanoi, F. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9665–9669
Gutierrez, L., Magee, A. I., Marshall, C. J., and Hancock, J. F. (1989) *EMBO J.* **8,** 1093–1098
Hancock, J. F., Magee, A. I., Childs, J. E., and Marshall, C. J. (1989) *Cell* **57,** 1167–1177
Hancock, J. F., Cadwallader, K., and Marshall, C. J. (1991) *EMBO J.* **10,** 641–646
He, B., Chen, P., Chen, S.-Y., Vancura, K. L., Michaelis, S., and Powers, S. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 11373–11377
Heikkila, R. E., and Cabbat, F. (1976) *Anal. Biochem.* **75,** 356–362
Hrycyna, C. A., and Clarke, S. (1990) *Mol. Cell. Biol.* **10,** 5071–5076
Hrycyna, C. A., Sapperstein, S., K., Clarke, S., and Michaelis, S. (1991) *EMBO J.* **10,** 1699–1709
Johnson, D. J., O'Brien, J. M., and Jacobs, C. W.(1991a) *Gene (Amst.)* **90,** 93–98
Johnson, D. J., O'Brien, J. M., and Jacobs, C. W.(1991b) *Gene (Amst.)* **98,** 149–150
Joly, A., Popjak, G., and Edwards, P. A. (1991) *J. Biol. Chem.* **266,** 13495–13498
Jones, E. W. (1991) *J. Biol. Chem.* **266,** 7963–7966
Jones, B. N., Paabo, S., and Stein, S. (1981) *J. Liq. Chromatogr.* **4,** 565–586
Kawata, M., Farnsworth, C. C., Yoshida, Y., Gelb, M. H., Glomset, J. A., and Takai, Y. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 8960–8964
Kinsella, B. T., Erdman, R. A., and Maltese, W. A. (1991) *J. Biol. Chem.* **266,** 9786–9794
Maltese, W. A. (1990) *FASEB J.* **4,** 3319–3328
Marcus, S., Caldwell, G., A., Miller, D., Xue, C.-B., Naider, F., and Becker, J. M., (1991) *Mol. Cell. Biol.* **11,** 3603–3612
Michaelis, S., and Herskowitz, I. (1988) *Mol. Cell. Biol.* **8,** 1309–1318
Moores, S. L., Schaber, M. D., Mosser, S. D., Rands, E., O'Hara, M. B., Garsky, V. M., Marshall, M. S., Pompliano, D. L., and Gibbs, J. B. (1991) *J. Biol. Chem.* **266,** 14603–14610
Nogae, I., and Johnston, M. (1990) *Gene (Amst.)* **96,** 161–169
Ohya, Y., Goebl, M., Goodman, L. E., Petersen-Bjorn, S., Friesen, J. D., Tamanoi, F., and Anraku, Y. (1991) *J. Biol. Chem.* **266,** 12356–12360

Ong, O. C., Ota, I. M., Clarke, S., and Fung, B. K.-K. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 9238–9242
Ota, I. M., and Clarke, S. (1989) *J. Biol. Chem.* **264,** 12879–12884
Perez-Sala, D., Tan, E. W., Canada, F. J., and Rando, R. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3043–3046
Pfeifer, K., Karol, R., Korpi, J., Burgoyne, R., and McCourt, D. (1983) *Am. Lab. (Fairfield, CT)* **15,** 78–82
Polakis, P. G., Uhing, R. J., and Snyderman, R. (1988) *J. Biol. Chem.* **263,** 4969–4976
Powers, S., Michaelis, S., Broek, D., Santa Anna-A., S., Field, J., Herskowitz, I., and Wigler, M. (1986) *Cell* **47,** 413–422
Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J., and Brown, M. S. (1990) *Cell* **62,** 81–88
Reiss, Y., Seabra, M. C., Armstrong, S. A., Slaughter, C. A., Goldstein, J. L., and Brown, M. S. (1991) *J. Biol. Chem.* **266,** 10672–10677
Rine, J., and Kim, S.-H. (1990) *New Biologist* **2,** 219–226
Russell, D. W., Smith, M., Williamson, V. M., and Young, E. T. (1983) *J. Biol. Chem.* **258,** 2674–2682
Seabra, M. C., Reiss, Y., Casey, P. J., Brown. M. S., and Goldstein, J. L. (1991) *Cell* **65,** 429–434
Stachelek, C., Stachelek, J., Swan, J., Botstein, D., and Konigsberg, W. (1986) *Nucleic Acids Res.* **14,** 945–963
Stephenson, R. C., and Clarke, S. (1989) *J. Biol. Chem.* **264,** 6164–6170
Stephenson, R. C., and Clarke, S. (1990) *J. Biol. Chem.* **265,** 16248–16254
Stephenson, R. C., and Clarke, S. (1992) *J. Biol. Chem.* **267,** in press
Stimmel, J. B., Deschenes, R. J., Volker, C., Stock, J., and Clarke, S. (1990) *Biochemistry* **29,** 9651–9659
Venkatasubramanian, K., Hirata, F., Gagnon, C., Corcoran, B. A., O'Dea, R. F., Axelrod, J., and Schiffmann, E. (1980) *Mol. Immunol.* **17,** 201–207
Volker, C., Miller, R. A., McCleary, W. R., Rao, A., Poenie, M., Backer, J. M., and Stock, J. B. (1991) *J. Biol. Chem.* **266,** 21515–21522
Wolf, D. H. (1986) *Microbiol. Sci.* **3,** 107–112
Worthington, C. C. (ed) (1988) *Worthington Manual, Enzymes and Related Biochemicals,* Worthington Biochemicals Corp., Freehold, NJ
Yamane, H. K., Farnsworth, C. C., Xie, H., Howald, W., Fung, B. K.-K., Clarke, S., Gelb, M. H., and Glomset, J. A. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5868–5872
Yamane, H. K., Farnsworth, C. C., Xie, H., Evans, T., Howald, W. N., Gelb, M. H., Glomset, J. A., Clarke, S., and Fung, B. K.-K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 286–290
Yokayama, K., Goodwin, G. W., Ghomashchi, F., Glomset, J. A., and Gelb, M. H. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5302–5306
Yoshida, Y., Kawata, M., Katayama, M., Horiuchi, H., Kita, Y., and Takai, Y. (1991) *Biochem. Biophys. Res. Commun.* **175,** 720–728
Yue, R. H., Noltmann, E. A., and Kuby, S. A. (1969) *J. Biol. Chem.* **244,** 1353–1364