Purification and Characterization of a Novel Metalloendopeptidase from *Saccharomyces cerevisiae*†

Christine A. Hrycyna and Steven Clarke*

Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California 90024-1569

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ABSTRACT: We previously identified an activity in the soluble fraction of the yeast *Saccharomyces cerevisiae* that is a candidate for catalyzing the proteolytic maturation of farnesylated -CXXX precursor polypeptides. We describe here a 1259-fold purification of this activity by chromatography on DEAE-cellulose, hydroxylapatite, phenyl-Sepharose, and Sephacryl S-200. Sodium dodecyl sulfate gel electrophoresis of this preparation demonstrated a single 68-kDa polypeptide chain. The experimentally determined N-terminal amino acid sequence was identical at all 20 positions with residues 28–47 of the deduced sequence of the *S. cerevisiae* YCL57w gene product. This analysis suggests that the YCL57w gene encodes this enzyme and that the initial translation product may contain a leader peptide. Its complete deduced amino acid sequence shows significant homology to a number of zinc metallopeptidases and is most closely related to rat metalloendopeptidase 24.15 (E.C. 3.4.24.15), an enzyme that preferentially cleaves after hydrophobic residues. Using the purified yeast enzyme, we show a unique cleavage site in the peptides bradykinin and β-neoendorphin four residues from the C-terminus on the carboxyl side of a hydrophobic amino acid. The cleavage pattern for neurotensin revealed a major site three residues from the C-terminus also on the carboxyl side of a hydrophobic residue and a minor site four residues from the C-terminus of the peptide. This specificity is similar to that of rat endopeptidase 24.15 and may explain why the farnesylated peptide employed in our studies is a good substrate for the yeast enzyme. However, subcellular localization experiments revealed that this activity does not appear to be cytosolic; its distribution follows that of Golgi and vacuolar marker enzymes. These results suggest that this enzyme may not be involved in the physiological processing of cytosolic -CXXX containing proteins but may be important in other types of protein or peptide metabolism.

In eukaryotic cells, a number of proteins and polypeptides whose initial translation product contain a -Cys-Xaa-Xaa-Xaa sequence at the C-terminus, where -Xaa represents a variety of amino acid residues, can be targeted for a series of posttranslational modifications that include sequential lipidation with either a C_{15} farnesyl or C_{20} geranylgeranyl moiety on the cysteine residue, proteolysis of the three terminal amino acids, and α-carboxyl methyl esterification of the newly exposed cysteine residue (Clarke, 1992; Cox & Der, 1992; Schafer & Rine, 1992). The initial lipidation step has been widely studied and is well characterized in several systems [for reviews, see Maltese (1990), Casey (1992), Glomset et al. (1992), and Sinensky and Lutz (1992)]. The final methylation step has also been characterized in yeast (Hrycyna & Clarke, 1990; Hrycyna et al., 1991) and mammalian cells (Stephenson & Clarke, 1990, 1992; Perez-Sala et al., 1991; Volker et al., 1991).

On the other hand, less is known about the intermediate processing step involving the proteolytic removal of the three terminal amino acids from the isoprenylated precursor polypeptide. Indirect evidence for this proteolytic event was inferred from the isolation of α-carboxyl methylated isoprenylcysteine derivatives from proteins whose initial translation products contain an additional three C-terminal residues (Ong et al., 1989; Ota & Clarke, 1989; Stimmel et al., 1990; Yamane et al., 1990, 1991). Guiterrez et al. (1989) directly showed the removal of the three terminal amino acids from the mammalian $p21^{ras}$ protein. By site-directed mutagenesis, a unique tryptophan residue was introduced into the protein precursor at each of the three C-terminal positions, and the loss of the residue was monitored during biosynthetic processing *in vivo*. Similarly, Fujiyama and Tamanoi (1990) demonstrated loss of the three C-terminal residues from the *Saccharomyces cerevisiae* RAS2 protein during its posttranslational biosynthetic processing. Finally, a membrane-associated proteolytic activity in canine microsomes has been identified that reportedly increases the membrane binding of farnesylated $p21^{H\text{-}ras}$ 2-fold over the nonproteolyzed species (Hancock et al., 1991).

Enzyme activities responsible for the cleavage of the three C-terminal amino acids in mammalian systems have recently been biochemically characterized. Ashby et al. (1992) identified a membrane-associated endoproteolytic activity in rat liver that releases the terminal three amino acids as a tripeptide and is specific for a farnesylated peptide substrate. A microsomal enzymatic activity from calf liver, specific for lipidated substrates, has also been identified that can specifically cleave a farnesylated tetrapeptide between the isoprenylated cysteine residue and the adjacent residue as well as the related farnesylated tri- and dipeptides (Ma et al., 1992; Ma & Rando, 1992). More recently, a distinct activity from brain microsomal membranes capable of sequentially removing the three terminal amino acids from a chemically synthesized C-terminal heptapeptide of mouse N-ras protein was identified (Akopyan et al., 1992). This activity has been characterized as a novel thiol-dependent, serine type carboxypeptidase that displays a higher affinity for the farnesylated substrate than its non-farnesylated analog (Akopyan et al., 1992). Inter-

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* To whom correspondence should be addressed.

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estingly, no soluble proteolytic activities capable of cleaving the three terminal amino acid residues from farnesylated precursors have been identified in mammalian systems.

Proteolytic activities recognizing synthetic isoprenylated peptide substrates have also been characterized in the yeast *S. cerevisiae*. We have identified at least three distinct activities in yeast that can catalyze the cleavage of the three COOH-terminal amino acids from the synthetic peptide substrate *N*-acetyl-KSKTK[*S*-farnesyl-Cys]VIM *in vitro*, a membrane-associated enzyme and two soluble activities, one of which has been identified as vacuolar carboxypeptidase Y (Hrycyna & Clarke, 1992). The membrane-associated enzyme is similar in its inhibitor specificity to an activity identified by Ashby et al. (1992) that catalyzes the removal of the three terminal amino acids as a tripeptide from a farnesylated peptide substrate *in vitro*. We characterized the partially purified soluble activity, also identified by Ashby et al. (1992), as a 110-kDa enzyme that initially appeared to be a metallocarboxypeptidase cleaving both farnesylated and nonfarnesylated -CXXX containing peptides but not unrelated peptides (Hrycyna & Clarke, 1992). In this work, we describe the purification of this soluble activity, its biochemical characterization, and the identification of the gene encoding this protein. Our results suggest that the activity is not, in fact, a carboxypeptidase but instead represents a novel noncytosolic zinc metalloendopeptidase encoded by the YCL57w gene on chromosome III in *S. cerevisiae*. The localization of this enzyme suggests that it may not play a role in the posttranslational maturation of isoprenylated protein precursors but instead may be important in peptide metabolism in yeast.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Growth Conditions. *S. cerevisiae* strains used in this study are ABYS1 (*MATa pra1 prb1 prc1 cps1 ade*) (Achstetter et al., 1984) and SM1188 (*MATa leu2 ura3 his4 can1 ste14Δ::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 Soluble Fraction from S. cerevisiae. Cells were grown to an OD_{600} of 0.9–1.2. The soluble fraction was prepared essentially as described previously (Hrycyna & Clarke, 1990) except 0.6 M mannitol in 10 mM Tris-HCl, pH 7.45, was used as the lysis buffer (Jazwinski, 1990).

Synthetic Peptide Substrates. The synthetic peptide *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 was synthesized as previously described (Hrycyna & Clarke, 1992). Bradykinin, β-neoendorphin, and neurotensin were purchased from Sigma.

In Vitro Coupled Peptidase Assay and Inhibition Assays. Peptidase enzyme activity assays and inhibition assays were performed essentially as previously described (Hrycyna & Clarke, 1992) except that the potential inhibitor was added directly to the reaction mixture without prior preincubation with the peptidase activity.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)[1] *Analysis.* SDS–PAGE was performed using the buffer system described by Laemmli (1970). Electrophoresis sample buffer was added to aliquots of the soluble fraction or active column chromatography fractions [final concentrations of 60 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 12% (v/v) glycerol, 0.001% Bromophenol Blue and 0.7 M β-mercaptoethanol] and heated for 5 min at 100 °C. Electrophoresis was carried out on 8% (w/v) acrylamide, 0.28% *N,N*-methylenebisacrylamide resolving gels using high molecular weight markers (Bio-Rad) or prestained high molecular weight markers (Bio-Rad) as standards. Gels were stained in 50% methanol (v/v), 10% acetic acid (v/v), and 0.1% Coomassie brilliant blue (w/v) for 1 h and destained overnight in 10% acetic acid (v/v), and 5% methanol (v/v). Subsequently, the protein bands were further visualized by silver staining (Ausubel et al., 1991).

Analysis of Cleavage of Synthetic Peptide Substrates. Bradykinin, neurotensin, and β-neoendorphin were dissolved in water to a final concentration of 1 mg/mL and purified on a C_4 Vydac reverse-phase column (300-Å pore diameter, 4.6 mm inner diameter, 250-mm length) monitored by UV absorption at 214 nm. The column was 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, 99% acetonitrile, and 0.9% water) and eluted with a linear 2.4% solvent B/min gradient at a flow rate of 1 mL/min. The amino acid composition of each peptide was determined by a modification of the method of Jones and Gilligan (1983). Solvent C is 0.1 M sodium acetate, pH 7.22/tetrahydrofuran/methanol (895:10:95), and solvent D is 100% methanol. The *o*-phthalaldehyde derivitizing solution is 0.4% (w/v) *o*-phthalaldehyde (Fluka), 10% (v/v) methanol, 0.4% β-mercaptoethanol, and 0.8 mg/mL Brij-35 (Sigma) in 0.4 M potassium borate, pH 10.4. The *o*-phthalaldehyde-derivatized amino acids were separated on a Waters Resolve C_{18} reverse-phase column (3.9-mm inner diameter × 150-mm length, 5-μm spherical silica) equilibrated in 100% solvent C. The column was eluted using the following gradient at a flow rate of 1.7 mL/min: 0–1 min, 0–20% solvent D; 1–12 min, isocratic with 20% solvent D; 12–17 min, 20–40% solvent D; 17–20 min, isocratic with 40% solvent D; 20–25 min, 40–60% solvent D; 25–31 min, isocratic with 60% solvent D; 31–32 min, 60–100% solvent D. The column was then washed for 5 min in 100% solvent D and re-equilibrated for 15 min in 100% solvent C prior to the next injection. Proline content was determined for each of the peptides by the method of Cooper et al. (1984) on an analytical Econosphere C_{18} reverse phase column (Alltech/Applied Scientific, 3.9-mm inner diameter × 150-mm length) except that the total volume of reagents used in the reaction was scaled down to 400 μL from 1.6 mL. In both analyses, fluorescence was monitored by a Gilson model 121 fluorometer. Quantification of the derivatized amino acids was based on the fluorescence of amino acid standards (Pierce Chemical Co., Standard H; 50–75 pmol).

Each of these peptides, bradykinin (9.4 nmol), β-neoendorphin (9.1 nmol), and neurotensin (6.0 nmol), was incubated in the presence of purified soluble peptidase (0.03, 0.09, and 0.09 μg, respectively) at 37 °C for 35 min, 1.75 h, and 3 h, respectively. The purified soluble peptidase used is the pool of Sephacryl S-200 fractions 33 and 34 in 100 mM Tris-HCl, pH 7.53, from a fractionation of pooled phenyl-Sepharose fractions 180 and 181. The total volume of the bradykinin-containing reaction was 30 μL whereas the total volumes of the reactions with β-neoendorphin and neurotensin were 40 μL. After the incubation, the entire reaction mixture was separated on a C_4 Vydac reverse-phase column and monitored by UV absorption at 214 nm. Each of the peptide products

[1] Abbreviations: HPLC, high-performance liquid chromatography; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, (ethylenedinitrilo)tetraacetic acid; pHMB, *p*-hydroxymercuribenzoate; PMSF, phenylmethanesulfonyl fluoride.

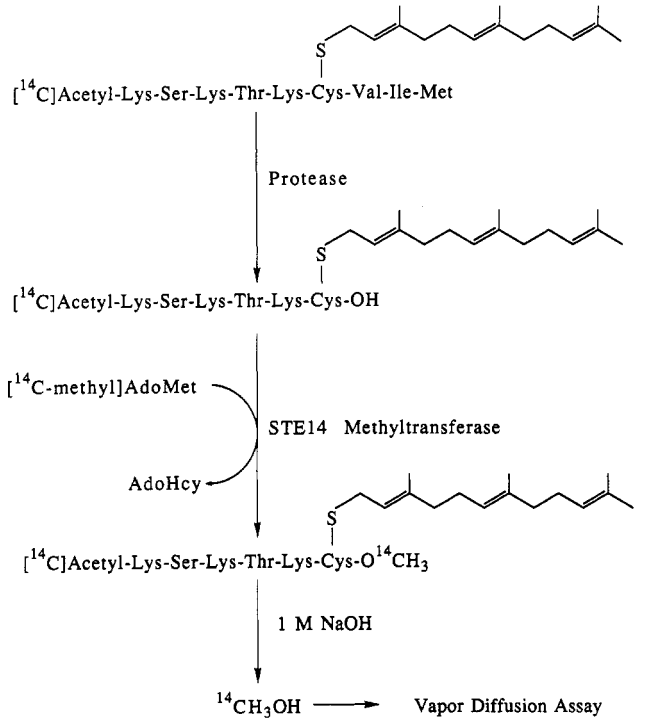

FIGURE 1: Assay for peptidase activity. Cleavage of the peptide bond between the farnesyl-cysteine and the valine residue of the synthetic peptide *N*-[^{14}C]acetyl-KSKTK[*S*-farnesyl-Cys]VIM forms a substrate for a C-terminal isoprenyl-cysteine methyltransferase (Hrycyna & Clarke, 1992). In the presence of excess *S*-adenosyl-L-[^{14}C-*methyl*]methionine ([^{14}C-methyl]AdoMet) and a preparation of the STE14 methyltransferase, [^{14}C]methyl esters are formed that can be base hydrolyzed to give [^{14}C]methanol. [^{14}C]Methanol is quantified by a vapor diffusion assay (Ota & Clarke, 1989).

was collected and subjected to amino acid analysis and determination of proline content as described above for the intact peptides.

Subcellular Fractionation. Enriched vacuolar fractions were prepared as described by Bankaitis et al. (1986) with the modifications of Conradt et al. (1992). Vacuoles were collected from the 0%/4% Ficoll interface. The remaining fractions (fraction 1, 4% layer; fraction 2, 4%/8% interface; fraction 3, 8% layer; fraction 4, 8%/15% interface; fraction 5, 15% layer; see Table IV) were collected sequentially from the top of the step gradient to the bottom. The enriched vacuolar fraction and each of the interface fractions were kept in an ice bath and subjected to sonication. Each of the fractions was assayed for the C-terminal peptidase activity as described above, glucose 6-phosphate dehydrogenase activity (Worthington, 1988), and dipeptidylaminopeptidase A and B activities (Suarez-Rendueles et al., 1981).

N-Terminal Amino Acid Sequence Analysis. Electroblotting of SDS–PAGE gels onto poly(vinylidene difluoride) (PVDF) membranes was performed essentially as described by Moos et al. (1988). The gel was presoaked in chilled buffer (25 mM Tris base, 10 mM glycine, 0.5 mM dithiothreitol, and 10% (v/v) methanol] for 20 min and then loaded into a Bio-Rad Mini Trans-Blot cell. Electrotransfer was run at 100 V (40 mA) for 40 min. Amino acid sequence analysis was performed by Dr. Audree Fowler (UCLA Protein Microsequencing Facility) with an Applied Biosystem Model 470A gas-phase sequencer with on-line HPLC detection.

RESULTS

Purification of a Soluble Activity from S. cerevisiae that Cleaves Farnesylated Peptides. Proteolytic activity was

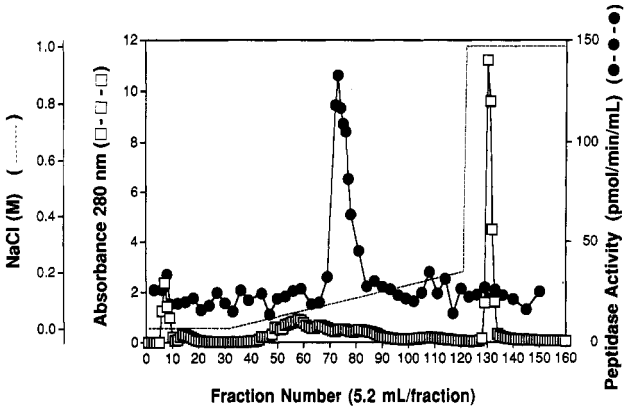

FIGURE 2: DE-52 ion exchange chromatography of soluble C-terminal peptidase activity from *S. cerevisiae* strain ABYS1. The 100000*g* supernatant (231 mg of protein) was applied to a column containing 52.5 mL of DE-52 resin (Whatman) equilibrated at 4 °C in 10 mM Tris-HCl, pH 7.75 and eluted at 0.43 mL/min. After the column was loaded (fractions 1–3) and washed with three column volumes (160 mL) of 10 mM Tris-HCl, pH 7.75 (fractions 3–32), a linear 500 mL gradient from 0 to 0.2 M NaCl in 10 mM Tris-HCl was used to elute the enzyme (fractions 33–121). This step was followed by a final wash with 1 M NaCl in the same buffer (fractions 122–160). A 3-μL aliquot of every third fraction was assayed for peptidase activity (●). The protein content of each fraction was determined by absorbance at 280 nm (□), and the NaCl gradient is shown by the dashed line (- - -).

followed throughout the purification using the synthetic peptide substrate *N*-acetyl-KSKTK[*S*-farnesyl-Cys]VIM in an *in vitro* coupled assay where the proteolytic removal of the three terminal amino acids renders it a substrate for the STE14 methyltransferase. This latter enzyme has been shown to recognize peptides containing a C-terminal isoprenylcysteine residue (Figure 1) (Hrycyna & Clarke, 1990, 1992). Preparations of the 100000*g* supernatant from disrupted ABYS1 cells, deficient in proteinase A, proteinase B, carboxypeptidase Y, and carboxypeptidase S activities, were used as the starting material for the purification procedure. The ABYS1 strain was utilized because we previously found that carboxypeptidase Y itself can catalyze the production of a methylatable substrate in this assay (Hrycyna & Clarke, 1992). The crude soluble fraction was first chromatographed on a DE-52 anion exchange column at pH 7.75. Enzyme activity was eluted as a single peak at a NaCl concentration of about 0.1 M (Figure 2). The active fractions were pooled and then fractionated on a hydroxylapatite column. The activity eluted in the initial portion of a 10–400 mM potassium phosphate gradient (Figure 3). These active fractions were pooled and fractionated by hydrophobic chromatography on a phenyl-Sepharose column. The proteins were eluted with a decreasing ammonium sulfate gradient from 1.7 to 0 M, and the peptidase activity eluted again as a single peak of activity at about 0.9 M salt (Figure 4).

As a final step in the purification, two active fractions from the phenyl-Sepharose column were pooled and applied to a Sephacryl S-200 column equilibrated in 100 mM Tris-HCl, pH 7.53. The activity eluted on the leading edge of a larger protein peak (Figure 5A) at the position expected for a 110-kDa species (Hrycyna & Clarke, 1992). The polypeptide composition of the S-200 column fractions was monitored by SDS–PAGE (Figure 5B). Fractions on the leading edge of the activity peak contained a single polypeptide migrating at 68 kDa, while later fractions were contaminated with a major polypeptide of 45 kDa. In all S-200 fractions, peptidase activity was found to elute coincidentally with the 68-kDa polypeptide (Figure 5B). A summary of the 1259-fold

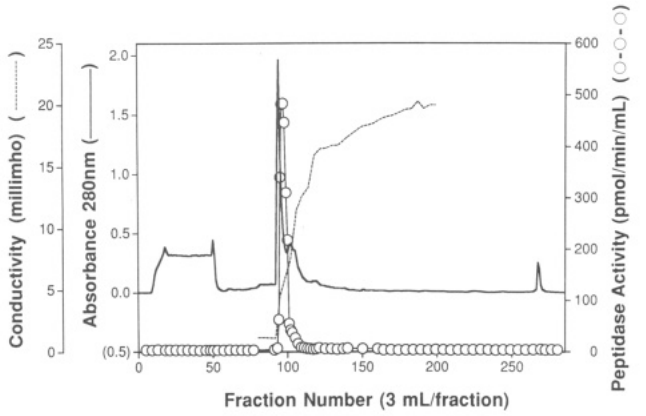

FIGURE 3: Chromatography of the soluble C-terminal peptidase activity on hydroxylapatite. The active fractions from two large scale DE-52 columns starting with 610.5 and 501.7 mg of the 100000*g* supernatant protein were pooled and filtered through a 0.2-μm syringe filter. The sample was applied in a volume of 120 mL (54 mg of protein) to a 29-mL Bio-Gel HT hydroxylapatite column equilibrated in 10 mM potassium phosphate, pH 8.04, at 4 °C (fractions 1–40). The column was then washed with 120 mL of 10 mM potassium phosphate, pH 8.04 (fractions 41–80), followed by a 500-mL gradient from 10 to 400 mM potassium phosphate established (fractions 81–257) at a flow rate of about 5 mL/h. The salt gradient was monitored by conductance readings and is shown by the dashed line (- - -). The column was then washed with 545 mM potassium phosphate, pH 8.04 (fractions 258–285). A 5-μL aliquot of each fraction shown (1 μL of fractions 95–100) was assayed for C-terminal peptidase activity (○), and each fraction was assayed for protein content by absorbance at 280 nm (—).

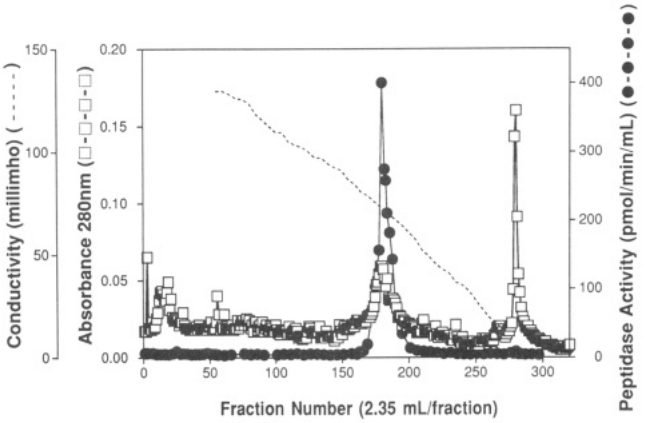

FIGURE 4: Phenyl-Sepharose chromatography of the C-terminal peptidase activity. Active fractions from the hydroxylapatite column (fractions 96–101) were pooled, and the concentration of ammonium sulfate was adjusted to 1.7 M by the addition of 4.1 g of ammonium sulfate to the 16.3 ml pool. The sample (4.9 mg of protein) was applied to a 30-mL phenyl-Sepharose column equilibrated in 10 mM Tris-HCl, pH 7.48, and 1.7 M ammonium sulfate at 4 °C, and the column was eluted at 0.24 mL/min. After the sample was loaded (fractions 1–10) and subsequently washed with 105 mL of 10 mM Tris-HCl, pH 7.48, and 1.7 M ammonium sulfate (fractions 10–54), a 500-mL linear gradient from 1.7 to 0 M ammonium sulfate in 10 mM Tris-HCl, pH 7.48, was established to elute the enzyme (fractions 55–265). The gradient was followed by a 130-mL wash with the 10 mM Tris-HCl, pH 7.48, buffer containing no salt (fractions 266–320). A 5-μL aliquot of each fraction shown (2 μL for fractions 172–192) was assayed for C-terminal peptidase activity (●), and each fraction was assayed for protein content by absorbance at 280 nm (□). The salt gradient was monitored by conductance readings as denoted by the dashed line (- - -).

purification is described in Table I, and the polypeptide composition of each step in the purification is shown in Figure 6.

Sequence Analysis of the Purified Enzyme Reveals a Unique Metallopeptidase Encoded by the YCL57w Gene on Chromosome III. Several active fractions from the Sephacryl S-200 step were concentrated and subjected to sodium dodecyl sulfate gel electrophoresis. The 68-kDa polypeptide was then electroblotted onto a PVDF membrane and was subjected to automated N-terminal Edman sequence analysis. Twenty residues were cleanly delineated (Figure 7). Using this sequence to search the NBRF-PIR (Release No. 34) database, we found identity at all 20 residues with the deduced amino acid sequence of the *S. cerevisiae* gene YCL57w, previously identified as an open reading frame in the DNA sequence of chromosome III, encoding a potential polypeptide of 82 kDa (Figure 7) (Oliver et al., 1992). These data strongly suggest that this open reading frame codes for the purified soluble proteolytic activity.

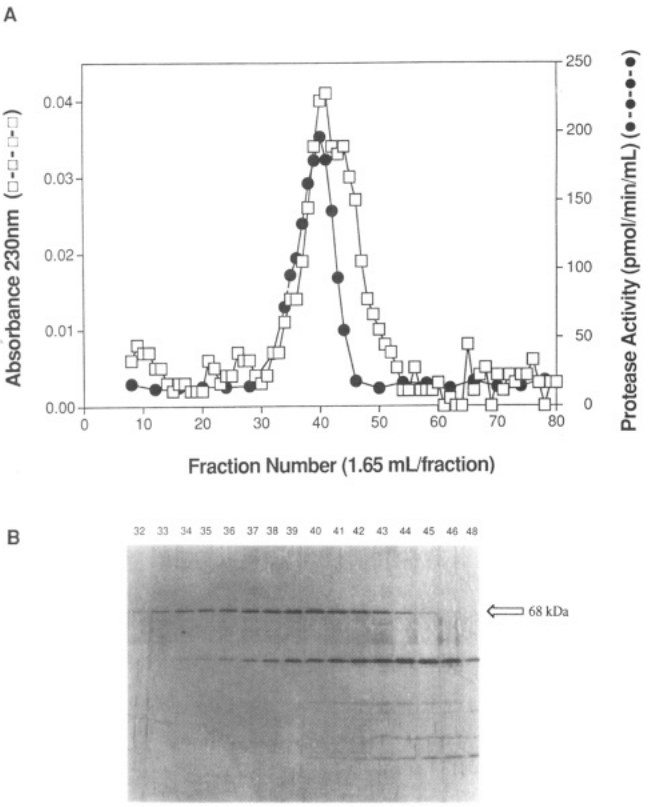

FIGURE 5: Sephacryl S-200 chromatography of the C-terminal peptidase activity. (A) Fractions 182 and 183 from the phenyl-Sepharose column were pooled in a volume of 4.7 mL. The sample (0.23 mg of protein) was applied to a 130-mL Sephacryl S-200 column equilibrated with 100 mM Tris-HCl, pH 7.53, at 4 °C. The column was run at 0.21 mL/min. A 2 μL aliquot of each fraction shown was assayed for C-terminal peptidase activity (●), and each fraction was assayed for protein content by absorbance at 230 nm (□). (B) Aliquots (20 μL) of active fractions 32–48 were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on an 8% polyacrylamide resolving gel as described under Experimental Procedures. Protein bands were first visualized by staining with Coomassie brilliant blue for 1 h and destaining overnight followed by silver staining. The migration position and approximate molecular weight of the soluble peptidase is indicated by the arrow.

The alignment of the experimentally obtained sequence with the deduced amino acid sequence of the YCL57w gene product begins at residue 28 of the encoded open reading frame (Figure 7). One possibility is that translation initiates at the methionine residue at position 27 and this residue is then proteolytically removed. However, in this case, one might expect that the N-terminal alanine residue would be acetylated (Lee et al., 1988, 1990), a result inconsistent with the Edman sequencing data. Alternatively, translation may initiate at the first methionine residue. The initial 27 residues may be removed

Table I: Purification of a Soluble Peptidase from *S. cerevisiae*

sample	volume (mL)	total protein (mg)[a]	total activity (pmol/min)	specific activity [pmol/(min·mg of protease)]	% recovery	purification (fold)
100000*g* supernatant	75.3	1112.2	35844	32.2	100	1
DE-52 ion exchange[b]	120	54	27810	515	77.6	16
hydroxylapatite	16.3	4.9	23932	4884	66.8	152
phenyl-Sepharose[c]	4.7	0.23	1545	6719	4.3	209
Sephacryl S-200[d]	1.65	0.00363	147.2	40540	0.41	1259

[a] Protein concentrations for the 100000*g* supernatant, the DE-52 pool, and the hydroxyapatite pool were determined by a modification of the Lowry procedure (Bailey, 1967) after precipitation with 1 mL of 10% (w:v) trichloroacetic acid. The concentrations for the phenyl-Sepharose pool and the S-200 fraction were determined by absorbance at 230 nm, assuming that 1 A = 0.2 mg of protein/mL. [b] Pools of two separate chromatography column runs. [c] Values from pool of fractions no. 182 and no. 183. [d] Values from fraction no. 34.

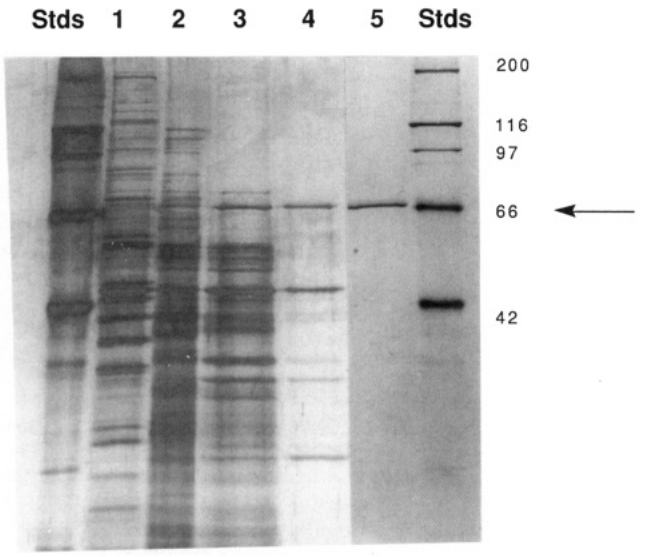

FIGURE 6: Purification of the soluble C-terminal peptidase from *S. cerevisiae* strain ABYS1. Samples from each of the purification steps were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on an 8% polyacrylamide resolving gel along with Bio-Rad high molecular mass markers as described under Experimental Procedures. The protein bands were visualized first by staining with Coomassie brilliant blue for 1 h and destaining overnight followed by silver staining. The polypeptide molecular mass standards include myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), and ovalbumin (42.7 kDa). The samples analyzed were crude 100000*g* supernatant (lane 1), DEAE-52 pool (lane 2), hydroxylapatite pool (lane 3), phenyl-Sepharose fraction no. 184 (lane 4), and Sephacryl S-200 fraction no. 35 (lane 5). The S-200 fraction (lane 5) was run on a separate gel under exactly the same conditions, and molecular mass markers are shown for both gels independently. The position of the soluble C-terminal peptidase is indicated with an arrow at approximately 68 kDa.

intact as an N-terminal leader peptide or may be cleaved in some combination of leader peptidase-dependent and -independent proteolytic step(s). The calculated mass of the polypeptide beginning at residue 28 is 79 kDa, a value comparable to the polypeptide chain mass of 68 kDa determined experimentally.

A comparison of the encoded amino acid sequence of the *YCL57w* gene with amino acid sequences in the NBRF-PIR (Release No. 34) and translated GenBank (Release No. 73) databases revealed homologies to several zinc metallopeptidases from a variety of organisms. These enzymes include the rat metalloendopeptidase 24.15 (EC 3.4.24.15) (Pierotti et al., 1990), the rabbit liver microsomal endopeptidase (Kawabata & Davie, 1993), the OpdA oligopeptidase A from *Salmonella typhimurium* (Conlin & Miller, 1992), the dcp dipeptidylcarboxypeptidase from *Escherichia coli* (Becker & Plapp, 1992), and the rat mitochondrial intermediate peptidase MIP (Isaya et al., 1992). The alignment of their most conserved region, the putative zinc-binding domain characterized by the sequence HEXXH, is shown in Figure 8 (Jongeneel et al., 1989; Vallee & Auld, 1990). The YCL57w gene product is most closely related to the rat metallopeptidase EP 24.15 (42% identity over the entire protein and 51% identity over the zinc binding domain) and the rabbit microsomal endopeptidase (38% identity over the entire protein and 50% identity over the zinc binding domain). Identity is defined as the number of matching residues in the alignments proposed by Kawabata et al. (1993) divided by the length of the shortest sequence aligned (Doolittle, 1986). Jiang and Bond have recently classified zinc metalloendopeptidases on the basis of the amino acid sequence in and around the zinc-binding site. Because the deduced sequence of YCL57w or its homologues (Figure 8) do not match any of the known consensus sequences, these proteins appear to represent a new family in this class of enzymes (Isaya et al., 1992; Jiang & Bond, 1992).

Inhibitor and Substrate Specificity Studies of the Purified Soluble Peptidase. The sequence comparisons shown in Figures 7 and 8 suggest that the purified soluble enzyme is a zinc metallopeptidase encoded by the *YCL57w* gene. We used inhibitor studies to provide experimental support for this idea. As shown in Table II, we found that the metal-chelating agent *o*-phenanthroline, but not EDTA, is a potent inhibitor of the enzyme. Other known metallopeptidases such as carboxypeptidase B also show no sensitivity to EDTA but are inhibited by *o*-phenanthroline (Folk et al., 1960). This differential sensitivity suggests that the anionic compound EDTA may have limited access to the metal-binding site as compared to the less polar *o*-phenanthroline (Dumas et al., 1989; Narasimhan & Miziorko, 1992). Partial inhibition was observed with the thiol protease inhibitor E-64 but only at relatively high concentrations (Table II). Inhibition was not seen with other thiol modifying compounds such as *N*-ethylmaleimide at 4 mM and *p*-hydroxymercuribenzoate at 1 mM (Table II). No effect was seen with pepstatin, leupeptin, aprotinin, phenylmethanesulfonyl fluoride, or dichloroisocoumarin.

Since sequence analysis revealed that the putative YCL57w protein is most closely related to the rat metalloendopeptidase 24.15, we sought to determine if the peptidase purified here and the rat enzyme had similar substrate specificities. The rat endopeptidase 24.15, highly active in brain, pituitary, and testis, has been shown to preferentially cleave bonds on the carboxyl side of hydrophobic amino acids (Orlowski et al., 1983, 1989; Chu & Orlowski, 1985). The specificity of the purified soluble peptidase from yeast was tested using several peptide substrates of the rat endopeptidase 24.15 including β-neoendorphin, bradykinin, and neurotensin (Table III). Each of the peptides was incubated with the purified soluble peptidase isolated from *S. cerevisiae* and subjected to reverse-

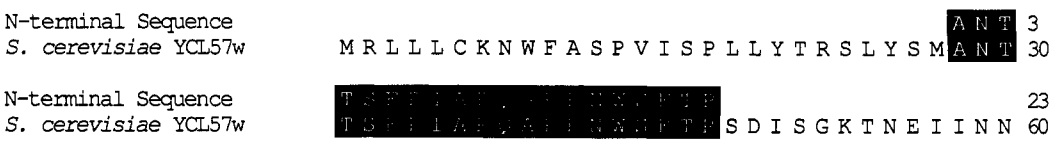

FIGURE 7: N-terminal sequencing of the purified soluble C-terminal peptidase from *S. cerevisiae*. Four active fractions (36–39) from a Sephacryl S-200 fractionation of phenyl-Sepharose fractions 180 and 181 were pooled to a total volume of 7.4 mL. This pool was concentrated in an Amicon Centricon 10 spin microconcentrator to 75 μL and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 1.5-mm thick 8% polyacrylamide resolving gel over three lanes along with prestained high molecular weight standards (Bio-Rad) as described under Experimental Procedures. The gel was then electroblotted to poly(vinylidene difluoride) (PVDF) as described under Experimental Procedures at 40 mA for 40 min. The membrane was stained with Coomassie brilliant blue for 1 min and subsequently destained to visualize the protein bands. The band at 68 kDa, clearly separated from a 45-kDa contaminant, was excised and used for N-terminal sequence analysis. The sequence is compared to that of hypothetical protein YCL57w from *S. cerevisiae* (see Results).

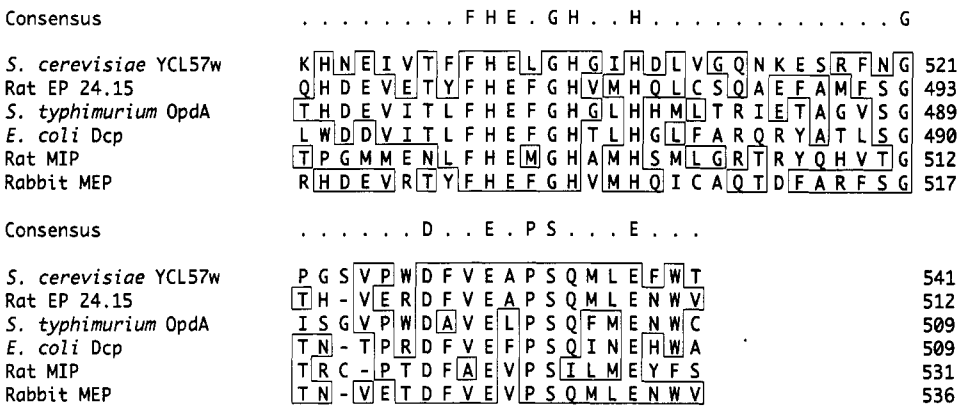

FIGURE 8: Alignment of the deduced amino acid sequences surrounding the putative zinc binding domains of *S. cerevisiae* YCL57w (NBRF-PIR S19387/S19745), rat endopeptidase 3.4.24.15 (NBRF-PIR A36165), *S. typhimurium* OpdA endooligopeptidase 3.4.22.19 (GenBank M84754), *E. coli* Dcp dipeptidyl carboxypeptidase (GenBank X57947), rat mitochondrial intermediate peptidase (MIP) (GenBank M96633), and rabbit liver microsomal endopeptidase (MEP) (GenBank D13310). The amino acid sequences of the proteins were aligned by the Clustal method in the program Megalign (DNASTAR). Residues common to two or more sequences are boxed. The consensus consisting of residues common to all of the sequences is shown above the alignment. The putative zinc binding domain is represented by the conserved sequence HEXXH, where X is any amino acid.

Table II: Inhibition of the Purified Soluble Peptidase Responsible for Cleavage of the Three Terminal Amino Acids from the Farnesylated Peptide *N*-Ac-KSKTKCVIM in Vitro

inhibitor	% of control[a]
none	100[b]
1 mM *N*-ethylmaleimide	99.4
2 mM *N*-ethylmaleimide	93.7
4 mM *N*-ethylmaleimide	83.8
0.5 mM pHMB	92.9
1 mM pHMB	89.8
0.5 mM pHMPS	98.9
20 μg/mL leupeptin	100.3
20 μg/mL pepstatin	110.8
20 μg/mL aprotinin	101.9
5 mM PMSF	98.0
2 mM *o*-phenanthroline	20.3[c]
20 μg/mL dichloroisocoumarin	99.6
0.75 mg/mL E-64	53.4[d]
2 mM EDTA	92.4

[a] Average of duplicate values. [b] Represents 0.249 pmol/min activity. [c] Represents 0.051 pmol/min activity. Control incubations with solvent alone (ethanol) gave 0.230 pmol/min activity (92.4% of control). [d] Represents 0.132 pmol/min activity. Control incubations with solvent alone (1:1 ethanol/water) gave 0.224 pmol/min activity (89.9% of control).

phase HPLC. The resultant peaks were then analyzed by amino acid analysis to determine the composition of each of the products (Table III). For the peptides β-neoendorphin and bradykinin, a cleavage site was observed with the yeast enzyme that was identical to that found for the rat enzyme. In each case, the peptide bond on the carboxyl side of a hydrophobic residue four residues from the C-terminus was cleaved (Orlowski et al., 1983, 1989; Chu & Orlowski, 1985). The cleavage pattern of the peptide neurotensin was more complicated and differed from that of the rat enzyme because two distinct cleavage sites were identified. A major site of cleavage was observed between the Pro and the Tyr residues, and a minor site was seen after the second Arg residue (Table III). The rat enzyme has been shown to cleave between the two Arg residues (Orlowski et al., 1983, 1989; Chu & Orlowski, 1985). Thus, these enzymes share similarity in both their amino acid sequences and in their substrate specificity. Importantly, these findings also explain why the farnesylated peptide *N*-acetyl-KSKTK[*S*-farnesyl-Cys]VIM can serve as a substrate for the yeast enzyme in our peptidase assay (Figure 1). Here, cleavage occurs following a hydrophobic farnesyl-cysteine residue three amino acids from the C-terminus of the peptide.

It is interesting to note that the minor cleavage observed after the Arg-Arg sequence in neurotensin using the purified yeast enzyme is at a site consistent with the specificity described for the rabbit liver microsomal endopeptidase (Kawabata & Davie, 1992). Although the degree of amino acid sequence identity of the yeast endopeptidase with the rat endopeptidase 24.15 (42%) and the rabbit microsomal metalloendopeptidase (38%) is similar, the specificity of the yeast enzyme appears to much more closely parallel that of the rat enzyme.

Subcellular Localization Studies. To determine if the soluble peptidase from *S. cerevisiae* actually is physiologically relevant in the processing of cytosolic isoprenylated protein precursors (Clarke, 1992), we explored its subcellular distribution in yeast. We fractionated gently lysed cells by Ficoll step gradient density ultracentrifugation and then assayed each fraction of the gradient for the C-terminal peptidase activity, dipeptidylaminopeptidase B (DPAP B) activity, a vacuolar marker enzyme, dipeptidylaminopeptidase A (DPAP A), a Golgi marker enzyme, and glucose 6-phosphate dehy-

Table III: Substrate Specificity of the Purified Endopeptidase from *S. cerevisiae*

substrate	sequence[a]	identified products (retention time: composition)[b]
β-neoendorphin[c]	Y-G-G-F-L↓R-K-Y-P	R-K-Y-P; 9.30 min; $Arg_{1.0}$ $Lys_{0.78}$ $Pro_{0.76}$ Tyr Y-G-G-F-L; 15.69 min; $Gly_{2.10}$ $Leu_{1.01}$ $Phe_{1.0}$ Tyr
neurotensin[d]	pE-L-Y-E-N-K-P-R-R↓P-Y-I-L	pE-L-Y-E-N-K-P-R-R; 12.14 min; $Arg_{2.32}$ $Asn_{0.87}$ $Glu_{2.85}$ $Leu_{1.0}$ $Lys_{0.68}$ $Pro_{0.77}$ Tyr pE-L-Y-E-N-K-P-R-R-P; 12.55 min; $Arg_{2.33}$ $Asn_{0.97}$ $Glu_{2.84}$ $Leu_{1.0}$ $Lys_{0.69}$ $Pro_{1.76}$ Tyr Y-I-L; 14.51 min; $Ile_{0.94}$ $Leu_{1.0}$ Tyr
bradykinin[e]	R-P-P-G-F↓S-P-F-R	S-P-F-R; 9.09 min; $Arg_{1.0}$ $Phe_{0.89}$ $Pro_{0.81}$ $Ser_{0.73}$ R-P-P-G-F; 11.60 min; $Arg_{1.0}$ $Gly_{0.96}$ $Phe_{0.81}$ $Pro_{1.70}$

[a] Solid arrows denote major cleavage sites whereas dotted arrows denote minor cleavage sites. [b] Tyrosine was detected in the peptides as indicated but not quantified due to oxidative losses during hydrolysis. [c] The purified peptide eluted at 14.78 min and had the following composition: $Arg_{1.0}$ $Gly_{1.65}$ $Leu_{0.85}$ $Lys_{1.21}$ $Phe_{0.82}$ $Pro_{0.93}$ Tyr. An additional product peak was detected at 12.69 min but accounted for only 4.3% of the total $A_{214\ nm}$ of the products and was not identified. [d] The purified peptide eluted at 16.28 min and had the following composition: $Arg_{2.23}$ $Asn_{0.90}$ $Glu_{2.83}$ $Ile_{0.97}$ $Leu_{2.0}$ $Lys_{1.47}$ $Pro_{1.55}$ Tyr. [e] The purified peptide eluted at 14.49 min and had the following composition: $Arg_{2.0}$ $Gly_{1.34}$ $Phe_{1.60}$ $Pro_{2.76}$

Table IV: Subcellular Localization of the Soluble Metalloendopeptidase from *S. cerevisiae*

	C-terminal protease		dipeptidyl aminopeptidase B		dipeptidyl aminopeptidase A		glucose-6-phosphate dehydrogenase	
fraction[a]	specific activity [pmol/(min·mg)]	total activity (pmol/min)	specific activity [nmol/(min·mg)]	total activity (nmol/min)	specific activity [nmol/(min·mg)]	total activity (nmol/min)	specific activity (units/mg)	total activity (units)
SM1188 vacuoles	267.9	94.5	92.3	32.6	48.7	17.2	0	0
SM1188 no. 1	128.0	61.7	11.8	5.7	4.8	2.3	0	0
SM1188 no. 2	114.8	50.6	6.7	3.0	4.6	2.0	0	0
SM1188 no. 3	63.8	63.2	2.7	2.7	1.3	1.3	0	0
SM1188 no. 4	12.1	79.7	1.0	6.6	0.0	0.0	0.23	1.5
SM1188 no. 5	8.0	190.7	0.3	7.2	0.26	0.60	0.21	5.0
ABYS1 vacuoles	150.3	35.3	44.4	10.4	31.0	7.3	0	0
ABYS1 no. 1	164.3	28.7	24.5	4.3	11.6	2.0	0	0
ABYS1 no. 2	114.7	30.2	15.9	4.2	12.2	3.2	0	0
ABYS1 no. 3	38.1	24.9	6.7	4.4	2.0	1.3	0	0
ABYS1 no. 4	16.8	41.6	1.1	2.7	0.18	0.44	0.16	0.4
ABYS1 no. 5	18.0	310.7	0.31	5.4	0.19	3.3	0.14	2.4

[a] DEAE-dextran lysed cells were applied to Ficoll gradients as described under Experimental Procedures. Fractions were removed from the top of the gradients.

drogenase, a cytosolic marker enzyme (Table IV). The data obtained revealed that the C-terminal peptidase activity is most highly enriched in the vacuole-containing fraction in both SM1188 cells and the carboxypeptidase Y-deficient strain ABYS1. The fact that there is no detectable contamination of this fraction with the cytosolic marker glucose 6-phosphate dehydrogenase suggests that the soluble C-terminal peptidase is not cytosolic. In fact, the distribution of the soluble peptidase activity follows those of both the vacuolar marker DPAP B and the Golgi marker DPAP A (Table IV).

DISCUSSION

We have purified and characterized a soluble proteolytic activity previously identified as a candidate for the post-translational processing of -CXXX containing peptides and proteins in the yeast *S. cerevisiae* (Hrycyna & Clarke, 1992). The purified enzyme contains a single 68-kDa polypeptide by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and elutes as a 110-kDa globular species on gel filtration chromatography. These data suggest that the native enzyme may exist as either a globular dimer or as an elongated monomer. N-terminal sequence analysis of the purified polypeptide indicates that the enzyme is encoded by the YCL57w gene on chromosome III of yeast. Comparison of the entire deduced amino acid sequence of the YCL57w open reading frame with other sequences reveals that this enzyme is homologous to several zinc metalloendopeptidases from a variety of organisms. Data from peptidase inhibition and substrate specificity experiments using the purified enzyme are consistent with the action of a novel metalloendopeptidase with a propensity for cleaving on the carboxyl side of hydrophobic amino acid residues. This latter finding can explain why the farnesylated peptide used in our *in vitro* peptidase assays serves as a substrate for this soluble enzyme.

Previously, characterization of a partially purified preparation of the soluble activity suggested that the enzyme behaved like a processive carboxypeptidase (Hrycyna & Clarke, 1992). The present results suggest that this enzyme preparation was contaminated with an aminopeptidase activity. In this case, endoproteolytic cleavage of the three C-terminal amino acids from the farnesylated peptide substrate may have resulted in the formation of a tripeptide that in turn became a substrate for the aminopeptidase. In fact, we did observe the release of valine and tyrosine from the synthetic peptide VYPNSA but no release of the C-terminal amino acids suggesting the presence of an active aminopeptidase (Hrycyna & Clarke, 1992; data not shown). Similarly, we also observed free tyrosine and isoleucine released when the peptide DRVYIHPF (angiotensin II) was incubated with the impure enzyme but no release of amino acids from the C-terminus of the peptide. These data suggested that endoproteolytic cleavage occurred on the C-terminal side of the valine residue and the contaminating aminopeptidase activity then partially cleaved the resultant peptide YIHPF.

Although the yeast peptidase was considered a candidate for catalyzing the proteolytic step in the maturation of cytosolic

isoprenylated protein precursors (Hrycyna & Clarke, 1992), two lines of biochemical evidence suggest that this enzyme may not be physiologically relevant in this pathway in *S. cerevisiae*. First, we find that the enzyme activity is not specific for farnesylated -CXXX peptide substrates since the non-farnesylated peptide analogue *N*-acetyl-KSKTKCVIM can inhibit the reaction (Hrycyna & Clarke, 1992) and because the non-farnesylated peptides bradykinin, β-neoendorphin, and neurotensin are substrates. Secondly, subcellular localization studies suggest that the soluble enzyme is not present in the cytosolic fraction but may be localized to a cellular compartment such as the vacuole or Golgi apparatus. A non-cytosolic localization is also consistent with the finding that the purified protein is not acetylated on the N-terminal alanine residue, a commonly modified residue of cytosolic eukaryotic polypeptides (Driessen et al., 1985; Persson et al., 1985). The deduced amino acid sequence of the open reading frame of *YCL57w*, the gene encoding this enzyme, contains an additional 27 N-terminal residues that may serve as a leader sequence for noncytosolic targeting.

A membrane-associated proteolytic activity specific for farnesylated -CXXX peptide substrates has been previously identified in *S. cerevisiae* both by our laboratory (Hrycyna & Clarke, 1992) and by Ashby et al. (1992). This activity may represent the physiologically relevant species in the posttranslational processing pathway of cytosolic farnesylated polypeptides *in vivo*. Similar membrane-associated activities have also been identified in mammalian cells (Ashby et al., 1992; Ma et al., 1992; Ma & Rando, 1992). Interestingly, mutants defective in this activity have not been described to date in *S. cerevisiae*. It is possible that multiple activities, including the soluble enzyme described here and carboxypeptidase Y, may be able to function to some extent in the processing of -CXXX containing polypeptides *in vivo*. We have recently constructed a mutant strain of yeast lacking both carboxypeptidase Y and the soluble endopeptidase described here (C. A. Hrycyna and S. Clarke, unpublished results). This strain (CH9100-2) was created by homologous recombination of a linear DNA fragment of the entire *YCL57w* gene where nucleotides 560–1576 were replaced by the selectable marker encoded by the *URA3* gene into the parental strain BJ2168 (*MATa prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52*, Yeast Genetic Stock Center, University of California, Berkeley). The resulting strain had less than 7% of the parental peptidase activity and thus may be useful in the search for a mutant in the membrane-bound activity.

The purification and characterization of this soluble activity not only represents the identification of a new metalloendopeptidase in *S. cerevisiae* but also adds to the description of a new class of enzymes in the larger family of zinc metallopeptidases (Jiang & Bond, 1992). The striking similarity of this activity to the rat metalloendopeptidase 24.15, an enzyme potentially involved in the processing and formation of some bioactive peptides and in the degradation of others (Orlowski et al., 1983, 1989; Chu & Orlowski, 1985, suggests that the yeast enzyme may also be important in some type of peptide metabolism *in vivo*. For example, a role is possible in the vacuolar breakdown of peptides. Since the deduced amino acid sequence of the yeast enzyme is also very similar to that of a microsomal endopeptidase from liver with a substrate specificity for processing proproteins (Kawabata & Davie, 1992; Kawabata et al., 1993), a role in protein metabolism is also possible for the yeast enzyme. The viability of the haploid deletion strain described above suggests that this endopeptidase is not essential for yeast cell growth, although we cannot rule out at this point the presence of a compensating suppressor mutation.

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