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Endoproteolytic processing of a farnesylated peptide *in vitro*

(CAAX motifs/prenylation/posttranslational processing/carboxyl-terminal protease)

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ABSTRACT **Numerous eukaryotic proteins containing a carboxyl-terminal CAAX motif (C, cysteine; A, aliphatic amino acid; X, any amino acid) require a three-step posttranslational processing for localization and function. The a mating factor of *Saccharomyces cerevisiae* is one such protein, requiring cysteine farnesylation, proteolysis of the terminal three amino acids, and carboxyl methylation for biological activity. We have used farnesylated a-factor peptides to examine the proteolytic step in the maturation of CAAX-containing proteins. Three distinct carboxyl-terminal protease activities were found in yeast cell extracts that could remove the terminal three residues of a-factor. Two of the proteolytic activities were in cytosolic fractions. One of these activities was a *PEP4*-dependent carboxypeptidase that was sensitive to phenylmethylsulfonyl fluoride. The other cytosolic activity was *PEP4*-independent, sensitive to 1,10-phenanthroline, and effectively inhibited by an unfarnesylated a-factor peptide. In contrast, a protease activity in membrane fractions was unaffected by phenylmethylsulfonyl fluoride, 1,10-phenanthroline, or unfarnesylated a-factor peptide. Incubation of membrane preparations from either yeast or rat liver with a radiolabeled farnesylated a-factor peptide released the terminal three amino acids intact as a tripeptide, indicating that this reaction occurred by an endoproteolytic mechanism and that the enzyme most likely possesses a broad substrate specificity. The yeast endoprotease was not significantly affected by a panel of protease inhibitors, suggesting that the enzyme is novel. Zinc ion was shown to inhibit the endoprotease ($K_i < 100$ μM). The specific activities of the a-factor carboxyl-terminal membrane endoprotease and methyltransferase clearly indicated that the proteolytic reaction was not rate-limiting in these processing reactions *in vitro*.**

Fungal mating pheromones were the first proteins shown to undergo carboxyl-terminal posttranslational processing involving modification with an isoprenoid compound (1–3). Since then a large number of eukaryotic proteins have been shown to undergo similar modifications. Such proteins include a-factor (4), the p21 family of Ras proteins (5–7), some subunits of trimeric GTP-binding proteins (8–10), and nuclear lamins (11, 12). A key determinant for proteins destined for this posttranslational processing is the carboxyl-terminal tetrapeptide sequence CAAX (C, cysteine; A, aliphatic; X, any amino acid). Processing includes prenylation of the cysteine residue with either a farnesyl or a geranylgeranyl moiety (1–12), proteolytic removal of the terminal three amino acids (13–15), and, finally, methylesterification of the newly exposed carboxylate group of the prenylated cysteine residue (16–20). The terminal (X) amino acid of the motif may be a determinant for distinguishing among protein prenyltransferases, since CAAX-containing proteins terminating in leucine are prenylated by a geranylgeranyl diterpenoid, whereas proteins terminating in cysteine, serine, alanine, or methionine are prenylated with a farnesyl sesquiterpenoid (21–24). The yeast a mating factor has the carboxyl-terminal sequence CVIA and has been shown to be farnesylated (4).

The isolation of yeast a-specific sterile mutants has identified several of the genes required for posttranslational processing of a-factor. *DPR1/RAM1* (also known as *STE16*) and *RAM2* have been shown to encode distinct subunits of the protein farnesyltransferase (25–27). Substrates of this protein farnesyltransferase include a-factor, RAS2, and the γ subunit of a trimeric GTP-binding protein (10). *STE14* encodes a protein methyltransferase that mediates carboxyl methylation of both a-factor and RAS2 in yeast (18, 28). Secretion of mature a-factor requires the *STE6* gene product (29). In contrast, no mutants defective in proteolytic maturation of RAS2 or a-factor have been isolated.

Studies on the maturation of a-factor and RAS peptides *in vitro* have shown that cytosolic fractions from yeast and mammalian cells contain protein farnesyltransferase activity (23, 25, 30, 31). Carboxyl-terminal methyltransferase activity is present in plasma membrane preparations (28). Removal of the terminal three amino acids of farnesylated $p21^{Ki\text{-}Ras(B)}$ translated *in vitro* will take place when incubated with membrane fractions from COS cells (15). We have begun to characterize the carboxyl-terminal protease involved in the maturation of a-factor and RAS prenylated precursors. We have identified comparable proteolytic activities in membrane preparations from both yeast and rat liver that will correctly process a farnesylated a-factor peptide. We have learned that this processing activity is an endoprotease that depends upon a prenylated substrate and may define a class of protease.

MATERIALS AND METHODS

Materials. *Saccharomyces cerevisiae* strain JRY2594 (*MATα ade2-101 his3-200 lys2-801 met ura3-52*) was the yeast strain used. *S*-adenosyl-L-[*methyl*-^{14}C]methionine (47 mCi/mmol; 1 Ci = 37 GBq) was from ICN. *L*-[4,5-^{3}H]isoleucine (100 Ci/mmol) was from Amersham. The peptides dansyl-WDPAC(*S*-*t*,*t*-farnesyl) and dansyl-WDPAC(*S*-*t*,*t*-farnesyl)VIA were a generous gift from S. Rosenberg and C. C. Yang (Protos, Emeryville, CA).

Peptide Synthesis. The radiolabeled peptide KWDPAC(*S*-*t*,*t*-farnesyl)V[4,5-^{3}H]IA was synthesized on *p*-hydroxymethylphenoxy-derivatized polystyrene/divinylbenzene resin by using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-activated 9-fluorenylmethoxycarbonyl (Fmoc) amino acids in the presence of 1-hydroxybenzotriazole and diisopropylethylamine in an Applied Biosystems 431A peptide synthesizer employing user-derived cycles. Protecting groups were *t*-butoxycarbonyl [K(Boc)], *t*-butyl [D(*t*-Bu)], and trityl [C(Trt)]. Fmoc-[^{3}H]Ile was synthesized from L-[4,5-^{3}H]isoleucine by using Fmoc *N*-succinimide ester in ethylene glycol dimethyl ether with carbonate as catalyst. Labeled peptide was cleaved and deprotected with reagent K (32) for

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Abbreviation: PMSF, phenylmethylsulfonyl fluoride.

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3 hr (room temperature), precipitated in *t*-butyl methyl ether, purified by reversed-phase HPLC with an acetonitrile gradient in 0.1% trifluoroacetic acid, and lyophilized. The peptide thiolate anion was generated with carbonate and alkylated with farnesyl bromide (85–90% yield). The radiolabeled farnesylated peptide was purified by reversed-phase HPLC to yield material of purity >96% (24.8 mCi/mmol). Structures were confirmed by derivative UV spectrometry and by electron spray ionization MS.

The peptide was then coupled to *N*-hydroxysuccinimide-activated CH-Sepharose 4B (Pharmacia) as instructed by the manufacturer. The lysine at the amino terminus of the peptide was utilized in place of the naturally occurring phenylalanine at this position to ameliorate the coupling reaction. Peptide coupling efficiency was ≈94%.

Crude Membrane and Cytosol Preparations. Yeast cultures were grown overnight at 30°C to an OD_{600} of ≈1. Cells were collected by centrifugation at 2500 × *g* for 10 min at room temperature, washed once in sterile distilled water, and pelleted again under the same conditions. The pellets were resuspended in 2 volumes of ice-cold 0.3 M sorbitol/0.1 M NaCl/5 mM Tris·HCl, pH 7.4 (28) and transferred to 15-ml glass centrifuge tubes. Cells were lysed by vortex mixing with 1 volume of glass beads for 1 min followed by incubation on ice for 1 min; this regimen was repeated five times. Glass beads and cell debris were removed by transfer of the lysates in a syringe with a 26-gauge needle to a 15-ml centrifuge tube and centrifugation at 1900 × *g* for 10 min at 2°C. The supernatant was further fractionated by centrifugation at 100,000 × *g* for 1 hr at 2°C. Aliquots of the resulting crude membrane (100,000 × *g* pellet) and cytosol (100,000 × *g* supernatant) fractions were stored at −80°C.

Crude membrane preparations from rat liver were kindly provided by L. Wood (University of California, San Francisco). Tissue was homogenized with a Polytron (Brinkmann) in 250 mM sucrose/10 mM Tris·HCl, pH 7.4/15 mM EDTA/15 mM EGTA/1 mM phenylmethylsulfonyl fluoride (PMSF) with aprotinin (1 μg/ml) and leupeptin (25 μg/ml). This membrane fraction contained those cellular components that pellet between 10,000 × *g* and 100,000 × *g*. The samples were stored in aliquots at −80°C.

Carboxyl-Terminal Methyltransferase Assays. *In vitro* methylation assays with farnesylated peptides as substrates were conducted as described (28) except that crude yeast membrane preparations were used instead of sucrose gradient-purified membranes and protease inhibitors were omitted. Reaction mixtures (60 μl) included 100 μg of crude yeast membrane protein, 1 nmol of *S*-adenosyl-L-[*methyl*-^{14}C]methionine, and 0–10 nmol of farnesylated peptide in 65 mM Tris·HCl, pH 7.4/100 mM sorbitol/32 mM NaCl. After incubation at 37°C for 1 hr, reactions were terminated by addition of 50 μl of 1 M NaOH/1% SDS, and the amount of base-labile ^{14}C was determined as described (17, 33).

Carboxyl-Terminal Protease Assays. Reaction mixtures (100 μl) included 3.5 nmol of dansyl-WDPAC(*S*-*t*,*t*-farnesyl)VIA in 80 mM Tris·HCl, pH 7.4. Reactions were initiated by addition of 20 μg of cellular protein and incubated at 37°C for 15 min. Farnesylated peptide products were extracted with an equal volume of 1-butanol, dried under nitrogen, and stored at 4°C. Samples were analyzed with a Hewlett–Packard 1090 HPLC and a Brownlee Lab RP300 C_8 reversed-phase column (2.1 mm × 25 cm). Peptides were eluted with a gradient of 35–65% acetonitrile over 20 min (0.3 ml/min).

Carboxyl-terminal protease assays were also carried out with the a-factor peptide KWDPAC(*S*-*t*,*t*-farnesyl)V[4,5-^{3}H]IA coupled to Sepharose as described above. The standard assay (100 μl) included 10 μl of Sepharose-coupled peptide (≈18,000 dpm) in 80 mM Tris·HCl, pH 7.4/60 mM sorbitol/20 mM NaCl. Protease inhibitors and other compounds were included as described in the figure legends. Reactions were initiated by addition of 1 μg of cell protein and incubated at 37°C for 15 min. For quantitative determinations the reaction mixtures were centrifuged at 10,000 × *g* for 2 min. Aliquots of the supernatant were taken and the soluble radioactivity was determined by liquid scintillation counting. Under these conditions the release of soluble [^{3}H]isoleucine was linear with respect to time and cell protein concentration. The concentration of the Sepharose-coupled peptide was in excess (data not shown). For qualitative analyses by HPLC, supernatants from five individual reactions were pooled and centrifuged through a Centricon-30 (Amicon) filter. Material passing through the filter (<30 kDa) was collected and analyzed by reversed-phase HPLC.

RESULTS

Carboxyl-Terminal Protease and Methyltransferase Activities in Crude Yeast Membrane Preparations. Hancock *et al.* (15) showed that pancreatic membrane fractions possessed an activity that removed the terminal three amino acids from Ki-Ras *in vitro*. We tested whether or not a similar protease activity existed in yeast membrane fractions that was capable of removing the terminal three amino acids from a yeast a-factor peptide. Since cysteinyl carboxyl methylation necessarily must occur after proteolysis of the a-factor precursor, cysteinyl methylation of an unproteolyzed a-factor substrate could be used to indirectly measure carboxyl-terminal proteolysis. The farnesylated a-factor peptide dansyl-WDPAC(*S*-*t*,*t*-farnesyl)VIA either including (octapeptide) or missing (pentapeptide) the carboxyl-terminal VIA extension was similarly methylesterified in a concentration-dependent manner (Fig. 1), indicating the presence of carboxyl-terminal protease activity in these fractions. Membrane preparations from *ste14* null mutants, which lack the RAS carboxyl-terminal methyltransferase (28), did not contain any detectable carboxyl-terminal methyltransferase activity with either a-factor peptide (data not shown). Control reactions in the absence of farnesylated peptide produced <2% of the base-labile ^{14}C produced in reactions containing peptide. Under these conditions, with 3.5 nmol of dansyl-WDPAC(*S*-*t*,*t*-farnesyl)VIA as a substrate, peptide carboxyl methylation was linear with respect to time and protein (crude membrane) concentration (data not shown). The specific activities were 11.3 pmol·min^{-1}·mg^{-1} for methylation of the farnesylated pentapeptide and 6.1 pmol·min^{-1}·mg^{-1} for the two-step proteolysis and methylation of the farnesylated octapeptide.

Distribution of Carboxyl-Terminal Protease Activity Between Yeast Cytosol and Membrane Fractions. Since the measurement of carboxyl-terminal protease activity through the action of the methyltransferase is indirect, we designed an *in vitro* assay to measure proteolysis of a farnesylated a-fac-

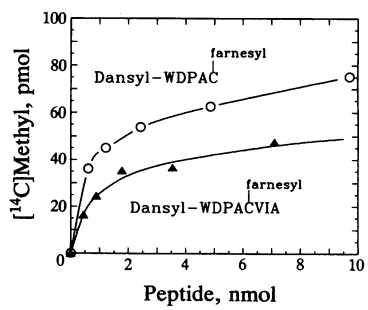

FIG. 1. Methylation of farnesylated a-factor peptides *in vitro*. Crude membrane fractions (100 μg) from wild-type *S. cerevisiae* strain JRY2594 were incubated with various amounts of dansyl-WDPAC(*S*-*t*,*t*-farnesyl) (○) or dansyl-WDPAC(*S*-*t*,*t*-farnesyl)VIA (▲). Methyl esters formed in the absence of added peptide substrate were subtracted to give the values shown.

tor peptide directly. When incubated in the absence of added cellular protein, dansyl-WDPAC(*S-t,t*-farnesyl)VIA remained intact and was eluted from the reversed-phase HPLC column at 16.9 min (Fig. 2*A*). Addition of membrane protein from the yeast strain JRY2594 resulted in nearly complete conversion of the farnesylated octapeptide to a species that was eluted at 15.7 min (Fig. 2*B*). This species was found to be farnesylated pentapeptide (octapeptide minus VIA) by MS of the HPLC-purified peptide and by coelution with an authentic farnesylated pentapeptide synthetic standard (data not shown). The doublet peaks at 12.7 and 12.9 min (Fig. 2*B*) were both identified as oxidized (M + 16) forms of the farnesylated pentapeptide by MS. The peak at 18.9 min was not identified.

The farnesylated a-factor octapeptide was incubated in the presence of yeast membrane (100,000 × *g* pellet; Fig. 3 *Left*) or cytosol (100,000 × *g* supernatant; Fig. 3 *Right*) fractions. Incubations of the farnesylated octapeptide with membrane fractions in the absence of protease inhibitors (Fig. 3*A Left*) resulted in processing of most of the peptide to the farnesylated pentapeptide. PMSF (Fig. 3*B Left*) or PMSF and 1,10-phenanthroline (Fig. 3*C Left*) did not affect protease activity in membrane preparations. The presence of 1,10-phenanthroline did prevent oxidation of the farnesylated pentapeptide (products at 12.7 and 12.9 min), suggesting that this oxidation was metal-catalyzed.

HPLC analysis of control incubations of yeast cytosol fractions with the farnesylated octapeptide in the absence of protease inhibitors (Fig. 3*A Right*) revealed four major peaks. Two of these major peaks, at 18.0 and 16.2 min, are distinct from those seen in the corresponding incubations with membrane fractions. These peaks were identified by MS as the farnesylated hepta- and hexapeptide, respectively. The existence of these intermediates indicated that the protease in cytosol fractions acted by a carboxyl-terminal exopeptidase mechanism. The serine protease inhibitor PMSF blocked nearly all detectable carboxypeptidase activity as judged by the absence of farnesylated hepta- and hexapeptide intermediates in incubations with cytosolic protein (Fig. 3*B Right*). PMSF and 1,10-phenanthroline abolished all detectable protease activity in cytosol fractions (Fig. 3*C Right*).

These data identified three distinct proteolytic activities capable of converting a farnesylated a-factor octapeptide to the corresponding pentapeptide by removal of the carboxyl-terminal three amino acids. One of these activities was present in the membrane fraction and was unaffected by PMSF and 1,10-phenanthroline. The absence of detectable

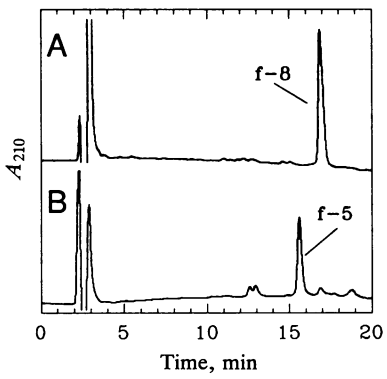

FIG. 2. A carboxyl-terminal farnesylated a-factor peptide was proteolyzed by a crude yeast membrane fraction. Dansyl-WDPA(*S-t,t*-farnesyl)VIA was incubated in the absence (*A*) or presence (*B*) of 50 μg of protein from a 100,000 × *g* pellet fraction from yeast strain JRY2594. Peptides were separated by reversed-phase HPLC. The identity of the labeled peaks was confirmed by electron spray ionization MS (data not shown). f-8, Farnesylated octapeptide; f-5, farnesylated pentapeptide.

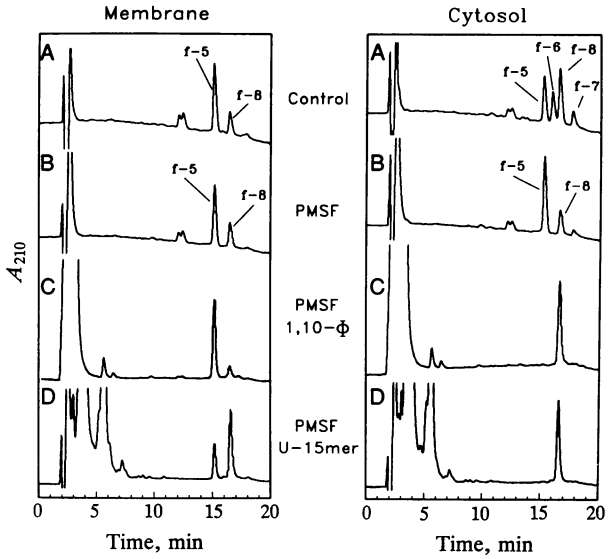

FIG. 3. A carboxyl-terminal farnesylated a-factor peptide was proteolyzed by three distinct enzyme activities in yeast extracts. Dansyl-WDPAC(*S-t,t*-farnesyl)VIA was incubated with 20 μg of protein from 100,000 × *g* pellet (crude membrane, *Left*) or supernatant (cytosol, *Right*) fractions from yeast strain JRY2594. Reaction mixtures contained the following additions: none (*A*); 1 mM PMSF (*B*); 1 mM PMSF and 4 mM 1,10-phenanthroline (1,10-φ) (*C*); 1 mM PMSF and 308 μM unfarnesylated a-factor peptide YIIKGVFWDPACVIA (U-15mer) (*D*). Peptides were separated by reversed-phase HPLC.

farnesylated hepta- and hexapeptide in incubations with membrane proteins suggested an endoprotease activity. Yeast cytosol fractions contained two distinguishable proteases. One was a PMSF-sensitive carboxypeptidase and the second was 1,10-phenanthroline-sensitive. Cytosol fractions from a *pep4-3* strain (data not shown) lacked PMSF-sensitive carboxypeptidase activity, indicating a dependence on protease A and thus a presumptive vacuolar origin for this enzyme. The phenanthroline-sensitive cytosol protease produced a similar HPLC peptide profile as the membrane protease (Fig. 3*B Left* vs. *Right*). A small amount of farnesylated heptapeptide was detected in the incubations of cytosol fractions, although this might be attributed to incomplete inactivation of the carboxypeptidase with PMSF. In addition to differential sensitivity to 1,10-phenanthroline, the cytosolic and membrane proteases could be distinguished by sensitivities to an unfarnesylated a-factor peptide. The cytosolic protease was completely inhibited by a 4-fold molar excess of an unfarnesylated a-factor peptide (Fig. 3*D Right*), whereas the membrane protease was inhibited only 40% (Fig. 3*D Left*). Although the mechanism of inhibition was not determined, this result suggested that the membrane protease, in contrast to the cytosolic enzyme, specifically recognized the farnesyl group on the cysteine.

Removal of the Carboxyl-Terminal Three Amino Acids Occurred by an Endoproteolytic Mechanism. The HPLC profiles of peptides from incubations of the farnesylated octapeptide with membrane fractions (Figs. 2 and 3*A*) did not reveal any intermediates indicative of an exopeptidase. To test conclusively whether the enzyme in the membrane fraction was processing the farnesylated a-factor peptide by an endoproteolytic mechanism, we designed an experiment to isolate the products of the proteolytic reaction. The peptide KWDPAC(*S-t,t*-farnesyl)V[4,5-^{3}H]IA was synthesized, covalently attached through the amino terminus to activated CH-Sepharose 4B, and used in a solid-phase protease assay. Radioactivity released from the Sepharose-coupled peptide after incubation with membrane prepara-

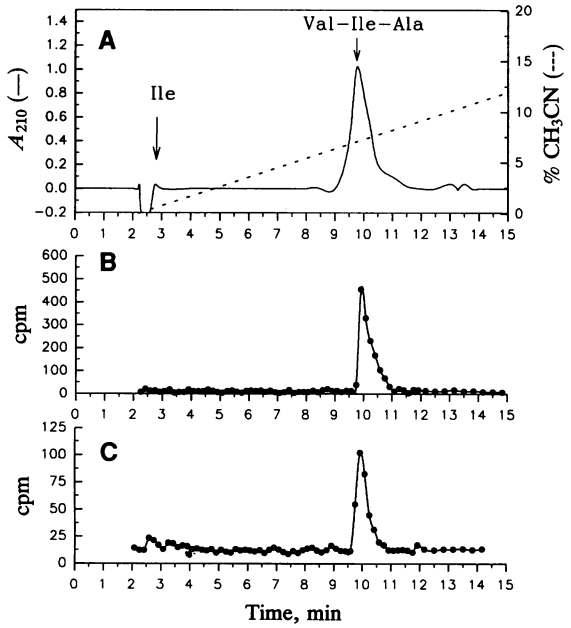

FIG. 4. Endoproteolytic processing of a farnesylated a-factor peptide by crude membrane preparations from yeast and rat. The synthetic tripeptide Val-Ile-Ala (VIA) was applied to a C_8 reversed-phase HPLC column. Retained material was eluted by a 0–12% acetonitrile gradient in water (*A*). The tripeptide was detected by absorbance at 210 nm. Under these conditions free L-isoleucine was not retained by the column and was eluted at the solvent front as indicated. Crude membrane fractions from yeast strain JRY2594 (*B*) or from rat liver (*C*) were incubated with the peptide KWDPAC(*S-t,t*-farnesyl)V[4,5-^{3}H]IA coupled to Sepharose gel (see *Materials and Methods*). Reaction mixtures also contained synthetic VIA tripeptide (0.1 mg/ml) and the following inhibitors: 1,10-phenanthroline (4 mM); EDTA (1 mM); leupeptin (5 μg/ml); pepstatin (5 μg/ml); aprotinin (2.1 μg/ml); E64 (10 μM, see ref. 34); and PMSF (1 mM). Released radioactivity was analyzed by HPLC as in *A* except that fractions were collected at 10-sec intervals between 2 and 12 min and at 20-sec intervals after 12 min.

tions was analyzed by reversed-phase HPLC. Fig. 4*A* shows the elution profile of L-isoleucine and the synthetic tripeptide Val-Ile-Ala (VIA). Incubations of membrane fractions from yeast released ^{3}H that was coeluted with the synthetic tripeptide VIA (Fig. 4*B*). Of the ^{3}H applied to the column, 93.5% was recovered in a single peak. Similarly, membrane preparations from rat liver (Fig. 4*C*) released a product that was coeluted with an authentic VIA standard. In this case nearly all of the detectable ^{3}H was recovered in a single peak; a small amount of free L-isoleucine was also observed.

DISCUSSION

Three distinct proteolytic activities were found in yeast subcellular fractions that could remove the carboxyl-terminal three amino acids from a farnesylated a-factor peptide *in vitro*. Two of these activities were present in cytosolic (100,000 × *g* supernatant) fractions and one in membrane (100,000 × *g* pellet) fractions. We believe the latter enzyme is the best candidate for the authentic *in vivo* a-factor processing protease for a number of reasons. One of the cytosolic activities was a serine carboxypeptidase that was *PEP4*-dependent. This enzyme was presumed to be a vacuolar contaminant and had properties consistent with carboxypeptidase Y (35), which is known not to have a role in a-factor maturation. The second cytosolic protease was phenanthroline-sensitive and exhibited a much higher degree of competition by an unfarnesylated a-factor peptide than did the protease in yeast membrane fractions. The phenanthroline-sensitive protease in fact appeared to prefer or selectively utilize the unfarnesylated substrate. Since farnesylation precedes carboxyl-terminal proteolysis in a-factor maturation (25, 36), the farnesyl moiety is likely to be a key determinant recognized by the physiological carboxyl-terminal protease. Hrycyna and Clarke (37) recently partially purified a 110-kDa protease from yeast with similar properties to the phenanthroline-sensitive enzyme described here. They found that in the presence of a farnesylated Ki-Ras peptide substrate the enzyme is a processive carboxypeptidase that, *in vitro*, could also remove the farnesylated cysteine unless this residue was methylesterified. The role of this activity *in vivo* and the identity of the bona fide carboxyl-terminal protease remain unclear.

We have shown that a likely candidate for an authentic carboxyl-terminal protease for prenylated proteins was present in membrane fractions from both yeast and rat liver. The carboxyl-terminal methyltransferase that acts on both a-factor and RAS proteins has also been found in yeast membrane fractions and, more specifically, in purified plasma membrane preparations (18, 28). Whether or not the protease and methyltransferase are physically coupled or even proximally situated *in vivo* is not known. The rates of processing of a-factor peptides, either proteolyzed or unproteolyzed at the carboxyl termini, by yeast membrane preparations revealed similar dependence on substrate concentration as well as on time (data not shown). Comparison of the specific activities of the carboxyl-terminal protease and methyltransferase either individually or together (Table 1) clearly showed that the proteolytic reaction was not rate-limiting in the proteolysis/methylation processing of a-factor. These data are consistent with coupled processing reactions or the assembly of these enzymes into a processing complex (18). The kinetic similarities in the processing of proteolyzed and unproteolyzed a-factor peptides could also be explained, perhaps more simply, by the substantially higher specific activity of the protease relative to that of the methyltransferase.

Both yeast and rat liver membranes possessed an analogous protease that correctly proteolyzed the carboxyl terminus of a farnesylated a-factor peptide *in vitro* by an endoproteolytic mechanism. The synthesis of precursor peptide hormones that require proteolytic processing by endoproteases is a common theme in both uni- and multicellular eukaryotes. For example, the yeast KEX2 protease, which processes α-factor from a 165-amino acid polyprotein precursor (38), and the mammalian neuroendocrine processing enzymes PC2 and PC3 (39) act through an endoproteolytic mechanism. Yeast a-factor is itself initially synthesized as a 36-amino acid precursor that undergoes proteolytic processing at both the amino and the carboxyl terminus, in addition to farnesylation and methylesterification, to produce a mature 12-amino acid lipopeptide (4, 40). The presence of an *in vitro* a-factor endoprotease in rat liver membrane fractions also suggests that the substrate specificity range of this enzyme is likely to include additional CAAX-containing proteins, as is the case with the protein farnesyltransferase (10, 21, 41) and the carboxyl-terminal methyltransferase (18). This hypothesis is further supported by the observed presence of this zinc-inhibited endoprotease in membrane preparations from a and α haploids and from a/α diploids (M.N.A. and J.R., unpublished data).

Table 1. Specific activities of a-factor carboxyl-terminal processing enzymes in crude yeast membrane preparations

Processing step	Specific activity, pmol·min^{-1}·mg^{-1}
Proteolysis	974 ± 147 (n = 4)
Proteolysis–methylation	6.1 ± 1.02 (n = 5)
Methylation	11.3 ± 1.75 (n = 5)

Values are presented as mean ± SD of n determinations.

The carboxyl-terminal endoprotease appears to be a novel enzyme on the basis of its insensitivity to a panel of protease inhibitors that generally distinguish the four mechanistic classes of proteases (data not shown). This hypothesis cannot be confirmed until the enzyme has been purified and the reaction mechanism elucidated. The endoprotease could, for example, be a member of one of the four protease families but possess unusual active-site stereochemistry. The endoprotease in yeast membrane fractions was inhibited by zinc ions but was unaffected by other cations we tested, including calcium and magnesium. A number of metalloproteases, such as carboxypeptidase A (42) and collagenase (43), require small amounts of zinc for catalysis but are inhibited by higher concentrations. The endoprotease described here did not exhibit similar zinc sensitivities. For example, dialysis of yeast membrane preparations in the absence of metal ions does not significantly effect endoproteolytic activity (M.N.A. and J.R., unpublished data). Additionally, there was no affect of metal chelators, nor did stimulation take place at lower zinc concentrations.

The contribution of carboxyl-terminal proteolysis to the function of a-factor and RAS protein has not been directly assessed. Without the availability of mutants or specific inhibitors of the protease, the functional significance of the proteolysis can be tested only indirectly. In a-factor processing, the absence of or defects in methylesterification result in an ≈100-fold decrease in pheromone bioactivity (44) and a-specific sterility (45), respectively. Since carboxyl-terminal proteolysis necessarily precedes carboxyl methylation, proteolysis must be required for mating of *MAT***a** cells. Methylesterification of a-factor is not required for secretion (46); whether carboxyl-terminal proteolysis of a-factor is required for secretion is not known. RAS proteins, like a-factor, require farnesylation for function (6). In yeast, carboxyl-terminal methyltransferase (*ste14*) mutants do not exhibit defects in RAS2 function despite a small decrease in membrane localization (18). Hancock *et al.* (15) were able to determine the relative contributions of $p21^{Ki\text{-}ras(B)}$ carboxyl-terminal proteolysis and methylation on membrane binding *in vitro*. They found that in the absence of methylation, carboxyl-terminal proteolysis of *in vitro* translated $p21^{Ki\text{-}ras(B)}$ increased membrane binding 2-fold (from 20% to 40%). Methylesterification caused another 2-fold increase in membrane binding. The *in vivo* contribution of carboxyl-terminal proteolysis to RAS protein function has not been determined, although *in vitro* data indicate it is likely to be important.

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