The Multispanning Membrane Protein Ste24p Catalyzes CAAX Proteolysis and NH₂-terminal Processing of the Yeast a-Factor Precursor*

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Saccharomyces cerevisiae Ste24p is a multispanning membrane protein implicated in the CAAX proteolysis step that occurs during biogenesis of the prenylated a-factor mating pheromone. Whether Ste24p acts directly as a CAAX protease or indirectly to activate a downstream protease has not yet been established. In this study, we demonstrate that purified, detergent-solubilized Ste24p directly mediates CAAX proteolysis in a zinc-dependent manner. We also show that Ste24p mediates a separate proteolytic step, the first NH₂-terminal cleavage in a-factor maturation. These results establish that Ste24p functions both as a bona fide COOH-terminal CAAX protease and as an a-factor NH₂-terminal protease. Importantly, this study is the first to directly demonstrate that a eukaryotic multispanning membrane protein can possess intrinsic proteolytic activity.

Eukaryotic proteins that terminate with a CAAX motif (C = cysteine, A = generally an aliphatic residue, X = one of a few amino acids) undergo three ordered post-translational maturation events referred to as CAAX processing. These events are: covalent attachment of an isoprenoid lipid (farnesyl or geranylgeranyl) to the cysteine, proteolytic removal of the AAX tripeptide, and carboxyl methylesterification of the newly exposed COOH-terminal prenlysystein (1, 2). These modifications can modulate the activity, stability, and/or membrane attachment of a protein (3–7). Examples of proteins that undergo CAAX processing (CAAX proteins) include small GTP-binding proteins, such as Ras and Rho, the γ subunit of some heterotrimeric G-proteins, nuclear lamins, and certain fungal mating pheromones, such as Saccharomyces cerevisiae a-factor.

Genetic analyses of S. cerevisiae have identified two genes that encode candidate CAAX proteases, STE24 (also designated AFC1) and RCE1 (8–10). Despite the lack of protein sequence similarity between Ste24p and Rce1p, both are predicted to have multiple membrane spans and are localized to the endoplasmic reticulum (ER) membrane (11). Consistent with its proposed role as a protease, Ste24p has a conserved zinc metalloprotease motif (HEXXH; where H is histidine, E is glutamate, and X is any amino acid). Mutation of this motif severely compromises in vivo a-factor production and thus the mating competence of MATα cells (9). Furthermore, in vitro Ste24p-dependent CAAX proteolysis is sensitive to the metalloprotease inhibitor 1,10-orthophenanthroline (8). Rce1p, on the other hand, lacks known protease motifs. Nevertheless, Rce1p-dependent CAAX proteolysis is sensitive to certain cysteine protease inhibitors (12). The specific combination of AAX residues influences whether proteolysis of a CAAX protein is carried out by Ste24p, Rce1p, or both (8, 13). Either Ste24p or Rce1p can promote a-factor CAAX proteolysis, whereas Ras proteolysis in yeast and mammalian cells is strictly Rce1p-dependent (8, 11, 13, 14).

In addition to promoting the COOH-terminal CAAX proteolysis of the a-factor protease, genetic studies indicate that Ste24p is involved in a separate proteolytic event during a-factor biogenesis (9, 10). The P1 a-factor intermediate, which is fully COOH-terminally modified (farnesylated, proteolyzed, and carboxyl methylated), contains an NH₂-terminal extension that undergoes two successive proteolytic cleavages to yield first P2, and finally mature (M) a-factor. The first cleavage (P1 → P2 conversion) occurs between residues Thr⁷ and Ala⁸ of P1 a-factor and is dependent on Ste24p (9, 15). Because of the redundant function of the Ste24p and Rce1 in COOH-terminal CAAX proteolysis, the observed defect of a ste24 mutant is inefficient cleavage at the first NH₂-terminal cleavage site, which leads to the accumulation of the P1 a-factor intermediate. The second NH₂-terminal cleavage event during a-factor biogenesis (P2 → M conversion) removes the remainder of the NH₂-terminal extension to complete the maturation of a-factor and is dependent upon Axl1p (16).

An important question that remains to be answered is whether Ste24p and/or Rce1p act directly as proteases, or whether they function indirectly as activators of another protease(s). Recent in vitro reconstitution experiments, using membranes prepared from wild-type and mutant yeast strains or insect cells expressing recombinant protein, firmly establish that both can function in CAAX proteolysis (8, 11, 12, 14). These studies, however, cannot answer whether Ste24p or Rce1p is sufficient for proteolysis, since crude membrane preparations were used in the reconstitution experiments rather than purified components. Thus, the possibility that Ste24p and Rce1p are limiting upstream activators of an unknown protease or are part of a multicomponent protease present in these membrane preparations cannot yet be excluded. Similarly, membrane reconstitution experiments have shown that Ste24p (but not Rce1p) is necessary for NH₂-terminal proteolysis of P1 a-factor; however, its sufficiency has not been addressed (17).

To determine if Ste24p directly mediates its two proposed...
activities, COOH-terminal CAAX processing and NH2-terminal proteolysis of P1 a-factor, we purified a histidine-tagged version of Ste24p from yeast. We demonstrate that Ste24p is sufficient to carry out CAAX proteolysis of a synthetic, farnesylated a-factor peptide in a zinc-dependent fashion. Hence, this report establishes that Ste24p is a bona fide CAAX protease. In addition, we show that purified Ste24p possesses the ability to cleave within the NH2-terminal extension of the P1 a-factor intermediate. Thus, Ste24p can catalyze two distinct cleavage events in a-factor biogenesis.

**EXPERIMENTAL PROCEDURES**

### Strains and Media

The yeast strains used in this study are listed in Table I. Strains were routinely grown at 30 °C on synthetic complete dropout (SC-) media, as previously described (18).

### Plasmids

The plasmids used in this study are listed in Table II. Plasmid pSM1191 (CEN URA3 STE24::HA) encodes yeast STE24 with three hemagglutinin (HA) epitopes inserted just before the stop codon. Plasmid pSM1282 ([2u] URA3 Poxc24 STE24::His::HA) encodes STE24 with 10 histidines codons followed by three HA epitopes inserted just after the start codon of STE24. Expression of STE24::His::HA is under the constitutive control of the phosphoglycerate kinase promoter (19).

### Coupled CAAX Proteolysis / Methylation Reactions Using Membrane Extracts

The substrate for the coupled reaction is a farnesylated 15-mer peptide (YIKGVFWDDPA(farnesyl)CVIA). The unmodified 15-mer peptide was enzymatically farnesylated in vitro using purified farnesyltransferase and farnesyl diphosphate according to published methods by Dr. J. Otto (Duke University) (14). A stock solution of this peptide was prepared at 165 μM in MeOH and stored at −80 °C. Membrane preparations derived from WT or mutant strains serve as the source of protease. A membrane preparation derived from a yeast strain that encodes STE14 on a high-copy plasmid and lacks chromosomal copies of STE24 and RCE1 (SM3614/pSM1317) serves as the source of carboxyl methyltransferase. All membrane preparations were typically prepared as previously described at 1–3 mg/ml in lysis buffer (50 mM Tris, 0.2 mM sorbitol, 1 mM EDTA, 0.02% NaN3, pH 7.5) and stored at −80 °C until needed (17). The methyl donor S-adenosyl-L-methionine and other reagents were purchased from Sigma.

**Proteolysis—CAAX** proteolysis reactions (typically 20 μl) were assembled on ice in a 96-well polystyrene microtiter plate by mixing 10 μl of farnesylated 15-mer (2 μM (38 ng) peptide in 200 mM HEPES, 200 mM NaCl, pH 7.5) and 10 μl of yeast membranes (diluted into lysis buffer at the indicated concentrations). Where noted, reaction mixtures contained the metal chelator, 1,10-orthophenanthroline. Proteolysis was initiated by shifting the reaction mixtures to 30 °C. After 10 min, proteolysis was terminated by heating the reaction mixtures to 95 °C for 1 min.

**Methylation—**Methylation reactions were assembled on ice by adding carboxyl methyltransferase-containing membranes (0.1 mg/ml final) and S-adenosyl-L-methionine (20 μM final) to the terminated CAAX proteolysis reaction mixtures. These components were prepared as a 4 × mixture prior to addition. Methylation was initiated by shifting the reactions to 30 °C. After 60 min, methylation was terminated by heating to 95 °C for 1 min or by adding copper acetate (2 mM final).

### Spot Halo Assay

To detect bioactive a-factor generated in coupled assays, published methods were followed (18, 20). Briefly, a portion of the coupled reaction mixture and serial 2-fold dilutions (into YPD media) thereof were spotted (2 μl/spot) onto a lawn of supersensitive strains. The yeast strains used in this study are listed in Table I. Strains Media, as previously described (18).

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Mass Spectrometry Analysis

Samples subjected to CAAX proteolysis (as described above) were adsorbed to Ziptip C18 beads (Millipore), washed three times with 0.1% trifluoroacetic acid, and eluted with 75% acetonitrile before analysis by matrix-assisted laser desorption ionization-time of flight mass spectrometry at the Johns Hopkins University School of Medicine Synthesis & Sequencing Facility.

PI a-Factor N-terminal Proteolysis Assay

PI a-factor was immunoprecipitated from yeast cells radiolabeled with [35S]cysteine, as previously described (17). The immunoprecipitate was resuspended into 0.1 N NaOH and incubated for 30 min at 30 °C to release PI a-factor from immune complexes. Prior to use in the proteolysis reaction, the pH of the eluted sample was neutralized by the addition of 0.1 M HEPES free acid.

N-terminal proteolysis reactions (25 μl final volume) were assembled on ice to contain purified radiolabeled PI a-factor, Escherichia coli lipid (0.025%) (Avanti Polar Lipid), and either purified Ste24p (4 μg/ml) (the E2 fraction in elution buffer 2), a mock-purified E2 fraction in elution buffer 2 obtained from a lysate lacking histidine-tagged Ste24p, or elution buffer 2 only. Where indicated, the reaction mixture also contained 1,10-orthophenanthroline (1 mM). Proteolysis was initiated by shifting the reaction mixtures to 30 °C. After 1 h, proteolysis was terminated by the addition of 6 × Laemmli sample buffer. Equivalent portions from each reaction mixture were analyzed by modified SDS-PAGE and PhosphorImager along with samples of the crude lysate and immunopurified PI a-factor (15, 17).

Proteinase K-Protection Assay

Proteinase K-protection experiments were performed as previously described (24), but using spheroplasts generated as above. Briefly, reactions contained clarified yeast membranes (3.2 mg/ml final) with or without Proteinase K (0.3 mg/ml final; Roche Molecular Biochemicals) in the presence or absence of Triton X-100 (0.4% final). After allowing the reaction to proceed for 5 min on ice, digestion was terminated by the addition of trichloroacetic acid (10% final). Equivalent portions of samples were separated by SDS-PAGE and subjected to immunoblot analysis using antibodies against HA (BabCo), Kar2p (gift of J. Brodsky), and the Ste24p COOH terminus (17).

RESULTS

A Coupled System and a Sensitive Bioassay to Measure CAAX Proteolysis—Because proteolysis is prerequisite for the carboxyl methylation of prenylated CAAX substrates, coupled proteolysis/methylation assays can be used to follow CAAX proteolysis in vitro (14, 21, 25). In such assays, the presence of a base-labile methyl group serves to indirectly monitor CAAX proteolysis. Typically, methylation is followed with radioactive tracers (e.g. 14C-methyl transfer from 14C-methyl-S-adenosyl-l-methionine).

Here we have developed a nonradioactive coupled system, schematized in Fig. 1A, that can be used in conjunction with a highly sensitive bioassay to indirectly monitor CAAX proteolysis. The substrate in this system is a farnesylated 15-mer peptide that contains the mature a-factor peptide sequence and the native a-factor CAAX motif. This substrate is biologically inactive. CAAX proteolysis and subsequent carboxyl methylaion convert this inactive 15-mer substrate into a farnesylated and carboxyl methylated 12-mer that is identical to biosynthetically generated active a-factor, which can be detected using an established bioassay. In this bioassay, samples containing a-factor generate a zone (also termed a halo) of growth inhibition when spotted onto a lawn of MATα sat2 yeast cells (18, 20). The minimum concentration of a-factor that can produce a halo is 12 pg/μl, which is termed the a-factor end point (6). Thus by determining the highest dilution at which a sample can still generate a detectable halo, the amount of mature a-factor produced in the reaction can be extrapolated. Under the conditions used here, CAAX proteolysis is rate-limiting. Thus, the amount of a-factor generated provides an indirect measurement of proteolytic activity.

We compared the sensitivity of this nonradioactive coupled assay to that of a traditional radioactive coupled assay (21). Overall, for both assays, we observed a clear linear correlation between the amount of protease-containing membranes added to the reaction mixture and the amount of active a-factor produced (Fig. 1, B and C, closed squares) or the amount of volatile radioactive methyl esters produced (Fig. 1C, open diamonds). As expected, the substrate becomes limiting in the presence of high levels of enzyme, as is evident at the highest concentration of membranes examined (400 μg/ml). An important advantage of the a-factor bioassay as compared with the radioactive assay is apparent at the lowest membrane protein concentrations (Fig. 1C, inset). The a-factor bioassay showed no background activity when membranes were absent from the reaction mixture, whereas the radioactive assay showed a relatively high level of background counts without added membranes. Since the very low levels of CAAX processing activity present in many biological samples can be obscured in the radioactive coupled system, the a-factor bioassay provides a more sensitive method of detection (26).

Using the nonradioactive coupled assay, we compared the CAAX proteolytic activity of yeast membranes derived from wild type and mutant yeast strains (Fig. 2, solid bars). Membranes lacking both Rce1p and Ste24p (rce1Δ ste24Δ) were unable to promote CAAX processing. On the other hand, membranes that contain both Rce1p and Ste24p (WT), Rce1p only (RCE1 ste24Δ), or Ste24p only (rce1Δ ste24Δ) were competent for CAAX processing. These results confirm previous in vivo and in vitro findings that either Rce1p or Ste24p can suffice for CAAX proteolysis of a-factor (8, 10). Similar to what was seen in an earlier study, we find that CAAX processing in a strain deleted for Ste24p is not significantly impaired, possibly because Rce1p is up-regulated under these circumstances (8). As expected, we also found that Ste24p-dependent a-factor production is inhibited by the metalloprotease inhibitor 1,10-orthophenanthroline, whereas Rce1p-dependent production was unaffected (Fig. 2, open bars). Importantly, our results validate the coupled system in conjunction with the a-factor bioassay as a reliable method for monitoring CAAX proteolysis. Purified Ste24p-His-HA N Has CAAX Proteolytic Activity—Thus far, no study has unequivocally demonstrated that either Ste24p or Rce1p directly mediates CAAX proteolysis. To address this issue, we sought to purify Ste24p and determine whether it retained proteolytic activity. To facilitate purification, Ste24p was tagged at its NH2 terminus with 10 histidine residues. A triply iterated HA epitope links the His tag to Ste24p and permits immunodetection. The mating defect of a ste24Δ strain is rescued to the same extent with plasmids expressing either wild-type Ste24p or Ste24-His-HA N, indicating that the tag does not disrupt Ste24p function in vivo.2

For the purification of histidine-tagged Ste24p, a detergent-solubilized yeast extract was prepared and subjected to nickel chelate chromatography. Fractions collected during a typical purification of Ste24p-His-HA N were analyzed by SDS-PAGE and silver staining (Fig. 3A). This purification procedure yields a single species (>95% purity) of ~60 kDa (Fig. 3A, lanes 6–7), which is immunoreactive with anti-HA and anti-Ste24p antibodies, is absent from a mock purification using a strain lacking Ste24p (ste24Δ), and is consistent with the calculated molecular mass of Ste24p-His-HA N (55 kDa).2 Ste24p represents ~0.5% of the total protein in the starting lysate.3 Approximately 10–15% of the Ste24p in the starting lysate (Fig. 3A, lane 1) was recovered in the elution fractions (Fig. 3A, lanes 6 and 7) as determined by immunoblot analysis.3

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2 A. Tam and S. Michaelis, unpublished data.

3 W. K. Schmidt and S. Michaelis, unpublished data.
A coupled proteolysis/methylation system that yields bioactive α-factor can be used to measure CAAX proteolysis. A diagram of the coupled system. The substrate for CAAX proteolysis is a farnesylated 15-mer peptide that is biologically inactive and contains the mature α-factor sequence (shaded in gray) and the α-factor CAAX motif. In the first step of the coupled system, a membrane-associated CAAX protease removes the three COOH-terminal residues (VIA). In the second step, membrane-bound Ste14p carboxyl methylates the substrate, yielding bioactive α-factor mating pheromone. Since bioactive α-factor contains in each sample was extrapolated after determining the α-factor end point for each sample, which is the highest dilution at which a halo can be generated (see “Experimental Procedures”). B, comparison of nonradioactive and radioactive coupled assays. The amounts of α-factor generated in the samples were calculated as described under “Experimental Procedures” and are represented graphically (closed squares; average of two experiments). Radioactive coupled reactions were carried out in parallel, as described under “Experimental Procedures,” and the base-labile methyl esters formed were quantified and are plotted (open diamonds; average of two experiments).

Fractions from the purification were assayed for CAAX proteolytic activity using the bioassay described above. We observed a 40-fold increase in specific activity upon comparison of the total starting lysate (T) and the peak elution (E2) fractions (Fig. 3B). As expected, 1,10-orthophenanthroline inhibited all of the measurable CAAX proteolytic activity in these fractions. Overall, these data provide strong support for the proposal that purified Ste24p-His-HA promotes CAAX proteolytic activity.

Ste24p Is Sufficient to Carry Out CAAX Proteolysis—Because the methylation step of the coupled proteolysis/methylation system required the addition of crude yeast membranes containing the Ste14p carboxyl methyltransferase, we could not yet exclude the possibility that purified Ste24p indirectly activated a “true” CAAX protease in the added membranes. To examine whether purified Ste24p alone was capable of catalyzing CAAX proteolysis, we carried out the CAAX proteolysis step of the coupled system and directly analyzed the proteolytic products by mass spectrometry (Fig. 4). Thus, the sole components present in this reaction were purified Ste24p and the synthetic farnesylated peptide.

As shown in Fig. 4A, the unreacted substrate is detected as a major peak at 1901 Da and a minor peak at 1737 Da. The larger species corresponds to a farnesylated 14-mer that lacks the NH2-terminal tyrosine of the 15-mer and was likely formed during the chemical synthesis of the 15-mer peptide. Both species have identical CAAX sequences and are thus substrates for CAAX proteolysis. Analysis of the reaction products generated by purified Ste24p reveals two new species (1618 and 1454 Da) that are shifted by 283 Da relative to those in the starting synthetic farnesylated peptide.

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producing Ste24p-His-HAN were solubilized in 1% dodecyl maltoside and the clarified lysate representing the total starting material (T) was incubated with Ni-NTA resin. The flow-through (FT) was discarded, and bound Ste24p-His-HA<sub>N</sub> was eluted (Elution 1 and 2). A fixed volume (5 μl) of each fraction was subjected to SDS-PAGE and silver staining, representing 0.06625% (T, FT), 0.025% (W1, W3), 0.1% (W5), 1% (E1), and 0.5% (E2) of the respective fraction. The binding conditions employed recover 10–15% of the total Ste24p in the lysate (87 μg from 1600 A<sub>280</sub> units of cells). The strain used for purification expresses STE24-His-HA<sub>N</sub> from a high copy plasmid (SM3103/pSM1282). The mobility of protein standards is indicated at the left. B, the specific activity of Ste24p increases upon purification. Samples before and after purification were examined for CAAX proteolytic activity by the coupled reaction and a-factor spot halo bioassay, as shown in Fig. 1. Specific activity corresponds to the units of a-factor generated/min/μg of protein added to the coupled reaction. The specific activity of the total (T), flow-through (FT), and peak elution (E2) fractions (5.0, 3.1, and 200 ng of a-factor/min/μg, respectively) is represented in graphical form. The specific activities of the wash (W1–5) and first elution (E1) fractions were not determined because the protein concentrations of these fractions were too low to quantify accurately.

substrate (Fig. 4B). The magnitude of the shift corresponds precisely to the mass of the three COOH-terminal CAAX residues (VIA), indicating that they have been removed. Thus, this experiment firmly establishes that purified Ste24p is sufficient to mediate CAAX proteolysis of a farnesylated peptide.

We also carried out the CAAX proteolysis reaction under conditions of substrate excess to examine whether partial exoproteolytic products might be evident (Fig. 4C). Under these conditions, we detected only the starting substrates (farnesylated 15-mer and 14-mer; 1901 and 1737 Da, respectively) and the corresponding proteolytic products lacking the three COOH-terminal residues (farnesylated 12-mer and 11-mer; 1618 and 1454 Da, respectively). Because no intermediates indicative of partial exoproteolysis were observed, these data corroborate that Ste24p endoproteolytically removes the COOH-terminal residues as a tripeptide, as has been previously shown in reconstitution studies using crude membranes containing Ste24p (8).

**Purified Ste24p Requires a Metal Cofactor for CAAX Proteolytic Activity**—Because Ste24p is predicted to be a metalloenzyme, we expected that purified Ste24p should require a supplemental metal cofactor for its CAAX proteolytic activity. However, Ste24p-mediated CAAX proteolysis occurs even in reaction buffer lacking metal ions (Figs. 3 and 4). This finding is not completely unexpected because a tight association between metalloproteases and metal cofactors has been reported (27).

To specifically determine whether a metal cofactor was required for Ste24p CAAX proteolytic activity, we examined if metal ions could reconstitute Ste24p that had been inactivated by pretreatment with the divalent metal ion chelator 1,10-orthophenanthroline, a known metalloprotease inhibitor (Fig. 5).
We sought to generate a working model of Ste24p cleavage site. The results presented in Fig. 6 indicate in vitro suggesting they are identical, although we cannot exclude the -factor was observed (Fig. 6 C a, calcium, and copper), only cobalt reactivated Ste24p CAAX proteolytic activity after 1,10-orthophenanthroline treatment, albeit to a much lesser extent (reactivation with 250 μM cobalt was only 25% of that seen with 250 μM zinc). These data indicate that the CAAX proteolytic activity of Ste24p optimally requires a zinc metal ion cofactor, and are consistent with the proposed classification of Ste24p as a zinc-dependent metalloprotease.

Purified Ste24p Is Sufficient to Carry Out NH2-terminal Proteolysis of P1 α-Factor—We have previously established using an in vitro reconstitution assay that Ste24p is necessary for the NH2-terminal proteolysis of P1 α-factor (Fig. 6A) (17). We therefore sought to determine if purified Ste24p is sufficient to cleave purified radiolabeled P1 α-factor, which was isolated by immunoaffinity purification (Fig. 6B). In the presence of purified Ste24p, significant NH2-terminal processing of purified P1 α-factor was observed (Fig. 6C, lane 3). Importantly, processing did not occur when the substrate was incubated with buffer alone, a mock purified fraction obtained from a yeast lysate lacking histidine-tagged Ste24p, or phenanthroline-treated Ste24p (Fig. 6C, lanes 1, 2, and 4, respectively). The P2 species produced in vitro comigrates with in vivo generated P2, suggesting they are identical, although we cannot exclude the possibility that in vitro processing occurs near, but not at, the in vivo cleavage site. The results presented in Fig. 6 indicate that purified Ste24p is capable of directly mediating NH2-terminal proteolysis of the P1 α-factor precursor. Combined with our previous data, this study clearly establishes that Ste24p directly mediates two distinct proteolytic steps in α-factor biogenesis, COOH-terminal CAAX proteolysis and NH2-terminal proteolysis.

Ste24p Has a Lumenal NH2 Terminus and Cytosolic COOH Terminus—We sought to generate a working model of Ste24p membrane topology, by determining the membrane sidedness of its NH2 and COOH termini. In addition to its multiple membrane spans (as predicted by Kyte and Doolittle hydropathy analysis), Ste24p also has a metalloprotease motif (HEXXH), and an ER-retrieval dilysine motif (KKXX) (Fig. 7A). We hypothesize that the HEXXH motif, which is likely to be the catalytic site of Ste24p, is in a cytosolic loop, since farnesylated CAAX substrates, including α-factor intermediates, reside on the cytosolic face of intracellular membranes (15). Likewise, ER-retrieval motifs are generally cytosolically disposed (30). To initiate the analysis of Ste24p topology, HA epitope tags were placed independently at the NH2 and COOH termini of Ste24p, and proteinase K protease-protection experiments were carried out on microsomal membranes to determine the disposition of these HA tags relative to the ER membrane in which Ste24p resides (Fig. 7B, top panel) (11, 24). A lumenally exposed tag should be protected from protease and a cytosolic tag should be protease sensitive. The fate of the ER luminal marker protein Kar2p was used to monitor vesicle integrity (Fig. 7B, middle panel). Blots were also probed using antibodies against the COOH terminus of Ste24p (Fig. 7B, bottom panel). Interest-
Purified Ste24p Has CAAX Proteolytic Activity

In control samples with no added protease or detergent, HA-tagged Ste24p and Kar2p are both detected by immunoblot analysis (Fig. 7B, lanes 1 and 5, top and middle panels). As expected, the addition of Triton X-100 alone to microsomes does not compromise the immunoreactivity of epitope-tagged Ste24p or Kar2p (Fig. 7B, compare lanes 1 and 2, and lanes 5 and 6, top and middle panels). Also as expected, the addition of Triton X-100 and proteinase K together results in the complete disappearance of immunoreactivity for Kar2p and HA-tagged Ste24p (Fig. 7B, lanes 4 and 8, top and middle panels). Notably, however, as mentioned above, the Ste24p portion of these molecules remains intact (Fig. 7B, lanes 4 and 8, bottom panel). Importantly, the addition of proteinase K alone does not affect the immunodetection of Ste24p tagged at the NH$_2$ terminus (Ste24p-HA$_N$), suggesting that the NH$_2$ terminus is lumenally disposed (Fig. 7B, lane 3, top panel). On the other hand, Ste24p tagged at the COOH terminus (Ste24p-HA$_C$) is no longer detected, suggesting that the COOH terminus is cytosolically disposed (Fig. 7B, lane 7, top panel). In both cases, the luminal marker protein Kar2p is detected, indicating that microsome membrane integrity is intact under these conditions (Fig. 7B, lanes 3 and 7, middle panel).

Based on these data, the NH$_2$ and COOH termini of Ste24p are predicted to lie on opposite sides of the ER membrane, suggesting that Ste24p must have an odd number of membrane spans. These results, taken together with the Kyte and Doolittle hydropathy analysis, predict that Ste24p is likely to possess seven membrane spans, with its NH$_2$ terminus in the ER lumen and its COOH terminus cytosolically disposed (Fig. 7C). This prediction rests on the view that region VII of Ste24p (Phe$^{355}$-Ser$^{366}$) represents a single span, rather than two. We favor a single span for several reasons. First, the region in question is less than 30 amino acids in length and contains two charged residues (Asp$^{360}$ and Glu$^{365}$), making it unlikely that it could form two separate membrane spanning segments. Second, hydropathy analysis of the Schizosaccharomyces pombe Ste24p homolog clearly predicts a single span for region VII (9). Finally, at least four other modeling programs indicate that region VII of Ste24p is likely to form only one span (31–34). These considerations and our data are consistent with a seven-span Ste24p topology. Importantly, in this working model, the zinc metalloprotease motif (HEXXH) is predicted to face the cytosol, where CAAX proteolysis must occur. Future experiments to directly determine the disposition of the internal loop containing the HEXXH motif and to assess the orientation of each span will require additional approaches, such as the construction of fusion proteins and/or internally tagged versions of Ste24p. While the results presented here are by no means definitive, they provide a starting point for further topology studies.

**DISCUSSION**

**Ste24p Is a Bona Fide Protease**—Genetic studies of *S. cerevisiae* have implicated Ste24p in CAAX proteolysis. Ste24p is predicted to contain multiple membrane spans, a feature typical of proteases, and until this study, direct *in vitro* proof establishing that Ste24p is sufficient to promote CAAX proteolysis has been lacking. Here we present the purification of histidine-tagged Ste24p and demonstrate that purified, detergent-solubilized Ste24p is indeed capable of directly mediating CAAX proteolysis *in vitro* (Figs. 3 and 4). In addition, we show that purified Ste24p catalyzes the NH$_2$-terminal proteolysis of the a-factor precursor, a proteolytic activity unrelated to CAAX cleavage (Fig. 6). These results rule out the possibility that Ste24p is simply a cofactor or upstream activator of an uniden-
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This study has additional significance. First, the purification of Ste24p sets a precedent for the purification of eukaryotic proteases containing multiple membrane spans. A growing number of multispanning membrane proteins are implicated in biologically diverse proteolytic events, yet direct demonstration of their proteolytic activity is lacking (35). One of these is Rce1p, which is also implicated in CAAX proteolysis and has functional overlap with Ste24p for some, but not all, CAAX substrates (8, 11, 13). Unlike Ste24p, Rce1p has no identifiable protease motifs, placing its classification as a protease in question. Using methodology analogous to that developed here for the purification of His-tagged Ste24p, we have recently found that yeast Rce1p, like Ste24p, has intrinsic protease activity.3 Other notable examples of putative proteases with multiple membrane spans are the mammalian site 2 protease, which is required for processing the sterol regulatory element-binding proteins, and the presenilins (PS1 and PS2), which are implicated in the processing of the amyloid precursor protein associated with Alzheimer’s disease (36, 37). However, a rigorous demonstration that any of these have proteolytic activity on their own has not been documented with purified components. To our knowledge, the only polytopic membrane-bound proteases that have been purified in active form are from prokaryotes (i.e. FtsH and OmpT) (38–40). In neither case is the topology like that of Ste24p, which has its catalytic HEXXH domain situated between predicted a-helical transmembrane segments. Second, in this study we have developed a new assay for CAAX proteolysis that is more sensitive and thus more versatile than traditional radioactive CAAX proteolysis assays (Fig. 1). The utility of this assay is demonstrated here, and also in a separate study where we have assessed the loss of CAAX proteolytic activity in membranes from a Ste24-deficient (Zmp-ste24−/−) mouse (26). Finally, a precise characterization of Ste24p proteolytic activity, as initiated here, may aid in identifying chemical agents that block CAAX proteolysis and therefore have potential chemotherapeutic value, as discussed below.

Ste24p Is a Zinc-dependent Metalloprotease—Previous studies have shown that mutation of the HEXXH motif of Ste24p abolishes its proteolytic activity (8, 9, 17). Here we demonstrate that the CAAX proteolytic activity of purified Ste24p is inhibited by the metal chelator 1,10-orthophenanthroline and that this inactivation is reversed by the addition of zinc (Fig. 5) and to a lesser extent by cobalt.2 Overall, these findings solidify the classification of Ste24p as a zinc-metalloprotease. We also present protease-protection experiments, which together with hydroxylation analysis, suggest that the HEXXH motif and the bulk of the Ste24p molecule likely face the cytosol (Fig. 7). Thus, Ste24p is properly positioned to interact with its cytosolically oriented prenylated CAAX substrates.

Interestingly, an emergent theme is that every step of CAAX processing (prenylation, proteolysis, and carboxyl methylation) appears to involve a metalloenzyme (this study, Ref. 41, and Ref. 42). While the ultimate biological significance of this observation is not clear, it suggests that metal (i.e. zinc) limitation could have profound affects on the biogenesis of CAAX proteins.

Ste24p Is a Bifunctional Protease—Ste24p is an unusual protease in that it can cleave the a-factor precursor at two distinct sites, between Thr7 and Ala8 (NH2-terminal processing) and between farnesylated Cys23 and Val134 (COOH-terminal CAAX processing). A comparison of the two Ste24p proteolytic activities reveals several similarities, suggesting that they may work through a shared mechanism. Both proteolytic activities are phenanthroline-sensitive, abolished by mutations in the metalloprotease motif of Ste24p, and require zinc (Fig. 5) (this study, Refs. 8, 9, and 17). Moreover, we have determined that the farnesylated 15-mer substrate for CAAX proteolysis used in this study can competitively inhibit NH2-terminal proteolysis of P1 a-factor.3 An important issue for future investigations is whether prenylation is directly required for both activities. In vivo studies indicate that the NH2- and COOH-terminal cleavages of the a-factor precursor are blocked in farnesyltransferase mutants, suggesting that prenylation is an important modification for proper a-factor processing (15, 43). However, these in vivo experiments do not establish whether farnesylation of the a-factor precursor is required for membrane association and/or for enzyme recognition. Further studies will also be required to understand how Ste24p can recognize and cleave its substrate at two dissimilar sites and to determine what is the minimum recognition site for each cleavage event.

The Physiological Role of Ste24p—The only known phenotype of the ste24 mutant is a mating defect in MATa cells, which results from impaired a-factor biogenesis. As discussed above, Ste24p has two roles in the production of this mating pheromone. As a CAAX protease, Ste24p performs a redundant function with Rce1p (8). In its second role, Ste24p promotes the first NH2-terminal cleavage in a factor-biogenesis. Because the CAAX proteases Ste24p and Rce1p function redundantly for a-factor biogenesis, the observed defect of a ste24 mutant is inefficient cleavage at the first NH2-terminal cleavage site, which leads to the accumulation of the P1 a-factor intermediate (9). In the absence of functional Ste24p (either by deletion of the gene or by mutational inactivation of the Ste24p HEXXH motif), mating of MATa cells is impaired because the production of mature secreted a-factor is severely compromised (9). In the absence of the Rce1p CAAX protease, Ste24p can faithfully carry out both CAAX and NH2-terminal proteolysis, permitting the production of mature a-factor; thus an rce1Δ mutant has no observable mating defect.

Thus far, the only known physiological substrate for either of the two proteolytic activities of yeast Ste24p is the a-factor precursor. The dual proteolytic specificities of yeast Ste24p appear to be conserved across species since mammalian (mouse and human) homologs of Ste24p can also faithfully cleave a-factor at both the NH2 and COOH termini (10, 17, 26). Mammalian a-factor-like substrates are, however, presently unknown. We expect that additional substrates are likely to exist in yeast and mammalian cells. In yeast, Ste24p is expressed in cell types that do not produce a-factor (i.e. MATα haploid and diploid cells) (9). In mammalian cells, Ste24p is expressed in a variety of cell types (26, 44, 45). Future studies are likely to identify the substrate(s) of Ste24p in these cell types and to help define the physiological role(s) of this protease. As a starting point, a viable Ste24 homozygous knockout mouse (Zmp-ste24−/−) has recently been generated (26), and studies are currently underway to examine the relationship between the loss of ZmpSte24 activity and potential phenotypes.4 Ultimately, it will be of interest to determine whether any as yet undiscovered substrates are subject to one or both of the proteolytic activities of Ste24p. Those that undergo both, in a manner similar to the processing of a-factor, may reflect a common theme linking yeast and human cells in the production of a novel type of farnesylated signaling molecule.

Many prenylated proteins have important roles in cellular transformation (e.g. Ras and Rho). Since proper CAAX processing is critical for modulating the activity of these proteins,
agents that interfere with CAAX processing are under development as cancer therapeutics. One such class of therapeutic agents, the farnesyltransferase inhibitors (FTIs), prevent the farnesylation step in the biogenesis of CAAX proteins. These agents have been shown to reverse or reduce tumor growth and are currently undergoing clinical trials (46–48). Recent studies reveal that CAAX proteins in addition to Ras are key FTI targets in transformed cells, yet which of these FTI targets will be relevant to cancer therapy has not been established (48). It is conceivable that some of these FTI targets are Ste24p substrates in transformed cells, yet which of these FTI targets will be relevant to cancer therapy has not been established (48). It is conceivable that some of these FTI targets are Ste24p substrates in transformed cells, yet which of these FTI targets will be relevant to cancer therapy has not been established (48).

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