The yeast mating pheromone α-factor precursor contains an N-terminal extension and a C-terminal CAAX motif within which multiple posttranslational processing events occur. A recently discovered component in α-factor processing is Ste24p/Afc1p, a multispanning endoplasmic reticulum membrane protein that contains an HEXXH metalloprotease motif. Our in vivo genetic characterization of this protein has demonstrated roles for Ste24p in both the N-terminal and C-terminal proteolytic processing of the α-factor precursor. Here, we present evidence that the N-terminal proteolysis of the α-factor precursor P1 can be accurately reconstituted in vitro using yeast membranes. We show that this activity is dependent on Ste24p and is abolished by mutation of the Ste24p HEXXH metalloprotease motif or by mutation of the α-factor P1 substrate at a residue adjacent to the N-terminal P1 cleavage site. We also demonstrate that N-terminal proteolysis of the P1 α-factor precursor requires Zn2+ as a co-factor and can be inhibited by the addition of the metalloprotease inhibitor 1,10-orthophenanthroline. Our results are consistent with Ste24p itself being the P1→P2 α-factor protease or a limiting activator of this activity. Interestingly, we also show that the human Ste24 homolog expressed in yeast can efficiently promote the N-terminal processing of α-factor in vivo and in vitro, thus establishing α-factor as a surrogate substrate in the absence of known human substrates. The results reported here, together with the previously reported in vitro reconstitution of Ste24p-dependent CAAX processing, provide a system for examining the potential bifunctional roles of yeast Ste24p and its homologs.

Preparations of the S. cerevisiae mating pheromones, α-factor and α-factor, undergo posttranslational processing (1–3). Surprisingly, the yeast mating pheromones do not share a common biosynthetic pathway even though the mature pheromones perform similar functions as secreted signaling molecules that stimulate cognate G-protein coupled receptors. The processing and export of α-factor involves translocation of its precursor into the endoplasmic reticulum (ER)1 and subsequent transport of the precursor through the luminal compartments of the classical secretory pathway, during which it is modified by resident processing enzymes of the ER, Golgi, and trans-Golgi network (4). In contrast, the processing of the α-factor precursor appears to occur on the cytosolic face of the ER (and possibly other intracellular membranes), yielding a cytosolically disposed mature pheromone (5). The eventual export of the mature α-factor pheromone across the plasma membrane is accomplished by Ste6p, a dedicated transporter that is a member of the ATP-binding cassette superfamily of transporters (3, 6, 7).

The S. cerevisiae α-factor mating pheromone, redundantly encoded by the MFA1 and MFA2 genes, is initially synthesized as a primary precursor (P0) that contains an N-terminal extension and a C-terminal CAAX motif (C = cysteine, A = an aliphatic amino acid, and X = one of several amino acids) (Fig. 1A). The ultimate formation of mature α-factor (M) requires multiple modifications at both ends of the primary precursor (Fig. 1B). The first set of modifications (isoprenylation, proteolytic removal of the AAX tripeptide, and carboxymethylation) are directed by the C-terminal CAAX motif (8, 9). These C-terminal modifications are common to all proteins bearing a CAAX sequence (e.g. Ras, Gγ, and nuclear lamin precursors). For α-factor, this processing yields an intermediate α-factor species (P1) that lacks the terminal three amino acids (VIA) and has a farnesylated and carboxymethylated C-terminal cysteine. The P1 intermediate is then trimmed of its N-terminal extension in two sequential proteolytic steps, yielding first the P2 intermediate and finally mature α-factor (M) (3, 10, 11). The initial N-terminal cleavage step (P1→P2) occurs between residues 7 (Thr) and 8 (Ala) of the α-factor precursor, and a subsequent cleavage step (P2→M) occurs between residues 21 (Asn) and 22 (Tyr) that removes the remainder of the extension. Thus, three distinct proteolytic processing events, one C-terminal and two N-terminal, are involved in α-factor maturation.

Recent genetic studies have identified several components involved in the proteolytic processing of α-factor (Fig. 1B). These include the Ste24p (also called Afc1p) and Rce1p proteins that are required for the C-terminal CAAX processing of α-factor and other proteins bearing a CAAX motif (12). Ste24p and Rce1p appear to be partially redundant for this function (12, 13). An unexpected finding was that Ste24p has a second distinct role in α-factor processing; namely, it also is required for the first N-terminal cleavage (P1→P2) event of α-factor biogenesis (11). The dual roles of Ste24p in promoting N- and C-terminal processing are notable given that these cleavage sites are spatially separated and have little sequence similarity (Fig. 1A). Finally, the Axl1p and Ste23p proteins were shown to have some partial overlapping function for the final N-terminal cleavage event (P2→M) that yields mature α-factor (14).

Further analysis of the α-factor proteolytic processing components revealed that Ste24p and Rce1p are ER-localized membrane spanning proteins (5). The localization of Axl1p and

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1 The abbreviation used is: ER, endoplasmic reticulum.
Ste24p-dependent Proteolysis in Vitro

and Rce1p were recently reconstituted in vitro using crude yeast membranes (12). Whereas membranes containing either Ste24p or Rce1p promoted the CAAX proteolysis of a particular farnesylated peptide substrate, only Rce1p-containing membranes could promote the CAAX proteolysis of yeast Ras2p. The Ste24p-dependent N-terminal proteolytic activity was not examined in this study.

In addition to its role in CAAX proteolysis, Ste24p is likely to act directly or indirectly as a protease in the first N-terminal cleavage of the α-factor precursor, but this has not yet been examined by direct in vitro studies (11). Accordingly, the aim of this study was to reconstitute an in vitro system that could be used to examine the role of Ste24p in the N-terminal processing (P1→P2) of α-factor. This would enable us to gain further insight into the role of Ste24p and to initiate a characterization of the biochemical properties of the N-terminal processing activity. Here, we have faithfully reconstituted in vitro the N-terminal proteolysis of the P1 α-factor precursor using yeast membranes as the source of Ste24p and the P1 α-factor precursor. We demonstrate biochemically that this activity requires Ste24p and a metal co-factor and that the cleavage event displays substrate specificity. Finally, we show in vivo and in vitro that the human homolog of Ste24p can properly direct the N-terminal processing of the yeast α-factor precursor.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The yeast strains used in this study are listed in Table I. All strains are isogenic to SM1058 (MATa trp1 leu2 ura3 his4 can1) and were routinely grown at 30 °C on synthetic-selective solid medium as described previously (15). Plasmid-bearing strains were created by transformation with the indicated plasmids according to published methods (11, 16). Plasmids pSM217 [2μ URA3], pSM219 [2μ URA3 MFA1], and pSM1093 [CEN URA3 STE24] have been previously described (10, 11). pSM1190 [CEN URA3 STE24:NotI] is essentially the same as pSM1093 except that a NotI site was engineered immediately before the stop codon of STE24, which results in a three-amino acid (Ser-Gly-Arg) insertion at the C terminus. This construct behaves normally as judged by functional complementation in a patch mating test.2 pSM1194 [2μ URA3 STE24] contains the XhoI-SacI STE24 fragment from pSM1069 (11) subcloned into the same sites of pSM217. pSM1543 [2μ URA3 HsSTE24] was constructed identically to pSM1194 except that the source for the insert was pSM1468 (13). pSM1566 [2μ URA3 MFA1] and pSM1606 [2μ URA3 MFA1:NotI] contain the EcoRI-NotI fragment from pSM478 [CEN URA3 MFA1] and pSM566 [CEN URA3 mfa1-A8G], respectively, cloned into the same sites of pSM217. pSM478 is essentially the same as pSM232 (10) except that unique restriction sites were introduced by site-directed mutagenesis in the untranslated region on either side of MFA1 (His at −50 and M1at at 130). pSM566 is derived from pSM478 and contains an A8G mutation that was generated by oligonucleotide-directed mutagenesis (11).3

Isolation of Yeast Membranes—Mid-log cells grown in synthetic-selective liquid medium were harvested (typically 50–500 A600) by centrifugation (500 × g for 5 min), washed with cold 10 mm NaCl, and resuspended to 500 A600/ml with cold lysis buffer (50 mm Tris, 0.2 mM orthovit, 1 mM EDTA, 0.02% NaN3, pH 7.5) containing protease inhibitors (3 μg/ml leupeptin, chymostatin, and pepstatin; 2 μg/ml aprotinin; and 1 mM phenylmethylsulfonyl fluoride). Cells were mechanically disrupted by vortexing in the presence of silica beads (six 2-min vortex bursts at 4 °C). Crude lysates were cleared twice of unbroken cells and large cellular fragments by centrifugation (500 × g for 10 min at 4 °C). Membranes were recovered from the cleared lysate by centrifugation (100,000 × g for 60 min at 4 °C), resuspended in lysis buffer to the original sample volume, adjusted to 4 mg of protein/ml by dilution with lysis buffer, and frozen as aliquots at −80 °C.

Radio labeling—The isolation of radiolabeled P1 α-factor precursor-containing membranes was performed as above except that the cells were radiolabeled prior to harvesting in synthetic minimal dextrose liquid medium supplemented with amino acids necessary for growth

Fig. 1. The biogenesis of α-factor. A, sequence of the precursor (P0) and mature (M) forms of the α-factor mating pheromone encoded by MFA1; the amino acid change in the mutant α-factor (A8G) used in Fig. 7 is shown in parentheses below the P0 sequence. The N-terminal extension and CAAX motif of the P0 precursor are indicated. Also shown are the proteolytic cleavage sites (arrows) and the components required for these events. B, linear diagram of the α-factor biogenesis pathway. The α-factor biosynthetic intermediates (P0, P0*, P1, P2, and M) and the cellular components that carry out the C-terminal CAAX modifications, N-terminal proteolytic processing, and export of α-factor are indicated (10, 11, 13). All α-factor biogenesis components are membrane-associated proteins, with the exception of the cytosolic farnesyltransferase complex (Ram1p/Ram2p). Ste24p, Rce1p, and Ste14p are specifically ER membrane-localized (5, 25). Ste23p has not been reported. Additionally, Ste24p, Ax1p, and Ste23p, but not Rce1p, possess consensus metallopeptide motifs that are conserved among many zinc-dependent metallopeases (e.g. HXXH or HXXXH). Not surprisingly, point mutations of the Ste24p metallopeptide motif (HXXH) resulted in defective CAAX and N-terminal processing of α-factor precursors (11, 12). Thus, the components genetically identified as being required for the proteolytic processing of α-factor may act directly on α-factor precursors as proteases, or alternatively could activate as yet unidentified downstream acting proteases. The CAAX proteolytic activities dependent on Ste24p

2 K. Fujimura-Kamada and S. Michaelis, unpublished data.
3 A. Kistler, F. J. Nouvet, and S. Michaelis, unpublished data.
Some reactions also contained 1–10 mM 1,10-orthophenanthroline, as noted. To examine metal ion specificity, reactions were assembled without membranes added, and reactions were initiated by incubation at 30 °C; the additions resulted in dilution of the 1,10-orthophenanthroline to a final concentration of 1 mM. The metal ions were added in the form of acetate salts from 25–100 mM stock solutions (Zn \( \text{II} \), Mg \( \text{II} \), Ca \( \text{II} \), Co \( \text{II} \), or Cu \( \text{II} \)) that were first acidified with 2 mM concentrated HCl and then neutralized with 1 mM HEPES, pH 7.5; this was especially necessary to achieve full solubilization of zinc acetate.

For analysis by immunoprecipitation, samples were diluted 10–20× with immunoprecipitation buffer (17) containing 0.1% SDS, cleared of insoluble material (13,000 × g for 5–10 min at 4 °C), and immunoprecipitated with an a-factor specific antibody (antibody 9-137) (10). Alternatively, samples were solubilized by SDS (final concentration, 0.5%), boiled for 3 min, and directly immunoprecipitated after dilution with immunoprecipitation buffer. Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis as described (10), fixed for 7 min with 20% trichloroacetic acid, dried, and exposed to a PhosphorImager screen for detection and quantitation (Molecular Dynamics, Sunnyvale, CA). Unless otherwise indicated, all of the above reagents were purchased from Sigma.

### Immunoblotting and Ste24p Antibody

Immunoblotting of yeast membrane samples (10 μg/lane) was carried out as described previously (5). The Kar2p rabbit polyclonal antibody was provided by Dr. J. Brodsky (University of Pittsburgh, PA) and used at a dilution of 1:10,000. The Ste24p rabbit polyclonal antibody (antibody 215) was generated against a GST-Ste24p fusion containing amino acids 223–306 of Ste24p (Covance, Denver, PA) and used at a dilution of 1:2500. Following incubation with the primary antibodies, immune complexes were detected by chemiluminescence (Roche Molecular Biochemicals) after incubation with an HRP-conjugated donkey anti-rabbit secondary antibody (Amersham Pharmacia Biotech).

### RESULTS AND DISCUSSION

**In Vitro Reconstitution of N-terminal Proteolysis (P1→P2) of the P1 a-Factor Precursor**—We have previously established that the P1 a-factor precursor accumulates in vivo in a ste24Δ strain, suggesting that Ste24p promotes the initial N-terminal (P1→P2) cleavage of the a-factor precursor (11). We have also determined that the P1 a-factor precursor and Ste24p are membrane-associated (5, 10). To gain further insight into the role of Ste24p in this cleavage event, we investigated whether we could reconstitute the N-terminal P1→P2 proteolysis of the P1 a-factor precursor in vitro by mixing membranes containing the a-factor P1 precursor (substrate) with membranes containing Ste24p.

We performed a reconstitution experiment and found that the addition of Ste24p-containing membranes to radiolabeled P1-containing membranes results in the efficient conversion of the P1 a-factor precursor to the P2 species (Fig. 2, lane 1). The P2 species generated in this in vitro assay appears to be bona fide P2 as it migrates similarly to the P2 species generated in vivo labeling of STE24 yeast (Fig. 2, lane 5). In some experiments, as seen in lane 1, we also observed the formation of a band that migrates slightly faster than the P2 species. This band may reflect Ste24p-dependent proteolysis of the P1 precursor at an alternative cut site as we have previously observed under certain circumstances in vivo (10). The addition of membranes from a yeast strain deficient for Ste24p (ste24Δ) did not result in any significant conversion to the P2 form (Fig. 2, lanes 3 and 4). Furthermore, no conversion was observed in reactions containing the corresponding cytosolic fraction derived from cells expressing STE24 (data not shown). These results indicate that the P1 protease activity is membrane-associated and STE24-dependent. Neither pretreatment of the membrane preparation with standard protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, chymostatin, leupeptin, and pepstatin) during membrane isolation nor the inclusion of protease inhibitors in the reaction mix prevented proteolysis, suggesting that the N-terminal protease is not a serine, thiol, or acid protease (data not shown). Conversion of P1 to the P2 form was inhibited by the metalloprotease inhibitor 1,10-orthophenanthroline (Fig. 2, lane 2), which is consistent with the role for the Ste24p metalloprotease in this processing event.

**Metal Dependence for N-terminal (P1→P2) Proteolysis**—We have previously demonstrated that mutation of the Ste24p HEXXH metalloprotease motif (Ste24p-E298D) results in a failure to process the P1 a-factor precursor in vivo (11). Similarly, we show in Fig. 3 that proteolysis of the P1 precursor is not observed in vitro with membranes prepared from cells.
FIG. 2. a-Factor proteolysis in vitro is dependent on STE24.
Unlabeled membranes isolated from a strain expressing high copy STE24 (SM4083; lanes 1 and 2) or lacking STE24 (ste24Δ) (SM3805; lanes 3 and 4) were mixed with membranes containing [35S]cysteine-radiolabeled a-factor P1 precursor (derived from strain SM3783) and incubated for 60 min at 30°C in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 mM 1,10-orthophenanthroline (1, 10-ϕ). Reactions contained 1.25 mg/ml each of unlabeled and labeled membranes. Each reaction was immunoprecipitated with an a-factor specific antibody (antibody 9-137), and immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and PhosphorImager analysis. For reference, immunoprecipitates of the radiolabeled a-factor intermediates generated in vivo from a wild-type strain expressing chromosomal STE24 are shown (SM3310; lane 5).

FIG. 3. Mutation of the Ste24p HEXXH metalloprotease motif results in loss of N-terminal proteolytic activity. Membranes isolated from yeast expressing wild-type STE24 or mutant ste24-E298D (SM3071 and SM3074, respectively) were assayed and prepared as in Fig. 2 except that reactions contained 0.5 mg/ml each of membranes containing Ste24p (derived from SM4083) and membranes containing radiolabeled P1 substrate (derived from SM3783).

expressing mutant Ste24p (E298D) (Fig. 3, lanes 3 and 4). The lack of proteolytic activity observed in vivo and in vitro suggests that an intact HEXXXH motif is important for the coordination of a metal co-factor that is critical for activity. However, significant misfolding caused by this mutation cannot be eliminated as an explanation for loss of function. The Ste24p-dependent CAAX proteolytic activity also requires metal ions in vitro (12) and an intact Zn2+-metalloprotease motif for in vivo function. Thus, the metalloprotease motif of Ste24p appears to be necessary for both the N-terminal and CAAX proteolysis of the a-factor precursor.

Because metalloproteases can utilize a broad range of metal co-factors, we examined whether the requirement for metal ions in the P1→P2 proteolysis reaction was restricted to zinc, as postulated, or other metal ions (Fig. 4). As a first step toward this analysis, we determined the fate of the P1 substrate in reactions lacking added metal ion. Surprisingly, P1→P2 conversion was observed without the supplemental addition of any metal ions (Fig. 4, lane 1) and even in the presence of up to 10 mM EDTA (data not shown). Typically, metalloproteases are purified with their metal co-factor, and our observation may simply reflect the high affinity that the P1 protease has for its metal co-factor (18). As expected, treatment with 1,10-orthophenanthroline had an inhibitory effect in reactions lacking added metal ion (Fig. 4, lane 2). The supplemental addition of zinc to the reactions containing 1,10-orthophenanthroline (final concentration, 0.5–1 mM) was sufficient to restore proteolytic activity in these reactions (lanes 3 and 4). However, higher levels of zinc had an inhibitory effect on the proteolysis reaction (lane 5). Other zinc-dependent metalloproteases are also inhibited by high levels of zinc (19). The addition of other divalent metal ions (1 mM) had either no effect (Co2+ and Cu2+) or a very slight effect (Mg2+ and Ca2+) on P1→P2 conversion (Fig. 4, lanes 6–9). These results thus confirm that P1→P2 proteolytic activity is directly or indirectly dependent on a zinc-dependent metalloprotease. As is evident in this experiment, we sometimes recovered less than the expected amounts of a-factor from the sample. In Fig. 4, only 40–55% of the input counts were detected in lanes 1, 3, and 4. We therefore cannot exclude the possibility that P1→P2 proteolytic activity is somewhat nonspecific, especially upon reactivation of the activity after pretreatment with a metalloprotease inhibitor.

Ste24p Is Limiting For N-terminal (P1→P2) Proteolysis In Vitro—The N-terminal assay described here relies on crude membranes as the source of protease and substrate. As such, we cannot formally establish whether Ste24p directly or indirectly participates in the processing event. To gain support for the possibility that Ste24p is indeed the P1 a-factor protease or at least a limiting upstream component for this reaction, we examined the proteolysis of the P1 a-factor precursor in response to changing levels of Ste24p (Fig. 5). Increasingly higher levels of Ste24p expression per mg of membrane proteins is achieved by encoding STE24 in the chromosome (single copy) or by encoding STE24 in CEN (low copy) and 2µ (high copy) plasmids, respectively (Fig. 5A, lanes 2–4). No Ste24p was detected in yeast deleted for STE24 (Fig. 5A, lane 1). For all of the membrane extracts examined, the amount of the gel loading control protein, Kar2p, remained constant (Fig. 5A). As the Ste24p level increased, we observed more complete conversion of the P1 precursor to the P2 a-factor species (Fig. 5, B–D). Whereas 1 mg/ml membranes is needed for complete conversion of the P1 a-factor substrate when the membranes are derived from a strain expressing chromosomal STE24 (Fig. 5B, lane 6), only 0.0625 mg/ml of membranes derived from a high copy STE24 strain is needed to fully convert the substrate (Fig. 5D, lane 1). As expected, an intermediate amount of membranes (0.25–0.5 mg/ml) is necessary for full conversion of the substrate when membranes are derived from a low copy CEN STE24 strain (Fig. 5C, lanes 4 and 5). These results establish a
correlation between increased Ste24p production and increased processing, which indicates that Ste24p is a limiting component in this event. These data are consistent with the hypothesis that Ste24p acts directly as the a-factor N-terminal protease, but we cannot exclude the possibility that Ste24p is an upstream activator of an as yet unidentified protease.

In a first step to examine some of the basic enzymatic properties of our in vitro activity, we sought to determine the fate of the P1 a-factor substrate over a time course. Shown as a graph and a PhosphorImager exposure (Fig. 6), as little as 0.02 mg/ml of protease containing yeast membranes was sufficient to nearly complete the proteolysis of 0.25 mg/ml of P1-containing yeast membranes within 20 min at 30°C. Increasing the substrate level (0.5–1.5 mg/ml) slowed the time needed for each reaction to go to completion in a manner that correlated with the increase in the amount of substrate. For all of the conditions tested, the progress curves are typical for the late stages of enzyme-catalyzed reactions, indicating that the conversion of the P1 a-factor substrate is a rapid event. At the highest substrate levels examined, late-stage steady-state reaction rates could be observed only during the first few minutes (<5 min) of the assay. Thus, the P1 a-factor substrate does not appear to be in excess in these time course experiments or in the experiments previously presented. We also noted that some proteolysis occurred at the initial time points (0 min), especially at higher substrate levels, reflecting activity that occurred while the reactions were assembled on ice.

N-terminal (P1→P2) Proteolysis Exhibits Substrate Specificity—We have isolated a-factor mutants that are defective for the P1→P2 proteolytic step that normally occurs between residues 7 (Thr) and 8 (Ala) of the P1 a-factor precursor (10, 11)3. To examine the in vitro proteolytic activity of the P1→P2 protease toward a mutant substrate (P1-A8G; see Fig. 1), we compared the fate of the P1-A8G a-factor under conditions where wild-type P1 a-factor is cleaved to the P2 form (Fig. 7). Notably, we did not detect significant conversion of the mutant P1-A8G a-factor under conditions where wild-type P1 a-factor is cleaved to the P2 form (Fig. 7, compare lanes 1 and 2 with lanes 3 and 4). In this experiment, the incomplete conversion of wild-type P1 a-factor simply reflects the reaction conditions examined. Lengthening the reaction period (≥60 min) and increasing the amount of protease in the reaction (0.5 mg/ml) results in the eventual conversion of the P1-A8G substrate to the P2 form (data not shown). This result is consistent with our previous observation that strains expressing the P1-
A8G mutant, or similar P1→P2 cut site mutants, are capable of producing some mature a-factor because they undergo mating to a lesser degree than strains expressing wild-type a-factor. Combined, our results indicate that the P1→P2 protease is not simply a protease that removes the first seven amino acids from the P1 a-factor precursor regardless of amino acid context. The P1→P2 protease is, in fact, highly discriminating because it can differentiate between two substrates that differ only in the side chain length of the amino acid adjacent to the cut site.

**Human Ste24 Can Promote N-terminal (P1→P2) Proteolysis**—We and others have recently reported the identification of human Ste24, which is expressed in a wide variety of tissues (13, 20, 21). We have also shown that human Ste24 expression rescues the mating defect of a yeast ste24-deficient strain in a patch mating assay (13). Thus the P1 a-factor substrate apparently can serve as a surrogate substrate for the human Ste24 protease. Because the patch mating assay is highly sensitive, requiring only a low amount of mature a-factor, it does not address the efficiency of a-factor processing. In order to further examine the role of human Ste24 in a-factor biogenesis, we compared the extent of a-factor N-terminal processing in vivo in a ste24Δ mutant bearing yeast or human STE24 plasmids (Fig. 8A). In cells that were subjected to pulse-chase radiolabeling, the overexpression of human Ste24 from a high copy plasmid (Fig. 8A, lanes 7 and 8) resulted in the efficient intracellular (I) processing of a-factor precursors to an extent similar to that observed for cells expressing low copy yeast STE24 (lanes 3 and 4). Low copy human Ste24 expression generated smaller amounts of the P2 intermediate (Fig. 8A, lanes 5 and 6). In yeast cells expressing human Ste24, subsequent proteolysis of P2 to mature a-factor (M) is evident by the presence of M in both intracellular (I) and secreted extracellular (E) samples (lanes 5–8). We extended this analysis to determine whether human Ste24 can also promote CAAX protease activity in a manner similar to yeast Ste24p (Fig. 8B). In the absence of both yeast RCE1 and STE24, neither CAAX nor N-terminal proteolysis is observed (13). Using a strain deficient for both yeast RCE1 and STE24 (recΔ ste24Δ), we determined in vivo by pulse-chase radiolabeling analysis that expression of high copy human Ste24 (Fig. 8B, lanes 3 and 4) restores a-factor processing (i.e. formation of mature a-factor) to a level similar to that observed for low copy yeast STE24 (Fig. 8B, lanes 1 and 2). Because the export of mature a-factor requires complete N- and C-terminal processing, the presence of extracellular mature a-factor indicates that human Ste24 can promote distinct proteolytic activities, much like yeast Ste24p.

As expected based on the observed processing of a-factor in vivo, yeast membranes containing human Ste24 were also functional for N-terminal proteolytic activity in our in vitro assay (Fig. 8C, lane 1). In the presence of 1,10-orthophenanthrolite (1 or 10 mM), P1→P2 conversion is inhibited in reactions that were incubated for 5 min at 30 °C (Fig. 8C, lanes 2 and 3).

Longer incubation times resulted in an overall loss of both P1 and P2 in the absence of 1,10-orthophenanthrolite (Fig. 8C, lane 4), suggesting that human Ste24-containing membranes have more significant nonspecific protease activity as compared with yeast Ste24p-containing membranes (see Fig. 2, lane 1). At the longer incubation time, we observed conversion of P1 in the presence of 1 mM 1,10-orthophenanthrolite (Fig. 8C, lane 5) but not in the presence of 10 mM 1,10-orthophenanthrolite (Fig. 8C, lane 6). These results indicate that...
1,10-orthophenanthroline is less effective on human Ste24-dependent P1→P2 proteolytic activity than it is on yeast Ste24p-dependent activity, because the latter activity is inhibited at a lower concentration of 1,10-orthophenanthroline under identical reaction conditions (see Fig. 2, lane 2). The decreased effectiveness of 1,10-orthophenanthroline on human Ste24-dependent proteolytic activity is not simply due to increased levels of human Ste24 relative to yeast Ste24p in the reaction because human Ste24 is detected at lower steady-state levels by immunoblot as compared with the yeast Ste24p (data not shown). The above in vivo and in vitro data establish that the yeast P1 a-factor precursor can serve as a surrogate substrate for a human Ste24-dependent proteolytic activity. Because a human Ste24-dependent proteolytic activity has not been previously identified, the assay used here will prove useful for future analyses of the role of human Ste24.

**Concluding Remarks**—We have previously presented in vivo genetic data suggesting a role for Ste24p in the N-terminal (P1→P2) processing of the P1 a-factor precursor. In this study, we provide compelling in vitro evidence that supports the view that Ste24p is itself the P1 a-factor N-terminal protease or that it is a limiting upstream activator of a P1→P2 protease. We use a reconstituted in vitro system to show that proteolysis of the P1 a-factor precursor is dependent on Ste24p (Fig. 2) and that the extent of proteolysis is Ste24p dosage-dependent (Fig. 5). Furthermore, we provide evidence that Ste24p acts as a metalloprotease, because proteolysis requires zinc as a co-factor (Fig. 4), and mutagenesis of the Ste24p metalloprotease motif (HEXXH) results in inactivation of proteolytic activity (Fig. 3). Although this data is consistent with the view that Ste24p is the P1 a-factor N-terminal protease, we cannot formally exclude the possibility that Ste24p is instead acting an upstream activator of an as yet unidentified membrane-associated protease.

Our data, taken together with a previously published in vitro reconstitution of Ste24p-dependent CAAX cleavage, lead us to consider the possibility that Ste24p may participate directly in the CAAX processing and N-terminal proteolysis steps of the yeast a-factor biogenesis pathway. Because Ste24p-dependent cleavages occur at sites on the a-factor precursor that are spatially separated and not conserved in primary amino acid sequence, Ste24p may be a bifunctional protease that recognizes distinct cut sites. In this sense, Ste24p would be unique among known proteases. The ability to carry out both CAAX and a-factor N-terminal proteolysis in vitro will allow us to rigorously compare and contrast these two distinct Ste24p-dependent activities (12, 22, 23). Such studies may prove insightful toward the identification of other potential substrates.

In this study, we have also provided evidence that human Ste24, when expressed in yeast, is also able to perform dual roles in promoting the N- and C-terminal processing of a-factor. Whether the dual activities of human Ste24, which is expressed ubiquitously, are exhibited in human tissues remains to be determined, because a native substrate has yet to be identified. An intriguing question is whether human Ste24 acts solely to promote cleavage of human CAAX substrates and/or on a human a-factor-like peptide hormone that awaits discovery.

Here we have shown that the first N-terminal processing step in a-factor biogenesis can be reconstituted in vitro. This system represents a significant step toward the eventual biochemical characterization of this cleavage event with purified components. Notably, however, other proteolytic cleavage events that are dependent on multi-spanning membrane proteins, such as the Rce1p-dependent CAAX proteolysis and the cleavage of sterol regulatory element-binding proteins by the Site 2 protease, have to date been difficult to reconstitute in vitro with purified components. In vitro analyses of these cleavage events have relied on reconstitution systems that utilize crude membranes (12, 22–24). The reconstitution of the N-terminal processing of a-factor with purified components may be equally challenging. Nonetheless, the only way to rigorously characterize this proteolytic event is to purify the multispansing protein Ste24p in active form and to chemically synthesize the highly hydrophobic and posttranslationally modified P1 a-factor substrate. Our ability to reconstitute the N-terminal processing of a-factor using crude membranes prompts us to undertake this challenge.

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