Dual Roles for Ste24p in Yeast a-Factor Maturation: NH_2-terminal Proteolysis and COOH-terminal CAAX Processing

Amy Tam, Franklin J. Nouvet, Konomi Fujimura-Kamada, Hilda Slunt, Sangram S. Sisodia, and Susan Michaelis

Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract. Maturation of the *Saccharomyces cerevisiae* **a**-factor precursor involves COOH-terminal CAAX processing (prenylation, AAX tripeptide proteolysis, and carboxyl methylation) followed by cleavage of an NH_2-terminal extension (two sequential proteolytic processing steps). The aim of this study is to clarify the precise role of Ste24p, a membrane-spanning zinc metalloprotease, in the proteolytic processing of the **a**-factor precursor. We demonstrated previously that Ste24p is necessary for the first NH_2-terminal processing step by analysis of radiolabeled **a**-factor intermediates in vivo (Fujimura-Kamada, K., F.J. Nouvet, and S. Michaelis. 1997. *J. Cell Biol.* 136:271–285). In contrast, using an in vitro protease assay, others showed that Ste24p (Afc1p) and another gene product, Rce1p, share partial overlapping function as COOH-terminal CAAX proteases (Boyartchuk, V.L., M.N. Ashby, and J. Rine. 1997. *Science.* 275:1796–1800). Here we resolve these apparently conflicting results and provide compelling in vivo evidence that Ste24p indeed functions at two steps of **a**-factor maturation using two methods. First, direct analysis of **a**-factor biosynthetic intermediates in the double mutant (*ste24*Δ *rce1*Δ) reveals a previously undetected species (P0*) that fails to be COOH terminally processed, consistent with redundant roles for Ste24p and Rce1p in COOH-terminal CAAX processing. Whereas **a**-factor maturation appears relatively normal in the *rce1*Δ single mutant, the *ste24*Δ single mutant accumulates an intermediate that is correctly COOH terminally processed but is defective in cleavage of the NH_2-terminal extension, demonstrating that Ste24p is also involved in NH2-terminal processing. Together, these data indicate dual roles for Ste24p and a single role for Rce1p in **a**-factor processing. Second, by using a novel set of ubiquitin–**a**-factor fusions to separate the NH_2- and COOH-terminal processing events of **a**-factor maturation, we provide independent evidence for the dual roles of Ste24p. We also report here the isolation of the human (Hs) Ste24p homologue, representing the first human CAAX protease to be cloned. We show that Hs Ste24p complements the mating defect of the yeast double mutant (*ste24*Δ *rce1*Δ) strain, implying that like yeast Ste24p, Hs Ste24p can mediate multiple types of proteolytic events.

Key words: CAAX processing • posttranslational modification • metalloproteinases • prenylated protein precursor • yeast mating pheromone

Many proteins are synthesized as precursors that undergo one or more maturation steps to attain their full activity, to acquire proper localization, or to facilitate protein–membrane or protein–protein interactions. The *Saccharomyces cerevisiae* mating pheromone **a**-factor provides an excellent model for dissecting several distinct types of posttranslational modification events. Fully mature **a**-factor (M)[1] is a prenylated, carboxyl-methylated dodecamer that is initially synthesized as a precursor encoded by the functionally redundant genes, *MFA1* and *MFA2*. The **a**-factor precursor (P0) consists of the mature **a**-factor (12 residues) flanked by an NH_2-terminal extension (21 residues for Mfa1p) and a COOH-terminal CAAX motif (C, cys; A, an aliphatic res-

Address all correspondence to Susan Michaelis, Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205. Tel.: (410) 955-8286. Fax: (410) 955-4129. E-mail: susan_michaelis@qmail.bs.jhu.edu

K. Fujimara-Kamada's current address is Department of Molecular Biology, Faculty of Science, Nagoya University, Furo-cho Chikusa-ku, Nagoya, Japan 464-01.

H. Slunt's current address is Department of Neuropathology, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

S.S. Sisodia's current address is Department of Pharmacological and Physiological Science, University of Chicago, Chicago, IL 60637

1. *Abbreviations used in this paper*: E, extracellular; EST, expressed sequence tag; Hs, *Homo sapiens*; I, intracellular; M, mature; ORF, open reading frame; Sc, *Saccharomyces cerevisiae*; SD, synthetic minimal medium; Sp, *Schizosaccharomyces pombe*; Ubi, ubiquitin; Ubp, ubiquitin-specific protease; YPD, yeast extract/peptone/dextrose medium.

idue; X, one of several residues) (Michaelis and Herskowitz, 1988). A goal of our laboratory is to define the biosynthetic intermediates and cellular components necessary for each step of **a**-factor biogenesis. Our current view of **a**-factor maturation, derived from pulse-chase and SDS-PAGE analysis of **a**-factor biosynthetic intermediates, is summarized in Fig. 1. Our studies have shown that **a**-factor biogenesis occurs in three ordered stages: (*a*) COOH-terminal CAAX processing, (*b*) NH_2-terminal proteolysis comprised of a pair of successive cleavage events, and (*c*) export (Chen et al., 1997*b*).

In addition to **a**-factor, the COOH-terminal CAAX motif is present on a number of eukaryotic proteins. Examples include nuclear lamins of multicellular organisms, the γ subunit of a heterotrimeric G protein, and most notably Ras proteins and the Ras-related Rho proteins (for reviews see Clarke, 1992; Schafer and Rine, 1992; Zhang and Casey, 1996). The CAAX motif directs three sequential posttranslational modification steps: (*a*) prenylation of the cysteine by farnesyl or geranylgeranyl, (*b*) proteolytic cleavage to remove the AAX residues (herein referred to as AAXing), and (*c*) carboxyl methylation of the newly exposed prenyl cysteine (Clarke, 1992; Schafer and Rine, 1992; Zhang and Casey, 1996). The CAAX modifications confer distinctive properties to the processed protein. For Ras, the prenyl group is required for its activity and more importantly, for its transforming activity (Casey et al., 1989; Schafer et al., 1989; Kato et al., 1992). Likewise for **a**-factor, the farnesyl group facilitates membrane association before export and is necessary for promoting growth arrest and mating activity by extracellular **a**-factor. Removal of the AAX tripeptide is required for methylation, and in turn, methylation of **a**-factor is important for its intracellular stability, export, and receptor interaction (He et al., 1991; Marcus et al., 1991; Sapperstein et al., 1994).

In *S. cerevisiae,* the genetic analysis of mutants defective in **a**-factor biogenesis has facilitated the identification and characterization of the yeast CAAX processing components. These enzymes include (*a*) the farnesyl transferase complex (Ram1p/Ram2p); (*b*) the CAAX prenyl proteases that carry out AAXing (Rce1p or Ste24p [see below]); and (*c*) the carboxyl methyltransferase (Ste14p) (Hrycyna and Clarke, 1990; Schafer et al., 1990; He et al., 1991; Boyartchuk et al., 1997). The genes encoding mammalian CAAX processing enzymes that have been cloned to date are the rat, bovine, and human farnesyltransferases; the rat and human geranylgeranyltransferase; and the human prenyl protein carboxylmethyltransferase (Kohl et al., 1991; Andres et al., 1993; Zhang, 1994 [no. 1610]; Dai et al., 1998). In contrast, no mammalian CAAX protease genes have as yet been identified.

For **a**-factor, COOH-terminal CAAX processing is only the first stage of its maturation. CAAX processing of the **a**-factor precursor (P0) produces an intermediate (P1) that is completely modified at the COOH terminus (farnesylated, AAXed, and carboxyl methylated) but contains an intact NH_2-terminal extension (see Fig. 1). The NH_2-terminal extension is removed in two sequential and obligatorily ordered NH_2-terminal proteolytic cleavages, the first one yielding the partially processed precursor (P2), and the second cleavage generating fully mature **a**-factor (M). We recently showed that Ste24p is required for the first NH_2-terminal proteolytic step (P1→P2 processing) (Fujimura-Kamada et al., 1997), as discussed below. The second step (P2→M) is mediated by Axl1p and can also be carried out redundantly by Ste23p (Adames et al., 1995). Upon completion of the NH_2-terminal processing steps, mature **a**-factor is exported from the cell via the Ste6p transporter, a member of the ATP-binding cassette superfamily (Kuchler et al., 1989; McGrath and Varshavsky, 1989; Michaelis, 1993).

The aim of this study is to clarify the role of Ste24p, a predicted multiple membrane-spanning zinc metalloprotease, in the biogenesis of **a**-factor. Interestingly, the *STE24* gene was identified by two independent genetic screens that assigned different functions for Ste24p in **a**-factor maturation (Boyartchuk et al., 1997; Fujimura-Kamada et al., 1997). Our laboratory isolated *STE24* as a mating-defective mutant (hence the designation *ste*) in a screen specifically aimed at identifying mutants with reduced mating efficiency (Fujimura-Kamada et al., 1997). A *ste24* mutant accumulates the **a**-factor intermediate P1 in vivo. Since P1 is fully COOH terminally modified but its NH_2-terminal extension is not proteolytically removed in the *ste24* mutant, we concluded that Ste24p is required for the first NH_2-terminal processing step (P1→P2) of **a**-factor maturation. In a separate screen using a mutant version of **a**-factor with an altered CAAX motif (CAMQ instead of CVIA), Boyartchuk et al. (1997) also identified *STE24* (called *AFC1* in their study, **a**-factor converting enzyme). Using an in vitro assay for release of the AAX tripeptide, *ste24* mutants showed reduced AAXing activity. Boyartchuk et al. (1997) concluded that Ste24p and a second functionally redundant protein, Rce1p, share overlapping roles in the COOH-terminal AAXing step of **a**-factor maturation. Rce1p, which is predicted to contain multiple membrane spans, bears no sequence similarity to Ste24p, and lacks any known protease motifs. Although *STE24* was identified in genetic screens based on defective extracellular **a**-factor production, the two reports reached surprisingly different conclusions regarding the role of Ste24p in **a**-factor maturation. Likely explanations for the divergent findings are that Boyartchuk et al. (1997) examined only COOH-terminal processing in their in vitro AAXing assay and our study did not detect an AAXing defect in vivo for the single *ste24* mutant because AAXing can be carried out redundantly by Rce1p.

In this study, we reconcile the apparently conflicting data for the roles of Ste24p in **a**-factor processing. We examine both NH_2- and COOH-terminal processing of **a**-factor in vivo in strains deleted for *STE24* and *RCE1*. By directly analyzing the **a**-factor biosynthetic intermediates produced by the mutant strains, we provide evidence that Ste24p indeed participates in both NH_2- and COOH-terminal processing steps. In contrast, Rce1p is involved only in CAAX processing, not NH_2-terminal cleavage of **a**-factor. We also use an independent method employing ubiquitin (Ubi)–**a**-factor fusions that uncouple the NH_2- and COOH-terminal processing steps to demonstrate the dual roles for Ste24p in **a**-factor maturation. Finally, we report here the cloning of the human Ste24p homologue, the first mammalian CAAX prenyl protease. We show that Hs Ste24p can complement the yeast double deletion (*ste24 rce1*) strain for mating.

Materials and Methods

Strains and Media

The yeast strains used in this study are listed in Table I. The *rce1-Δ1::TRP1* deletion allele, referred to as *rce1Δ*, replaces codons 116 to the stop codon 315 with *TRP1*. Strains (SM3613, SM3614, SM3689, and SM3691) harboring the *rce1Δ* allele were constructed using one-step gene disruption by transforming SM1058, SM3103, SM2331, and SM3375, respectively, with a BamHI-XhoI fragment from pSM1285 bearing *rce1-Δ1::TRP1*, and selecting for Trp+ transformants. The *ste24Δ::LEU2* deletion allele, referred to as *ste24Δ*, is a γ disruption of *ste24* that eliminates nearly the entire coding sequence (codons 1–444 of 453 total). Strains (SM3375 and others) harboring this allele were constructed by transformation of SM2331 with linearized pSM1072 and selection of Leu^+ transformants, as described (Fujimura-Kamada et al., 1997). All deletion strains were confirmed by Southern analysis. *MAT*α strains bearing the single (*rce1*Δ) and the double (*ste24Δ rce1Δ*) deletions were constructed by mating-type switching of *MAT***a** strains SM3613 and SM3614, respectively, using the HO endonuclease as described (Herskowitz and Jensen, 1991). Yeast transformations were performed by the Elble method (Elble, 1992). All strains were grown at 30°C in complete yeast extract/peptone/dextrose (YPD) media, synthetic complete drop-out (SC-URA, TRP, LEU), or synthetic minimal media (SD) (Michaelis and Herskowitz, 1988; Kaiser et al., 1994).

Patch Mating Test and a-Halo Assay

To assay mating, we used the semiquantitative patch mating test as described previously (Fujimura-Kamada et al., 1997). In this assay, master plates containing patches of strains to be tested are replica plated onto a mating tester lawn of the opposite mating type that has been spread on an SD plate. The lawn is prepared by resuspending the mating tester strain in YPD and spreading 0.3 ml of this suspension onto an SD plate. The stringency for the patch mating test was increased by resuspending the mating tester lawns in decreasing concentrations of YPD diluted in sterile water (100% YPD is permissive, 1% YPD is stringent) since limiting nutrients decrease plate mating efficiency, and thereby increase the mating test sensitivity. The strains tested are Trp^-, Leu^-, or His^- auxotrophs, whereas the mating testers (SM1067 and SM1068) are Lys^-. Only cells that mate to generate prototrophic diploids are able to grow on SD minimal media after incubation for 2 d at 30°C.

To compare the level of extracellular **a**-factor for wild-type and mutant strains, we performed the **a**-factor spot halo assay as described previously (Nijbroek and Michaelis, 1998). Serial dilutions of concentrated **a**-factor (2 μl) were spotted onto a lawn of supersensitive halo tester cells (SM1086) spread on a YPD plate. Plates were incubated for 1 d at 30°C.

Plasmid Constructions

The plasmids used in this study are listed in Table II. The single-step gene disruption plasmid, pSM1285, used to generate *rce1-Δ1::TRP1* alleles, was constructed as follows: A HindIII-MluI fragment containing the *RCE1* open reading frame (ORF) from pHY01 (provided by A. Toh-e, University of Tokyo, Tokyo, Japan) (Yashiroda et al., 1996) was rendered blunt-ended with Klenow and subcloned into the EcoRV-SmaI sites of pBluescriptIISK (Stratagene, La Jolla, CA) to yield pSM1284. Plasmid pSM1284 was digested with EcoRI to remove a fragment corresponding to the last 200 codons of the *RCE1* ORF, which was replaced with a *TRP1* EcoRI fragment from pUC18-TRP1 (Sapperstein et al., 1994) to generate pSM1285.

Ubi–**a**-factor fusion constructs encode chimeric proteins consisting of ubiquitin (76 residues) fused either to the full-length **a**-factor precursor encoded by *MFA1*, to the NH_2 terminally truncated **a**-factor (P2) (see Fig. 1), or to mature **a**-factor (M). All of these fusions contain the intact COOH-terminal CAAX motif and are expressed under the control of the *MFA1* promoter. These constructs are designated Ubi-P1, Ubi-P2, and Ubi-M, where the **a**-factor segments correspond to codons 1–36 (full-length), codons 8–36, and codons 21–36 of *MFA1*-encoded **a**-factor, respectively. The fusion constructs were generated by recombination-mediated PCR cloning, a method in which a linearized or gapped acceptor plasmid serves as the target for homologous recombination directed by a donor PCR fragment in yeast (Muhlrad et al., 1992; Oldenburg et al., 1997). The PCR fragments were amplified with oligonucleotides encoding precise fusion junctions between the COOH terminus of ubiquitin and the NH_2 terminus of the various species of **a**-factor.

The linear target vector and the donor PCR fragment were cotransformed into a strain deleted for the chromosomal **a**-factor genes *mfa1Δ* and *mfa2Δ* (SM2331). Subsequently, candidate plasmids were screened by yeast colony PCR. In brief, crude yeast extracts were prepared by incubating a small amount of yeast cells in 60 μl of lysis buffer (0.45% NP-40, 0.45% Tween 20, 50 mM KCl, 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$, 0.1% gelatin, 0.3 mg/ml zymolyase) for 90 min at 37°C. The cleared lysate (5 μl) was used as the template for the PCR screening. Plasmids were recovered from yeast as described (Robzyk and Kassir, 1992). Ubi-P1, Ubi-P2, and Ubi-M are encoded by pSM1368, pSM1369, and pSM1366, respectively. All Ubi-**a**-factor fusion plasmids were amplified in *Escherichia coli* strain DH5α, prepared by alkaline lysis, analyzed by restriction digests, and then confirmed by DNA sequencing.

Metabolic Labeling, Immunoprecipitation, and SDS-PAGE

To examine **a**-factor biosynthetic intermediates, extracts were prepared from metabolically labeled cells and radiolabeled proteins were immunoprecipitated with anti–**a**-factor antiserum and analyzed by SDS-PAGE, essentially as previously described (Chen et al., 1997*b*; Fujimura-Kamada et al., 1997). In brief, 5 OD_{600} U log phase cells were harvested and resuspended in 250 μl of SD media supplemented with the appropriate amino acids. Cells were pulse labeled with 150 μCi [^{35}S]cysteine for the indicated times, and the labeling was stopped on ice by the addition of an equal volume of 2× azide buffer (40 mM methionine, 40 mM cysteine, 20 mM NaN_3, 500 mg/ml BSA). For pulse-chase experiments, chases were initiated by addition of 10 μl of 1 M cysteine per time point. Intracellular (I) and extracellular (E) fractions were processed as described (Chen et al., 1997*b*; Fujimura-Kamada, et al., 1997). Radiolabeled **a**-factor was immunoprecipitated with anti–**a**-factor antiserum 9-137 or 9-497, and the immunoprecipitated material was subjected to SDS-PAGE and PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) analysis as described (Chen et al., 1997*b*). To maximize the resolution between partially processed **a**-factor precursors, we used 16% polyacrylamide separating gels that were 13 cm in length. The half-life of each **a**-factor precursor (P0* or P1) was determined by quantitation of the [^{35}S] counts corresponding to the **a**-factor signal using ImageQuant software (Molecular Dynamics, Inc.). The 0 min time point was used as the 100% reference value for each time course experiment.

a-Factor Carboxyl Methylation Assay

The carboxyl methylation levels of the immunoprecipitated **a**-factor intermediates that had been cut out of a dried polyacrylamide gel were measured (Fujimura-Kamada et al., 1997). In general, the procedure involves generating double-labeled **a**-factor with [^{3}H] at the carboxyl methyl group and with [^{35}S] at the prenyl cysteine. For each **a**-factor species (P0*, P1, P2, or M), the ratio of [^{3}H]/[^{35}S] cpm reflects the absolute methylation level. The relative methylation level is then determined by dividing the absolute methylation level of the mutant strain by that of the wild-type strain and converting this number to a percentage value.

Specifically, this procedure was carried out in two steps. First, cells were radiolabeled, immunoprecipitated, and subjected to SDS-PAGE, as described in the previous section, with the following changes: cells were double-labeled with 50 μCi S-*adenosyl*-L-[^{3}H-methyl]methionine and 150 μCi [^{35}S]cysteine for 6 min. For this assay, labeled cells were not lysed by base treatment since base hydrolysis could release the methyl esters which we wish to detect in this assay (see below). Instead, protein extracts were prepared by vortexing labeled cells at 4°C with zirconium beads in breaking buffer (50 mM potassium phosphate buffer, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM 2-mercaptoethanol, 1 mM PMSF). After separation by SDS-PAGE, the radiolabeled and immunoprecipitated **a**-factor intermediates were visualized by autoradiography.

Second, to determine the extent of carboxyl methylation for each **a**-factor intermediate, we measured the levels of [^{3}H] and of [^{35}S] incorporation by scintillation counting. The **a**-factor gel bands were excised from dried gels. The [^{3}H] labeling was determined by the vapor-phase diffusion assay, which detects volatile [^{3}H]methyl esters (i.e., carboxyl methyl) cleaved by base hydrolysis (Xie et al., 1990; Hrycyna et al., 1991). Gel slices (P0*, P1, P2, and M) were placed in an open microfuge tube containing 150 μl of 1 M NaOH. The microfuge tube was placed inside a tightly capped 20 ml scintillation vial that contained 5 ml of scintillation fluid such that the contents

of the microfuge tube did not mix with the scintillation fluid. After incubation at 37°C for 24–36 h, the released volatile [^{3}H]methanol was measured by scintillation counting. The total amount of [^{35}S] incorporated into each **a**-factor species was then determined. First, the contents of the microfuge tube (including the gel slice) were neutralized with 100 μl of glacial acetic acid and then dissolved in 1 ml of Solvable (New England Nuclear Research Products, Boston, MA), a tissue solubilizer, in capped microfuge tubes at 65°C for 6 h. The [^{35}S] counts of the entire sample were then measured by liquid scintillation counting. The ratio of [^{3}H] cpm to [^{35}S] cpm was calculated for each **a**-factor species to give the absolute methylation level. The relative methylation level was determined by dividing the absolute methylation level of the mutant strain by the absolute methylation level of the corresponding wild-type **a**-factor species.

Human STE24 Cloning

A BLAST search (Altschul et al., 1990) of the database of expressed sequence tags (ESTs) revealed several human ESTs with high amino acid similarity to *S. cerevisiae* (Sc) Ste24p. These ESTs represented cDNAs from many cell types. A 703-bp HindIII fragment from EST N76181 National Center for Biotechnology Information (NCBI) (no. 69928) was used to screen ~3 × 10^6 clones from a human B cell cDNA library (provided by S. Elledge, Baylor College of Medicine, Houston, TX) by standard methods (Ausubel, 1987). Nine positive clones were obtained and sequenced, each of which encoded partial ORFs corresponding to the 3′ end of human *STE24*. The most complete *Homo sapiens* (Hs) clone contained an ORF encoding 383 residues and included the poly A tail. To obtain the remaining 5′ Hs *STE24* ORF, this clone was used in 5′ rapid amplification of cDNA ends (RACE) PCR procedure with a fetal brain library according to the Marathon-Ready cDNA kit (Clontech, Palo Alto, CA). The full-length *STE24* ORF was subcloned by recombination-mediated PCR cloning into a yeast plasmid such that the coding sequence of Sc *STE24* was precisely replaced with that of Hs *STE24*. The resulting plasmid (pSM1468) contains the 5′ upstream and 3′ downstream sequences of the yeast *STE24* ORF flanking the human *STE24* ORF.

Results

Mating Phenotype and Extracellular a-Factor Production by ste24Δ, rce1Δ, and ste24Δ rce1Δ Mutants

To examine the roles of Ste24p and Rce1p in **a**-factor maturation, we first compared the extracellular **a**-factor produced from strains deleted for *STE24* and *RCE1*. We used the spot halo dilution assay, which provides a semiquantitative measure of the amount of mature **a**-factor secreted by these strains. In this assay, extracellular **a**-factor causes the growth arrest of a lawn of supersensitive *MAT*α *sst2* cells, resulting in a clear zone (Michaelis and Herskowitz, 1988; Nijbroek and Michaelis, 1998). The relative amounts of **a**-factor produced by the different strains are compared by determining the highest dilution that yields a clear spot (Fig. 2 *A*). Whereas the *ste24Δ* mutant produces significantly decreased levels of extracellular **a**-factor (eightfold less than wild-type), the *rce1Δ* mutant is virtually indistinguishable from wild-type (Fig. 2 *A*). In contrast, we detect no extracellular **a**-factor for the double *ste24Δ rce1Δ* mutant. This result is consistent with the notion that Ste24p and Rce1p play redundant roles in the COOH-terminal AAXing step of **a**-factor maturation, such that only one gene must be intact for AAXing to occur, as proposed by Boyartchuk et al. (1997). However, the more severe phenotype of the *ste24Δ* single mutant versus the *rce1Δ* single mutant most likely reflects the additional role of Ste24p in the NH_2-terminal processing of **a**-factor.

A comparison of the *MAT***a** strains in a plate mating assay indicates that their mating efficiencies parallel the results of the **a**-factor spot dilution assay. As shown in Fig. 2 *B*, the *rce1Δ* mutant mates like wild-type as indicated by the confluent growth of diploids, whereas the *ste24Δ* mutant has a leaky mating defect as indicated by growth of fewer diploids. The double mutant fails to mate altogether, consistent with its complete lack of extracellular **a**-factor production (Fig. 2 *B*). Importantly, the mating defects exhibited by these mutants is *MAT***a** cell type-spe-

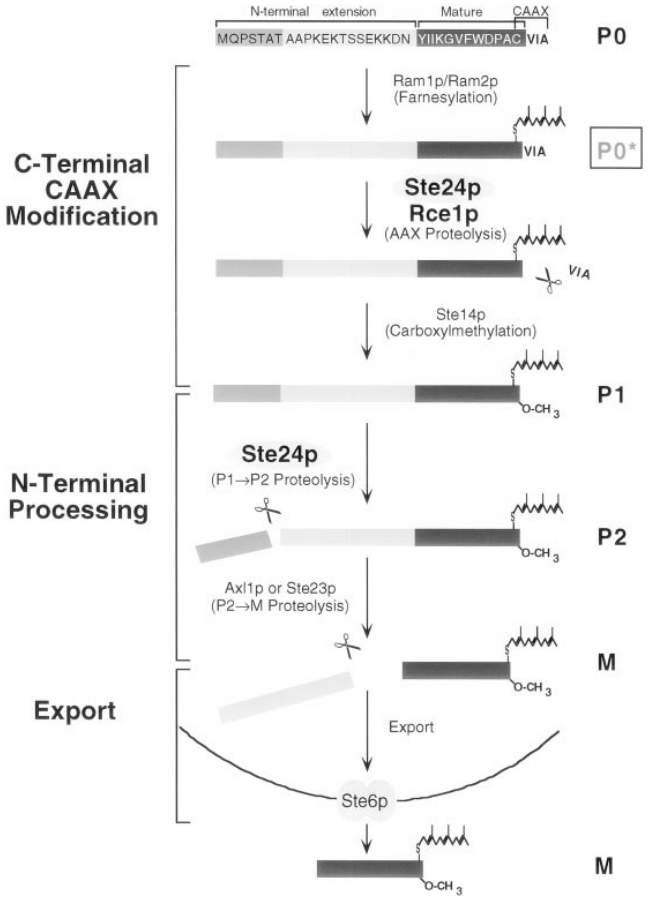

Figure 1. Pathway of **a**-factor biogenesis. The **a**-factor precursor, biosynthetic intermediates, and components of the biogenesis machinery are shown. The biogenesis of **a**-factor is comprised of three stages, indicated at left: (*a*) COOH-terminal CAAX processing, (*b*) two sequential NH_2-terminal proteolytic cleavages, and (*c*) export. The **a**-factor precursor (*P0*, *top*) consists of the mature, bioactive portion (*black*) flanked by an NH_2-terminal extension (*two shades of gray*) and a COOH-terminal CAAX motif. Mature **a**-factor is a prenylated and carboxyl methylated dodecapeptide (*M*, *bottom*). The known **a**-factor biosynthetic intermediates that can be directly visualized by SDS-PAGE are designated P0, P1, P2, and M (Chen et al., 1997*b*). In this study, we present the previously undetected intermediate P0*. The cellular components that mediate COOH-terminal CAAX modification events (prenylation, AAX proteolysis, carboxyl methylation) are the Ram1p/Ram2p complex, Rce1p and Ste24p, and Ste14p, respectively (refer to text for more details). NH_2-terminal processing involves two successive proteolytic steps. The first NH_2-terminal cleavage step (P1→P2) is mediated by Ste24p (as confirmed by this study), and the second step (P2→M) requires Axl1p or Ste23p. The dual roles of Ste24p in COOH-terminal AAXing and the first NH_2-terminal (P1→P2) processing step is the subject of this study.

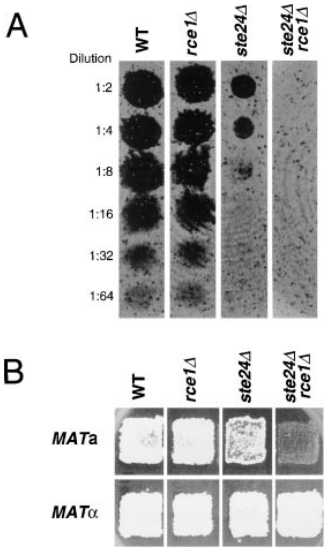

Figure 2. STE24 and RCE1 are required for the efficient production of **a**-factor by *MAT***a** cells. (*A*) **a**-Factor spot dilution assay. The **a**-factor–induced growth arrest of a *MAT*α supersensitive (*sst2*) tester lawn results in a clear zone or spot. Dilutions of concentrated, extracellular **a**-factor produced by a wild-type strain, the single (*rce1Δ* and *ste24Δ*) and double (*ste24Δ rce1Δ*) mutants were prepared as described in the Materials and Methods and spotted onto a lawn of the *MAT*α halo tester (SM1086) on YPD. The highest dilution yielding a clear spot is the titer of **a**-factor for that strain. The papillae arising in the clear spots are revertants arising from the *sst2* lawn (Chan and Otte, 1982). (*B*) Patch mating assay. Strains bearing the single and double *rce1Δ ste24Δ* mutations were tested for the ability to mate with cells of the opposite mating type. Patches of the *MAT***a** or *MAT*α strains to be tested for mating were replica-plated from a patch master plate onto a lawn of auxotrophic mating tester cells spread on minimal SD media. The growth of prototrophic diploids is indicative of mating. *MAT*α strains do not exhibit mating defects even under stringent mating conditions (5% YPD) (data not shown). *MAT***a** strains tested are wild-type (SM1058), *rce1Δ* (SM3613), *ste24Δ* (SM3103), and *rce1Δ ste24Δ* (SM3614); the isogenic *MAT*α strains tested are wild-type (SM1059), *rce1Δ* (SM3811), *ste24Δ* (SM3102), and *rce1Δ ste24Δ* (SM3812). The mating tester lawns are *MAT*α (SM1068) and *MAT***a** (SM1067) and were resuspended in 5% YPD and 50% YPD, respectively, as described in the Materials and Methods.

cific, since *MAT*α strains bearing either the single (*ste24Δ*) or double (*ste24Δ rce1Δ*) mutations did not have any mating defects (Fig. 2 *B*). Because *MAT***a** and *MAT*α cells differ mainly by pheromone and receptor expression, the *MAT***a** mating defects seen in the *ste24Δ* and *ste24Δ rce1Δ* mutants are likely to reflect specific effects on **a**-factor biogenesis.

A Novel a-Factor Intermediate (P0) Is Observed in the Double ste24Δ rce1Δ Mutant*

To directly examine the roles of *STE24* and *RCE1* in **a**-factor maturation, we compared the **a**-factor biosynthetic intermediates produced in strains deleted for *STE24* and *RCE1*. Cells were metabolically labeled, and the **a**-factor biosynthetic intermediates were immunoprecipitated and separated by SDS-PAGE. For the wild-type strain, the typical profile of the intracellular (I) forms of **a**-factor includes the partially processed precursor species (P1 and P2) and mature (M) **a**-factor (Fig. 3 *A*, lane *1*; refer to Fig. 1 for a description of each band). The band migrating slightly faster than M is the **a**-factor–related peptide (AFRP), whose biogenesis involves mechanisms and machinery distinct from those used to generate mature **a**-factor (Chen et al., 1997*a*). The extracellular (E) fraction contains exported mature (M) **a**-factor (Fig. 3 *A*, lane *1*). For the *rce1Δ* mutant, the biosynthetic profile is similar to wild-type (Fig. 3 A, lane *2*). Because the stepwise removal of the NH_2-terminal extension appears to be normal in the *rce1Δ* mutant, Rce1p does not play a major role in NH_2-terminal processing of **a**-factor. Furthermore, this apparently normal processing in the *rce1Δ* mutant is expected if Ste24p and Rce1p play redundant functions in COOH-terminal AAXing of **a**-factor (see below), since Ste24p would compensate for the lack of Rce1p. In contrast, the *ste24Δ* mutant has a dramatic phenotype of P1 accumulation (Fig. 3 *A*, lane *3*), as we have also shown previously (Fujimura-Kamada et al., 1997). In the *ste24Δ* mutant, COOH-terminal AAXing is complete but the cleavage of the NH_2-terminal extension is defective, indicating that Ste24p is necessary for the first NH_2-terminal cleavage step (P1→P2) in **a**-factor maturation (see also Fig. 3 *B*). Notably, only a tiny amount of mature **a**-factor produced by the *ste24Δ* mutant is detected in the extracellular fraction by SDS-PAGE and autoradiography after a long exposure (Fujimura-Kamada et al., 1997); the more sensitive spot halo assay detects this low level of extracellular mature **a**-factor (refer to Fig. 2 *A*).

If Ste24p does indeed function redundantly in COOH-terminal AAXing with Rce1p, then we predict that the double mutant (*ste24Δ rce1Δ*) would be completely defective for **a**-factor AAXing. Such a processing block should produce a new intermediate (P0*) that is prenylated but retains its AAX and thus cannot be methylated (refer to Fig. 1). In the double mutant (*ste24Δ rce1Δ*), we observe a single band, designated P0*, that shows a subtle mobility shift compared with P1 (Fig. 3 *A*, compare lane *4* with *3*). Since this slight mobility difference is difficult to detect consistently, we used a methylation assay, described below, to show that the intermediate generated by the double mutant is truly distinct from P1.

Methylation can serve as an indirect AAXing assay, since AAXing is required to expose the methylation substrate, the free carboxyl of prenyl cysteine (Hrycyna and Clarke, 1992; Ashby and Rine, 1995; Hrycyna et al., 1995). We measured the degree of carboxyl methylation for each intracellular **a**-factor species made in wild-type and mutant strains. Cells were metabolically double-labeled with S-*adenosyl*-L-[^{3}H-methyl]methionine and [^{35}S]cysteine, which label the COOH-methyl and prenyl cysteine of **a**-factor, respectively. Methylation levels were first normalized to protein levels ([^{3}H]/[^{35}S]) and then calculated as a fraction of the corresponding **a**-factor intermediate from the wild-type strain (refer to Materials and Methods). As we have previously shown, the P1 intermediate produced by the *ste24Δ* mutant is completely methylated relative to the methylation levels of the wild-type strain (Fig. 3 *B*) (Fujimura-Kamada et al., 1997), indicating that this strain has AAXing activity, presumably mediated by Rce1p. In the *rce1Δ* mutant, we also detect a significant amount of methylation of P2 and M, although the levels are somewhat reduced, and the slowest migrating species (Fig. 3 *B*, *top band*) is not detectably methylated (see below). The methylation that occurs in the *rce1Δ* mutant most likely reflects the AAXing activity mediated by Ste24p. In contrast to the single mutants, the double *ste24Δ rce1Δ* mutant produces a single intermediate that is completely unmethylated (Fig.

Table I. S. cerevisiae Strains Used in This Study

Strain	Relevant genotype*	Reference
SM1058	*MAT***a** *trp1 leu2 ura3 his4 can1*	Michaelis and Herskowitz, 1988
SM1059	*MAT*α *trp1 leu2 ura3 his4 can1*	Michaelis and Herskowitz, 1988
SM1067	*MAT***a** *cry1 lys1*	Michaelis and Herskowitz, 1988
SM1068	*MAT*α *lys1*	Michaelis and Herskowitz, 1988
SM1086	*MAT*α *sst2-1 his6 met1 can1 cyh2*	Michaelis and Herskowitz, 1988
SM1871	*MAT***a** *ste14:: TRP1 [CEN URA3 MFA1]*	Sapperstein et al., 1994
SM2331	*MAT***a** *mfa1-Δ1 mfa2-Δ1*	Chen et al., 1997
SM3102	*MAT*α *ste24Δ::LEU2*	Progeny of SM3095 (Fujimura-Kamada, et al., 1997)
SM3103	*MAT***a** *ste24Δ::LEU2*	Fujimura-Kamada et al., 1997
SM3286	*MAT***a** *ste24Δ::LEU2 [CEN URA3 MFA1]*	Fujimura-Kamada et al., 1997
SM3310	*MAT***a** *[CEN URA3 MFA1]*	Transformant of SM1058 with pSM233
SM3375	*MAT***a** *ste24Δ::LEU2 mfa1-Δ1 mfa2-Δ1*	This study
SM3613	*MAT***a** *rce1Δ::TRP1*	This study
SM3614	*MAT***a** *rce1Δ::TRP1 ste24D::LEU2*	This study
SM3644	*MAT***a** *rce1Δ::TRP1 [CEN URA3 MFA1]*	Transformant of SM3613 with pSM233
SM3650	*MAT***a** *rce1Δ::TRP1 ste24Δ::LEU2 [CEN URA3]*	Transformant of SM3614 with pSM316
SM3651	*MAT***a** *rce1Δ::TRP1 ste24Δ::LEU2 [CEN URA3 MFA1]*	Transformant of SM3614 with pSM233
SM3653	*MAT***a** *rce1Δ::TRP1 ste24Δ::LEU2 [CEN URA3 STE24]*	Transformant of SM3614 with pSM1093
SM3683	*MAT***a** *mfa1-Δ1 mfa2-Δ1 [CEN URA3 MFA1]*	Transformant of SM2331 with pSM1036
SM3684	*MAT***a** *mfa1-Δ1 mfa2-Δ1 [CEN URA3 UBI-M]*	Transformant of SM2331 with pSM1366
SM3685	*MAT***a** *mfa1-Δ1 mfa2-Δ1 [CEN URA3 UBI-P1]*	Transformant of SM2331 with pSM1368
SM3686	*MAT***a** *mfa1-Δ1 mfa2-Δ1 [CEN URA3 UBI-P2]*	Transformant of SM2331 with pSM1369
SM3689	*MAT***a** *rce1Δ::TRP1 mfa1-Δ1 mfa2-Δ1*	This study
SM3691	*MAT***a** *rce1Δ::TRP1 ste24Δ::LEU2 mfa1-Δ1 mfa2-Δ1*	This study
SM3714	*MAT***a** *ste24Δ::LEU2 mfa1-Δ1 mfa2-Δ1 [CEN URA3 MFA1]*	Transformant of SM3375 with pSM1036
SM3715	*MAT***a** *ste24Δ::LEU2 mfa1-Δ1 mfa2-Δ1 [CEN URA3 UBI-P1]*	Transformant of SM3375 with pSM1368
SM3716	*MAT***a** *ste24Δ::LEU2 mfa1-Δ1 mfa2-Δ1 [CEN URA3 UBI-P2]*	Transformant of SM3375 with pSM1369
SM3721	*MAT***a** *rce1Δ::TRP1 mfa1-Δ1 mfa2-Δ1 [CEN URA3 UBI-P2]*	Transformant of SM3689 with pSM1369
SM3726	*MAT***a** *rce1Δ::TRP1 ste24Δ::LEU2 mfa1-Δ1 mfa2-Δ1 [CEN URA3 UBI-P2]*	Transformant of SM3691 with pSM1369
SM3811	*MAT*α *rce1Δ::TRP1*	This study
SM3812	*MAT*α *rce1Δ::TRP1 ste24Δ::LEU2*	This study
SM3814	*MAT***a** *rce1Δ::TRP1 ste24Δ::LEU2 [CEN URA3 Hs STE24]*	Transformant of SM3614 with pSM1468

*All strains are isogenic to SM1058 (*trp1 leu1 ura3 his4 can1*) with the exception of SM1067, SM1068, and SM1086.

3 *B*), and thus represents P0*, a prenylated but unAAXed biosynthetic intermediate of **a**-factor. We previously have not detected P0* probably because it is converted to P1 very quickly. Unexpectedly, the *rce1Δ* mutant produces a precursor species (Fig. 3 A, lane *2*, *top band*) that is likely to be P0* because it is unmethylated (Fig. 3 *B*). In this case, and in contrast to P0* of the double *ste24Δ rce1Δ* mutant, P0* in the *rce1Δ* mutant is converted to P2 and then to M. A likely explanation is that when Ste24p recognizes P0* in the *rce1Δ* mutant, the dual roles of Ste24p permit it to cleave P0* at both the NH_2 and COOH termini to generate a P2-like intermediate that is subsequently methylated. Our conclusions from this analysis of **a**-factor intermediates generated in vivo are consistent with AAXing roles for both Ste24p and Rce1p, and an additional NH_2-terminal processing role for Ste24p only.

Table II. Plasmids Used in This Study

Plasmid	Genotype	Reference
pConstructXIV	*CEN TRP1 UBI4-DHFR-HA*	Johnsson and Varshavsky, 1994
pGAL-*HO*	*CEN URA3 GAL-HO*	Herskowitz and Jensen, 1991
pHY01	YEp24 *URA3 RCE1*	Yashiroda et al., 1996
pRS316	*CEN URA3*	Sikorski and Hieter, 1989
pSM233	*CEN URA3 MFA1*	Chen et al., 1997*b*
pSM236	*CEN URA3 MFA1;* BamHI site inserted at codon 17	This study
pSM1036	*CEN URA3 MFA1*	This study
pSM1093	*CEN URA3 STE24*	Schmidt et al., 1998
pSM1284	Bluescript-*RCE1*	This study
pSM1285	*rce1-Δ1::TRP1* (*Δ116–315*)	This study
pSM1366	*CEN URA3 UBI-M*	This study
pSM1368	*CEN URA3 UBI-P1*	This study
pSM1369	*CEN URA3 UBI-P2*	This study
pSM1468	*CEN URA3 Hs STE24*	This study
pUC18-TRP1	*TRP1* EcoRI fragment	Sapperstein et al., 1994

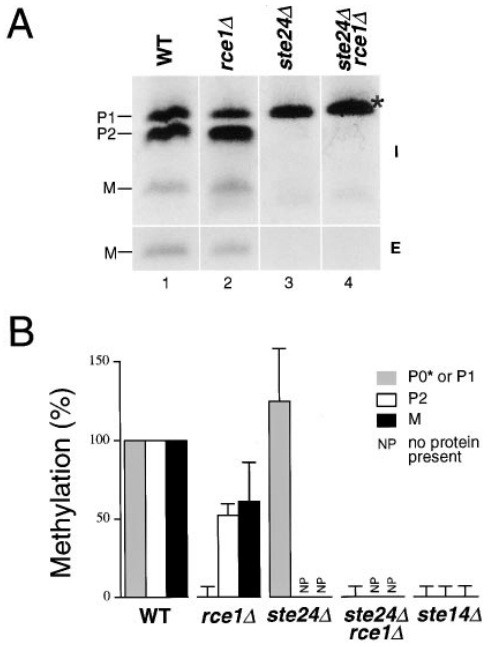

Figure 3. The *rce1Δ ste24Δ* double mutant produces a novel **a**-factor intermediate, P0*, that is unmethylated. (*A*) To examine the **a**-factor biogenesis profile in wild-type and mutant strains, cells were metabolically labeled with [^{35}S]cysteine for 5 min. Intracellular (*I*) and extracellular (*E*) fractions were separated by centrifugation and extracts were prepared as described in the Materials and Methods. Protein extracts were immunoprecipitated with anti–**a**-factor antiserum no. 9-137, and subjected to SDS-PAGE and PhosphorImager analysis. The previously characterized precursor species (*P1*, *P2*) and mature **a**-factor (*M*) are shown. The band migrating slightly faster than M is the **a**-factor–relabeled peptide (AFRP) and is discussed elsewhere (Chen et al., 1997*a*). The asterisk (*) marks the new **a**-factor intermediate P0* (refer to Fig. 1). (*B*) Carboxyl methylation levels of **a**-factor were used as an indirect measure of AAXing activity in wild-type and mutant strains. Cells were double-labeled with S-*adenosyl*-L-[^{3}H-methyl]methionine and [^{35}S]cysteine for 6 min. Total cell extracts were prepared, immunoprecipitated with anti–**a**-factor antiserum 9-137, and then subjected to SDS-PAGE and autoradiography. Bands representing each **a**-factor species (*P0** or *P1*, *P2*, and *M*) were excised from the dried gel. The degree of methylation for each band was measured by determining the [^{3}H]/[^{35}S] cpm ratio. The relative methylation level was then calculated as a fraction of the corresponding species from the wild-type strain (100%). The *ste14Δ* strain is a control that lacks carboxyl methylation activity. *NP*, no protein was detected by autoradiography. The data are averaged from three independent experiments. Strains are wild-type (SM3310), *rce1Δ* (SM3644), *ste24Δ* (SM3286) *rce1Δ ste24Δ* (SM3651), and *ste14Δ* (SM1871).

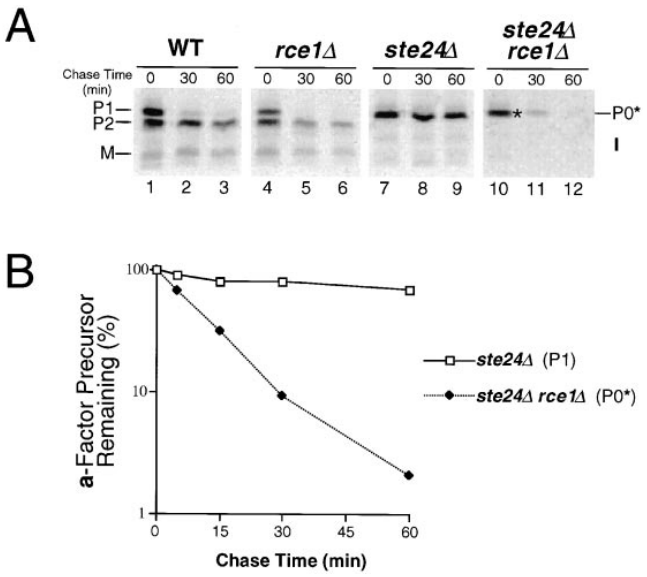

Figure 4. P0* is metabolically unstable. (*A*) Pulse-chase analysis of **a**-factor in wild-type and mutant strains. Cells were pulse labeled with [^{35}S]cysteine for 5 min and chased with excess cold cysteine for the indicated times (*min*). Protein extracts were prepared, immunoprecipitated with anti–**a**-factor antiserum 9-137, and then analyzed by SDS-PAGE. Strains are wild-type (SM3310), *rce1Δ* (SM3644), *ste24Δ* (SM3286), and *rce1Δ ste24Δ* (SM3651). (*B*) Graph of **a**-factor precursor stability versus time. The amount of the single **a**-factor species (either P1 or P0*) present at each time point in the single (*ste24Δ*) and double (*rce1Δ ste24Δ)* mutants, respectively, was quantitated by PhosphorImager analysis and graphed as shown.

The Biosynthetic Intermediate, P0, Is Metabolically Unstable*

We have shown elsewhere that a defect in the carboxyl methylation of **a**-factor correlates with the metabolic instability of **a**-factor biosynthetic intermediates (Sapperstein et al., 1994). We compared the metabolic stability of P0* and P1 generated by the double (*ste24Δ rce1Δ*) and single (*ste24Δ*) mutants, respectively, by pulse-chase analysis (Fig. 4 *A*). This experiment reveals that the methylated P1 intermediate that accumulates in the *ste24Δ* mutant is metabolically stable with a half-life greater than 60 min (Fig. 4, *A,* lanes *7–9* and *B*). In contrast, the half-life of the unmethylated P0* species present in the double *ste24Δ rce1Δ* mutant is dramatically less (half-life of ~9 min) (Fig. 4, *A*, lanes *10–12* and *B*). The biosynthetic profile and metabolic stability of the **a**-factor intermediates in the single *rce1Δ* mutant does not appear to be significantly different from that of the wild-type strain (Fig. 4 *A*, compare lanes *4–6* with *1–3*).

Taken together, the experiments carried out in Figs. 3 and 4 indicate that although the gel mobility difference between P0* and P1 is small, they can be discriminated by a large difference in their methylation level and metabolic stability. P0* is unstable and unmethylated whereas P1 is metabolically stable and methylated. The CAAX processing of **a**-factor in the double mutant (*ste24Δ rce1Δ*) fails to proceed beyond P0*, and thus is consistent with redundant roles for Ste24p and Rce1p in COOH-terminal AAXing. In contrast, the single *ste24Δ* mutant accumulates the stable, methylated P1 intermediate, indicating that COOH-terminal AAXing can be carried out via Rce1p and that Ste24p is needed to mediate the first NH_2-terminal P1→P2 processing step in **a**-factor biogenesis.

Characterization of a-Factor Species Produced by Ubiquitin–a-Factor Fusions

Since Ste24p appears to have dual roles in **a**-factor maturation involving both NH_2- and COOH-terminal steps, we

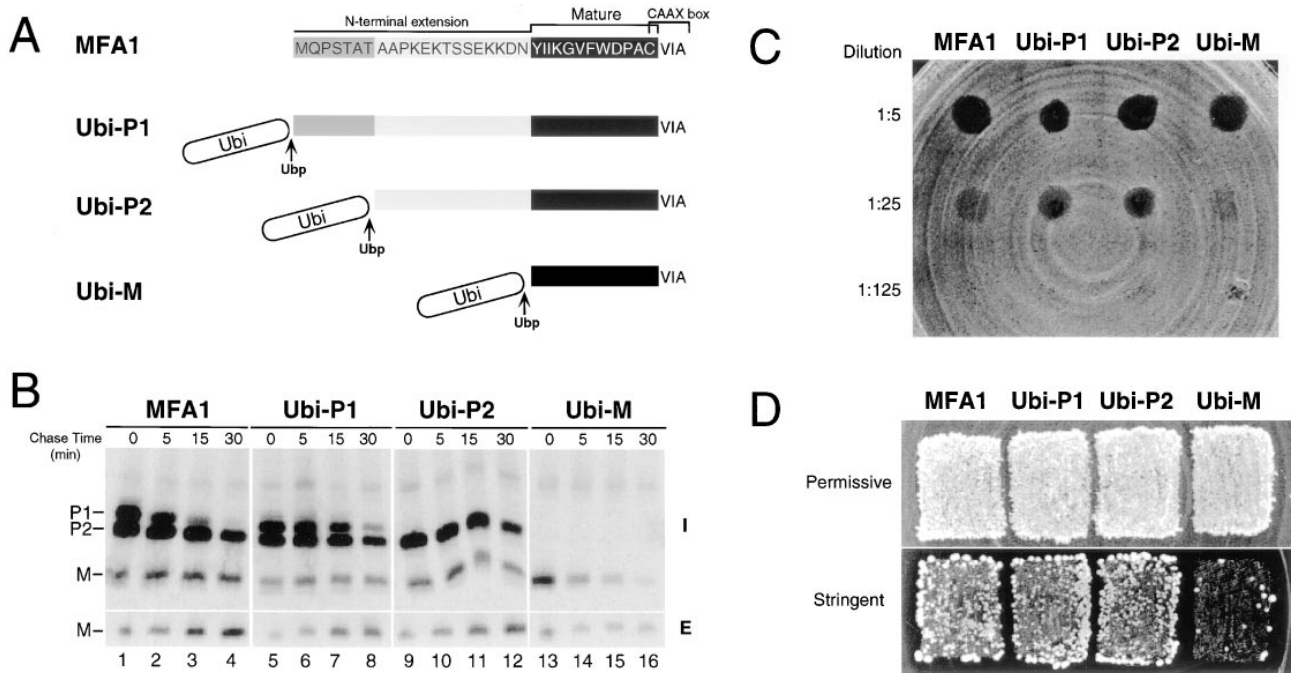

Figure 5. Processing of Ubi–**a**-factor fusion proteins generate the expected **a**-factor species. (*A*) Ubi–**a**-factor fusions. To generate Ubi–**a**-factor fusions, the sequence encoding a single copy of ubiquitin (*Ubi*) was fused precisely to the full-length **a**-factor gene *MFA1* or to truncated *MFA1* corresponding to P2 or mature **a**-factor, yielding Ubi-P1, Ubi-P2, and Ubi-M, respectively. In all cases, the CAAX motif was retained in the fusion construct. The site of cleavage by the ubiquitin-specific proteases (Ubp) is indicated. The shading of regions within **a**-factor correspond to that shown in Fig. 1. (*B*) Pulse-chase analysis of **a**-factor derived from wild-type *MFA1* and Ubi–**a**-factor fusions. The pulse-chase experiment was carried out as described in Fig. 4, except that the anti–**a**-factor antiserum 9-497 was used. Chase times (*min*) are indicated. It should be noted that we do not detect the full-length forms of the Ubi–**a**-factor fusions, presumably because processing by the Ubps is cotranslational. We have observed the expected full-length Ubi–**a**-factor fusion upon introduction of a COOH-terminal G76→V mutation in ubiquitin that abolishes the normal cut site for the Ubps (Nouvet, F. and S. Michaelis, unpublished data). Strains bear deletions of the chromosomal **a**-factor genes (*mfa1Δ mfa2Δ*) and contain plasmids harboring wild-type *MFA1* (SM3683), Ubi-P1 (SM3685), Ubi-P2 (SM3686), or Ubi-M (SM3684). (*C*) **a**-Factor spot dilution assay. The amount of extracellular active **a**-factor produced by the Ubi–**a**-factor fusions was measured as described in the Fig. 2 legend. (*D*) Patch mating assay of Ubi–**a**-factor fusions. For permissive and stringent mating conditions, the mating tester lawn is *MAT*α (SM1068) resuspended in 100% YPD and 1% YPD, respectively, as described in the Materials and Methods.

sought a method to uncouple the two processing events. This goal necessitated a way to produce mature active **a**-factor independent of the normal NH_2-terminal processing steps. The expression of NH_2-terminally truncated *MFA1* to directly generate the desired **a**-factor intermediates posed two problems: (*a*) translation requires an initiator methionine and thus would yield abnormal versions of the **a**-factor intermediates and (*b*) such *MFA1* truncation mutants were expressed poorly (Nouvet, F., and S. Michaelis, unpublished data). Instead, we used a method for expressing polypeptides that does not require the initiator methionine (Bachmair et al., 1986). In this system, the ubiquitin structural gene is fused to the gene of interest, **a**-factor in our case. During translation, the ubiquitin moiety is recognized by ubiquitin-specific proteases (Ubp), which cleave precisely after the COOH-terminal residue of ubiquitin (Tobias and Varshavsky, 1991; Baker et al., 1992), and thus should yield the desired **a**-factor intermediates.

For this study, we made three ubiquitin–**a**-factor fusion constructs, designated here as Ubi–**a**-factor fusions (Fig. 5 *A*). We fused the ubiquitin gene to the full-length *MFA1* gene (Ubi-P1), and to the truncated *MFA1* corresponding either to the P2 intermediate (Ubi-P2) or to mature **a**-factor (Ubi-M). Ubi-P2 lacks the first seven residues of the MFA1 precursor and Ubi-M lacks the entire NH_2-terminal extension. As shown in Fig. 5 *B*, Ubi-P1, Ubi-P2, and Ubi-M directly generate the respective **a**-factor species that are depicted diagrammatically in Fig. 5 *A*.

These Ubi–**a**-factor fusions show normal production of mature exported **a**-factor, as determined by pulse-chase metabolic labeling, immunoprecipitation, and SDS-PAGE. As expected, the biogenesis profiles of Ubi-P1 and wild-type MFA1 are indistinguishable from one another (Fig. 5 *B*, compare lanes *5–8* with lanes *1–4*). The ubiquitin moiety appears to be rapidly and effectively cleaved. Likewise, Ubi-P2 produces P2 directly, since it lacks the first seven residues of the NH_2-terminal extension. P2 is then processed correctly to M, which is exported (Fig. 5 *B*, lanes *9–12*). The Ubi-M construct directly yields the predicted mature **a**-factor, which is exported normally (Fig. 5 *B*, lanes *13–16*). However, the intracellular mature species derived from Ubi-M is metabolically unstable (Fig. 5 *B*, lanes *13–16*). By the **a**-factor spot dilution assay and by mating assays carried out under stringent conditions, a strain bearing Ubi-M produces somewhat reduced extracellular **a**-factor activity compared with Ubi-P1 and Ubi-P2

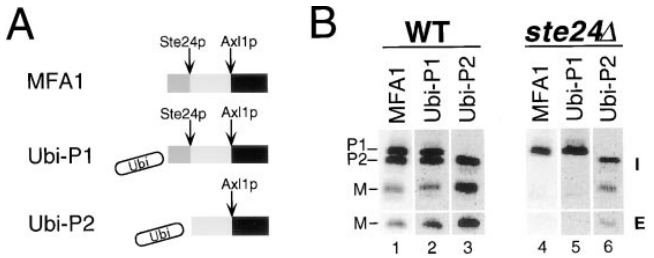

Figure 6. Ubi–**a**-factor fusions provide independent evidence that Ste24p plays a critical role in NH_2-terminal processing of **a**-factor. (*A*) Schematic representation of wild-type **a**-factor, Ubi-P1, and Ubi-P2, with the expected cleavages are shown. (*B*) Processing of Ubi–**a**-factor fusions is compared in the wild-type and *ste24Δ* mutant strains, as indicated. Radiolabeling and immunoprecipitation with anti–**a**-factor antiserum 9-497 was carried out as described in Fig. 3. Strains are wild-type and *ste24Δ* with *MFA1* (SM3683 and SM3714), Ubi-P1 (SM3685 and SM3715), and Ubi-P2 (SM3686 and SM3716), respectively.

(Fig. 5, *C* and *D*). Presumably this is due to the lower steady-state amount of exported **a**-factor, which in turn possibly reflects inefficient methylation or perhaps a protective role normally played by the NH_2-terminal extension (see Discussion). Since the Ubi-P1 and Ubi-P2 constructs produce mature **a**-factor that is stable and supports mating (Fig. 5), we used these two constructs to study specific processing steps in **a**-factor biogenesis.

Ubi–a-Factor Fusions Provide Independent Evidence that Ste24p Plays a Critical Role in the NH_2-terminal Processing of a-Factor

We used the Ubi–**a**-factor fusion constructs to confirm the role of Ste24p in the first NH_2-terminal cleavage step of the **a**-factor precursor. To do so, we compared the processing of Ubi-P1 versus Ubi-P2 in the *ste24Δ* mutant (Fig. 6). As expected, MFA1 and Ubi-P1, which both possess the Ste24p NH_2-terminal cut site (P1→P2), are not properly processed to mature **a**-factor in the *ste24Δ* mutant (Fig. 6, lanes *4* and *5*). In contrast, mature **a**-factor expressed from the Ubi-P2 construct bypasses the requirement for *STE24* in the *ste24Δ* mutant (Fig. 6, lane *6*). The *STE24* requirement in the production of mature **a**-factor by Ubi-P1, but not by Ubi-P2, provides strong independent evidence for the role of Ste24p in the first NH_2-terminal cleavage step of **a**-factor. Interestingly, these data also verify the obligatory sequential order of the two NH_2-terminal processing steps of the **a**-factor precursor, reinforcing the notion that the first seven residues of the NH_2-terminal extension must be removed before Axl1p can act in the second NH_2-terminal processing step.

Ubi-P2 Provides Independent Evidence for the Overlapping Roles for Ste24p and Rce1p in COOH-terminal AAXing of a-Factor

Since Ubi-P2 can bypass the need for *STE24* in NH_2-terminal processing, we could study the involvement of Ste24p in the COOH-terminal AAXing of **a**-factor in a system where its NH_2-terminal processing activity was not required to generate mature **a**-factor. Thus, we were able

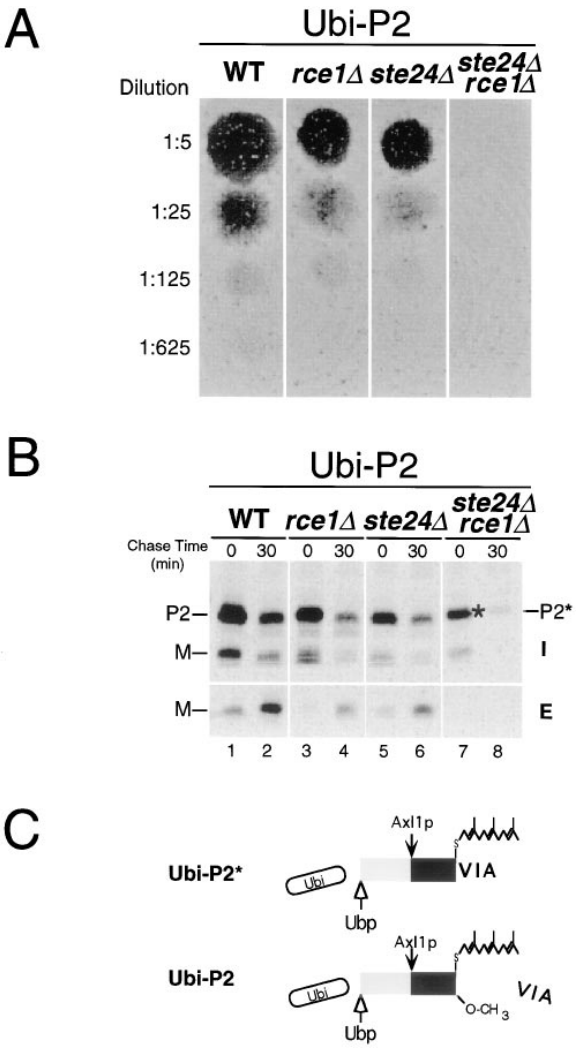

Figure 7. Ubi-P2 confirms overlapping roles for Ste24p and Rce1p in COOH-terminal AAXing of **a**-factor; similar extracellular **a**-factor levels are produced from Ubi-P2 in the single ste24Δ and rce1Δ mutants. (*A*) The **a**-factor spot dilution assay was carried out as described in Fig. 2. The amount of **a**-factor derived from Ubi-P2 is roughly equivalent when the endpoint dilutions of the single *rce1Δ* and *ste24Δ* strains are compared. It is notable that the amount of **a**-factor derived from *MFA1* differs markedly in the single *rce1Δ* and *ste24Δ* mutants (compare with Fig. 2). (*B*) Pulse labeling and immunoprecipitation of **a**-factor expressed from Ubi-P2 in wild-type and mutant strains. Cells were pulse labeled with [^{35}S]cysteine for 2 min and the label was chased with excess cold cysteine for the indicated times (*min*). Immunoprecipitation of **a**-factor was carried out with anti–**a**-factor antiserum 9-497. Strains contain a plasmid harboring Ubi-P2 in the wild-type (SM3686), *rce1Δ* (SM3721), *ste24Δ* (SM3716), or *rce1Δ ste24Δ* (SM3726) backgrounds. (*C*) The diagram illustrates the predicted structure of the Ubi-P2 and Ubi-P2* species.

to probe the individual contributions of Ste24p and Rce1p solely with respect to the AAXing of **a**-factor. We examined the amount of extracellular **a**-factor derived from Ubi-P2 in wild-type and mutant strains by the spot halo assay. As shown in Fig. 7 *A*, compared with the wild-type, the single mutants harboring Ubi-P2 show reduced but equivalent levels of **a**-factor activity. This result with Ubi-P2

contrasts with our findings with *MFA1* where the single *ste24Δ* and *rce1Δ* mutants differ markedly in extracellular **a**-factor levels (refer to Fig. 2 *A*). Apparently this difference is due to the additional role of Ste24p in NH_2-terminal processing of the full-length *MFA1*-encoded **a**-factor. The use of Ubi-P2 thus allows us to dispense with the need for the NH_2-terminal processing activity and permits us to conclude that Ste24p and Rce1p can play roughly equivalent roles in contributing to the AAXing of **a**-factor. As expected, the double mutant fails to produce any extracellular **a**-factor from Ubi-P2, confirming the redundant roles for Ste24p and Rce1p in **a**-factor AAXing.

We also examined the production of mature **a**-factor from Ubi-P2 by pulse-chase analysis in wild-type and mutant strains (Fig. 7 *B*). As predicted by the spot dilution test, extracellular **a**-factor is generated by the single mutants at a reduced level and is absent altogether for the double mutant (Fig. 7 *B*). Notably, in the intracellular fractions of all strains including the double mutant, we observe the conversion of P2 to M (from Ubi-P2), indicating that Axl1p processing is intact. A reasonable explanation for the presence of intracellular but not extracellular **a**-factor in the double mutant is that AAXing fails to occur and consequently methylation is blocked. The lack of methylation would in turn prevent recognition by the Ste6p transporter and therefore block export (Sapperstein et al., 1994). To ascertain if AAXing is defective for Ubi-P2 in the double mutant, we compared the methylation levels of **a**-factor when the wild-type and mutant strains are expressing Ubi-P2 (Fig. 8). For the double mutant, we detect no methylation for the slowest migrating band and thus conclude that this band is P2*, not P2 (Fig. 7 *C*). This verifies the overlapping roles of Ste24p and Rce1p in **a**-factor AAXing. The single mutants bearing Ubi-P2 show slightly different patterns of methylation from one another. The methylation profile for Ubi-P2 in the *ste24Δ* mutant is similar to that of the wild-type strain (Fig. 8), indicating that Rce1p can mediate AAXing very efficiently. The *rce1Δ* mutant strain also shows a methylation profile similar to wild-type, but only 30 min after labeling. At the early time point, the level of methylation is about half that of wild-type (Fig. 8). Thus, the AAXing mediated by Ste24p in the *rce1Δ* mutant may be somewhat less efficient than that mediated by Rce1p in the *ste24Δ* mutant. Nevertheless, either Ste24p or Rce1p can mediate sufficient AAXing to produce a substantial amount of steady-state mature **a**-factor.

The Human Ste24p Homologue Complements the S. cerevisiae ste24Δ Mutants

CAAX processing is evolutionarily conserved. Multicellular organisms as well as yeast express and process CAAX proteins, such as Ras. Of the trio of human CAAX-processing enzymes, only the CAAX prenyl protease(s) has not been cloned to date. Towards this end, we searched for homologues of Ste24p in the database of expressed sequence tags and identified multiple ESTs with amino acid similarity to *S. cerevisiae* Ste24p (Boyartchuk et al., 1997, Fujimura-Kamada et al., 1997). Using a fragment from one of these ESTs, N76181, we screened a human B cell cDNA library and identified several positive clones encoding the 3′ partial ORFs with sequence similarity to *S. cerevisiae* Ste24p. The most complete clone encoded an ORF of 383 residues and contained the poly A tail. We obtained the remaining 5′ ORF sequence using the longest clone for 5′ RACE PCR with a human fetal brain library.

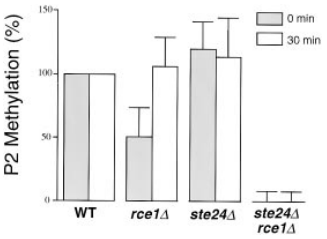

Figure 8. Relative methylation levels of Ubi-P2 in wild-type and mutant strains. Carboxyl methylation of Ubi-P2–derived **a**-factor was used as an indirect measure of AAXing activity in wild-type and mutant strains, as described in Fig. 3 *B*, except that the radiolabel was chased for 0 and 30 min and immunoprecipitation of **a**-factor was carried out with anti–**a**-factor antiserum no. 9-497. The P2 or P2* bands were excised from dried gel. The data are averaged from two independent experiments. Strains contain a plasmid harboring Ubi-P2 in the wild-type (SM3686), *rce1Δ* (SM3721), *ste24Δ* (SM3716), or *rce1Δ ste24Δ* (SM3726) backgrounds.

The human clone, which we designate Hs *STE24*, encodes a protein of 475 amino acids with 36% identity and 51% similarity to the *S. cerevisiae* Ste24p (Fig. 9 *A*). Like Ste24p of *S. cerevisiae* and *S. pombe*, Hs Ste24p has a characteristic zinc metalloprotease motif (HEXXH: H, histidine; E, glutamate; X, any amino acid), several corresponding transmembrane spans as predicted by hydropathy analysis (Fig. 9 *B*), and regions I, II, and III that are conserved among the homologues of this subfamily of zinc metalloproteases (Fig. 9 *A*) (Fleischman et al., 1995; Fujimura-Kamada et al., 1997; Kornitzer et al., 1991). Interestingly, Hs *STE24* has a short stretch of residues within region I that is not present in either yeast homologue (Fig. 9 *A*, *dashed lines*). Hs Ste24p does not possess a consensus dilysine ER localization signal at its COOH terminus but does contain lysines at positions −3 and −6 from the COOH terminus (Fig. 9 *A*), which may reflect a degenerate ER localization signal.

We expressed the human *STE24* clone in *S. cerevisiae* strains deleted for *STE24* and *RCE1* to examine whether it can complement their mating defects. We find that Hs *STE24* partially corrects the mating defect of the double *ste24Δ rce1Δ* mutant strain (Fig. 9 *C*) and fully complements the modest mating defect of the single *ste24Δ* mutant under stringent mating conditions (data not shown). These data suggest that Hs Ste24p can carry out both the NH_2-terminal processing and the COOH-terminal AAXing steps in **a**-factor maturation.

Discussion

Ste24p Is Involved in Two Distinct Proteolytic Maturation Steps of the a-Factor Precursor: NH_2- (P1→P2) and COOH-terminal (CAAX) Processing

The analysis of biosynthetic intermediates in **a**-factor biogenesis has facilitated the characterization of processing activities required for several posttranslational modifications, including the CAAX farnesyltransferase (Ram1p/Ram2p), the prenyl cysteine carboxyl methyltransferase

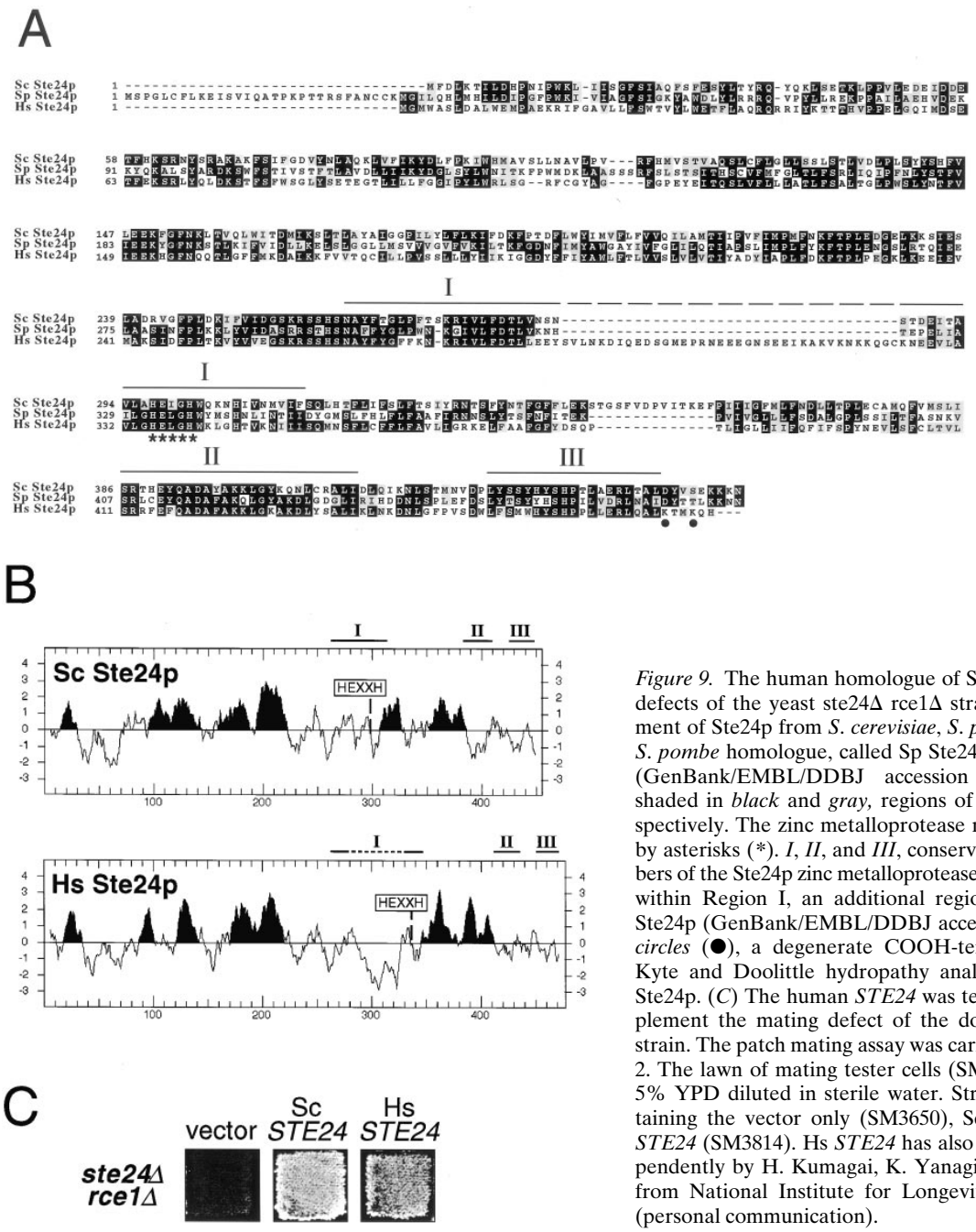

Figure 9. The human homologue of Ste24p complements mating defects of the yeast ste24Δ rce1Δ strain. (*A*) Amino acid alignment of Ste24p from *S. cerevisiae*, *S. pombe,* and *H. sapiens*. The *S. pombe* homologue, called Sp Ste24p here, is known as YAN5 (GenBank/EMBL/DDBJ accession no. Q10071). Residues shaded in *black* and *gray,* regions of identity and similarity, respectively. The zinc metalloprotease motif (HEXXH) is marked by asterisks (*). *I*, *II*, and *III*, conserved regions unique to members of the Ste24p zinc metalloprotease subfamily. *Dashed line* (---) within Region I, an additional region found solely within Hs Ste24p (GenBank/EMBL/DDBJ accession no. AF064867). *Solid circles* (●), a degenerate COOH-terminal dilysine motif. (*B*) Kyte and Doolittle hydropathy analysis of Sc Ste24p and Hs Ste24p. (*C*) The human *STE24* was tested for the ability to complement the mating defect of the double *rce1Δ ste24Δ* mutant strain. The patch mating assay was carried out as described in Fig. 2. The lawn of mating tester cells (SM1068) was resuspended in 5% YPD diluted in sterile water. Strains are *ste24Δ rce1Δ* containing the vector only (SM3650), Sc *STE24* (SM3653), or Hs *STE24* (SM3814). Hs *STE24* has also been recently cloned independently by H. Kumagai, K. Yanagisawa, and H. Komano (all from National Institute for Longevity Sciences, Aichi, Japan) (personal communication).

(Ste14p), and the **a**-factor P2→M protease (Axl1p) (Hrycyna and Clarke, 1990; Schafer et al., 1990; He et al., 1991; Adames et al., 1995). The present study focuses on the most recently discovered **a**-factor processing component, Ste24p, a transmembrane protease that has been previously assigned distinct, and seemingly disparate roles in **a**-factor processing, by us and others. We showed that Ste24p is required for the first NH_2-terminal cleavage step in **a**-factor biogenesis, the P1→P2 cleavage, by characterizing the **a**-factor intermediate that accumulates in a *ste24* mutant (Fujimura-Kamada et al., 1997). In contrast, Boyartchuk et al. (1997) provided evidence that Ste24p and a dissimilar, but functionally redundant gene product (Rce1p) are involved at a different step, COOH-terminal CAAX processing of **a**-factor, using a biochemical assay. Here we investigate and present compelling evidence for the unifying hypothesis that Ste24p mediates dual steps in the maturation of the **a**-factor precursor: COOH-terminal CAAX processing and NH_2-terminal (P1→P2) processing. We show that Ste24p functionally overlaps with Rce1p only for CAAX processing. Our evidence for the roles of Ste24p and Rce1p in **a**-factor maturation is summarized below.

We demonstrate that Ste24p and Rce1p are functionally redundant for COOH-terminal CAAX processing of **a**-factor by the characterization of biosynthetic intermediates generated in both the *ste24* and *rce1* single and double mutants. A significant amount of AAXing occurs (indicated

by methylation) in each of the single mutants (*ste24* or *rce1*) but completely fails to occur in the double mutant (*ste24 rce1*) (refer to Fig. 3 *B*) (see below for a discussion of P0*). Since either *STE24* or *RCE1* is sufficient for AAXing, and deletion of both genes results in a complete defect in AAXing, we conclude that Ste24p and Rce1p share redundant roles in **a**-factor AAXing and appear to be the only CAAX proteases for **a**-factor in yeast.

A key finding here is the identification of the novel **a**-factor biosynthetic intermediate, P0*, in the double mutant (*ste24 rce1*). P0* is an early **a**-factor intermediate that is incompletely COOH terminally processed (prenylated but not AAXed or methylated). P0* is formed before P1, from which it shows only a subtle difference by gel mobility (refer to Figs. 1 and 3). The lack of methylation and metabolic instability of P0* distinguish it from P1, which is fully methylated and metabolically stable. These methylation differences between P1 and P0* stem from the absence or presence of the AAX tripeptide, respectively, which must be absent for the methyltransferase to act. The P0* species of **a**-factor is transient, and thus is not readily apparent in a wild-type strain. Presumably this is because each step of CAAX processing occurs in rapid succession, resulting in the fast conversion of P0* to P1; we can detect fully processed **a**-factor (M) even after a short 5 min of pulse labeling. The accumulation of the unmethylated (and unAAXed) P0* in the double mutant (*ste24 rce1*) provides the basis for our conclusion that Ste24p and Rce1p have redundant roles for the COOH-terminal AAXing of **a**-factor.

A role for Ste24p in NH_2-terminal processing of **a**-factor was implicated by a block in P1→P2 processing that results in P1 accumulation (neither P2 nor M is formed) in our initial study of the *ste24* mutant (Fujimura-Kamada et al., 1997). In the present study, we provide independent confirmation that Ste24p is in fact necessary for this step by use of Ubi–**a**-factor fusions. The observation that production of mature **a-**factor from a construct that lacks the P1→P2 cut site (Ubi-P2) can bypass the Ste24p requirement for NH_2-terminal processing is consistent with the role of Ste24p in the first NH_2-terminal processing step of **a**-factor maturation (Fig. 6). Interestingly, although Ste24p and Rce1p function redundantly for CAAX processing of **a**-factor, Rce1p is unlikely to contribute to NH_2-terminal processing, based on the finding that P1 fails to be converted to P2 in the *ste24* mutant where Rce1p is present (refer to Fig. 3 *A*).

As described above, Ste24p has dual roles in **a**-factor processing and functionally overlaps with Rce1p in one of these steps. This predicts that Rce1p should be dispensable for **a**-factor processing. Indeed, we observe that in the *rce1* single mutant, the **a**-factor biogenesis profile is similar to wild-type; P2 and M are generated at similar rates in the *rce1* mutant as in a wild-type strain and export of M is also indistinguishable in these two strains (refer to Fig. 3 *A* and Fig. 2).

Having established here that Ste24p participates in two distinct proteolytic processing steps for **a**-factor, we can explain the previous discrepant findings for the role of Ste24p (Boyartchuk et al., 1997; Fujimura-Kamada et al., 1997). These were due to (*a*) the use of two different assays and (*b*) the complexity arising from the situation that one protein, Ste24p, carries out two functions and that two proteins, Rce1p and Ste24p, mediate a common step. Our combined approach of examining the NH_2- and COOH-terminal processing steps of **a**-factor in strains deleted for *STE24* and *RCE1* has now resolved this issue.

a-Factor Production and Mating in ste24 and rce1 Single and Double Mutants

In light of our current view of dual functions for Ste24p (COOH-terminal AAXing and NH_2-terminal cleavage) and a single function for Rce1p (COOH-terminal AAXing), we can reevaluate the mature **a**-factor production and mating phenotypes of the *ste24* and *rce1* single and double mutants (Fig. 2). The double mutant (*ste24 rce1*) is completely sterile because it can carry out neither COOH- nor NH_2-terminal processing, and thus does not produce any mature **a**-factor. As expected, the *rce1* mutant has a wild-type mating efficiency because Ste24p mediates both NH_2- and COOH-terminal processing of **a**-factor in this strain. In contrast, the *ste24* mutant has a dramatically decreased mating efficiency (5% of wild-type) (Fujimura-Kamada et al., 1997), that is due solely to a block in NH_2-terminal processing, since COOH-terminal AAXing can be carried out by Rce1p in the *ste24* mutant.

The residual mating exhibited by the *ste24* mutant is significant since it reflects a low level of mature **a**-factor production. What gene product is responsible for the residual **a**-factor processing in the *ste24* mutant? An unknown enzyme, or possibly even Rce1p, could mediate a very low level of P1→P2 cleavage. Alternatively, the P1→P2 processing step might be bypassed at a low level by a one-step removal of the entire NH_2-terminal extension from P1. A candidate for this latter possibility is Axl1p, whose normal role is to mediate the second NH_2-terminal (P2→M) cleavage step (between N21 and Y22) in **a**-factor processing. Axl1p may be able to generate mature **a**-factor directly from P1 somewhat inefficiently (P1→M cleavage). We favor the second hypothesis because overexpression of *AXL1* can significantly suppress the *ste24* mating defect (Fujimura-Kamada, K., and S. Michaelis, unpublished results).

Overlapping Roles for Ste24p and Rce1p

As discussed above, the SDS-PAGE and phenotypic analyses of the *rce1* and *ste24* single and double mutants indicate that either Ste24p or Rce1p is sufficient for the COOH-terminal AAXing of **a**-factor. Using the Ubi-P2 construct to circumvent the Ste24p requirement for NH_2-terminal processing, we could investigate the individual contributions of Ste24p and Rce1p solely to the COOH-terminal AAXing of **a-**factor. Based upon **a-**factor spot dilution assays and SDS-PAGE analysis (refer to Fig. 7), we concluded that Ste24p and Rce1p each can carry out a substantial amount of the AAXing of **a**-factor, since the single mutants produced approximately equivalent amounts of the bioactive mature species. Although the levels of P2 methylation in the single mutants expressing Ubi-P2 are indistinguishable from one another at a later time point, at an early time point their methylation levels differ by ~50% (i.e., a twofold lower level of methylation in the *rce1* versus the *ste24* strains for P2 [refer to Fig. 8]). This is consistent with findings for the somewhat asymmetric roles of Ste24p and Rce1p in **a**-factor AAXing (35 versus

60%, respectively) using an in vitro AAXing assay with a synthetic peptide substrate and membrane extracts from mutant strains (Boyartchuk et al., 1997).

It is rather surprising that Ste24p and Rce1p function redundantly in the CAAX processing of **a**-factor, since they are neither homologues nor do they share common consensus sequences. Whereas Ste24p possesses the HEXXH zinc metalloprotease motif, Rce1p lacks any known protease motifs. The sole resemblance between Ste24p and Rce1p is that both are extremely hydrophobic with several predicted membrane spans. This situation contrasts with another redundant pair of **a**-factor processing components, Axl1p and Ste23p, that are structurally similar to each other and contain the same protease motif (Adames et al., 1995). Both Axl1p and Ste23p can mediate the final cleavage step (P2→M) in **a**-factor maturation, accounting for >90% and <10% of processing, respectively (Adames et al., 1995).

Given the dissimilarity of Ste24p and Rce1p and that their roles do not overlap completely, it is possible that one (or both) of these proteins acts indirectly in **a**-factor maturation. Ste24p and/or Rce1p may regulate another protease(s) that cleaves **a**-factor. Because Rce1p lacks any currently known protease motifs, it may either be a cofactor involved in AAXing or it may represent a novel protease. The development of an in vitro assay with purified Ste24p (or Rce1p) and its substrate will resolve this issue. Since both candidate proteases, Ste24p and Rce1p, as well as the potential substrate, prenylated **a**-factor, are quite hydrophobic and likely membrane bound, the purification and assay development will be challenging.

STE24 likely encodes a protease, however, because the conserved protease motif of Ste24p is necessary to complement the mating and **a**-factor production defects of a *ste24* mutant (Boyartchuk et al., 1997, Fujimura-Kamada et al., 1997). Furthermore, mutations in the HEXXH motif are sufficient to abolish AAXing as well as NH_2-terminal processing (data not shown). Aside from the Ste24p subfamily of unusually hydrophobic zinc metalloproteases (Fujimura-Kamada et al., 1997), only one other subclass of HEXXH multispanning membrane proteases is known, the unrelated S2P protease involved in cholesterol homeostasis (Rawson et al., 1997). Both the S2P protease and its substrate are very hydrophobic (Rawson et al., 1997). S2P releases the sterol regulatory element binding protein transcription factor by cleaving within a transmembrane span (Hua et al., 1996; Sakai et al., 1996). It will be interesting to see how such a hydrophobic protease recognizes and processes its similarly hydrophobic substrate and whether Ste24p and S2P use analogous processing mechanisms.

If Ste24p directly cleaves **a**-factor, then how does Ste24p cut its substrate at two distinct sites? The COOH-terminal AAXing site (between residues C32, which is prenylated, and V33) bears no resemblance to the NH_2-terminal P1→P2 processing site (between residues T7 and A8). One explanation is that the prenyl cysteine serves as a landmark or recognition site for recruitment of Ste24p and that cleavage occurs at certain nearby sites. Alternatively, Ste24p could have broad substrate specificity, like the signal peptidases that recognize hydrophobic residues (for review see Dalbey et al., 1997). Ferreting out the rules for Ste24p cleavage will require assaying a large number of defined substrates.

Since Ste24p and Rce1p are not homologues but do function redundantly in **a**-factor processing, it is reasonable to postulate that each enzyme recognizes subsets of overlapping substrates. Various CAAX-containing proteins possess different CAAX sequences. Specific CAAX sequences can direct farnesylation, geranylgeranylation, or even both (Moores et al., 1991; Trueblood et al., 1993, 1997; Caplin et al., 1994, 1998), which could also provide another level of substrate discrimination for Ste24p and Rce1p. Although Ste24p and Rce1p mediate processing of the **a**-factor CAAX box, each is also likely to promote AAXing of other yeast CAAX proteins, such as Ras or the γ subunit (Ste18p) of the heterotrimeric G protein that transduces the pheromone response. For example, it has been suggested that Rce1p is involved in AAXing of Ras2p, whereas Ste24p is not (Boyartchuk et al., 1997; Schmidt et al., 1998). The function of another CAAX protein, Ste18p, does not require Ste24p and Rce1p since the *MAT*α *ste24 rce1* mutant does not have a mating defect (Fig. 2 *B*). This suggests that (*a*) Ste18p does not require AAXing by Ste24p and Rce1p for its function, (*b*) AAXing is not necessary for Ste18p function, or (*c*) another unidentified CAAX protease processes Ste18p. The substrate specificities of Ste24p and Rce1p remain to be elucidated.

Insight into the Role of the NH_2-terminal Extension of a-Factor by Ubi–a-Factor Fusions

In addition to reaffirming the role of Ste24p in NH_2-terminal processing, the Ubi–**a**-factor fusions provided a method to verify the order of **a**-factor processing events and also to examine the role of the NH_2-terminal extension in the production and export of mature **a**-factor. First, the NH_2-terminal extension must be removed in two successive steps for production of mature **a**-factor. This sequence of events is based on the finding that the second NH_2-terminal processing step by Axl1p can proceed efficiently only after completion of the first NH_2-terminal cleavage, either via Ste24p or as encoded by Ubi-P2. The P1→P2 cut could expose the substrate recognition site for Axl1p. Second, since the NH_2-terminal extension is ultimately removed from **a**-factor before its export, a reasonable hypothesis is that the NH_2-terminal extension could play a transient role, such as targeting **a**-factor to its transporter, Ste6p. However, our results with Ubi-M suggest that this is not the case because Ubi-M, which lacks the NH_2-terminal extension, can still produce mature **a**-factor that is properly exported (refer to Fig. 5 *B*). Instead, the major difference between mature **a**-factor generated conventionally from *MFA1* or unconventionally from Ubi-M is that the latter is highly metabolically unstable. An interesting possibility is that the NH_2-terminal extension plays a role in protecting **a**-factor from degradation. A candidate for a degradation pathway is the N-end rule pathway since the first residue (tyrosine) of mature **a**-factor is a destabilizing residue. Another possibility is that the NH_2-terminal extension acts as a chaperone to stabilize mature **a**-factor. Alternatively, the mature species derived from Ubi-M may not be methylated as efficiently as wild-type **a**-factor, and thus could be degraded by the same machinery that degrades P0*.

Model for Intracellular a-Factor Processing and Trafficking

Most secreted molecules, such as the other *S. cerevisiae* mating pheromone **a**-factor, undergo posttranslational processing in the lumenal compartments of the secretory pathway. In contrast, **a-**factor and other CAAX proteins are thought to be COOH terminally processed in the cytosol or on the cytosolic face of membranes. For example, it has been suggested that most of unprocessed Ras2p accumulates on intracellular membranes in yeast strains deleted for the CAAX proteases (*ste24 rce1*) (Boyartchuk et al., 1997). We have sought elsewhere to define the intracellular site where **a-**factor CAAX processing and NH_2-terminal processing take place (Schmidt et al., manuscript submitted for publication). In those studies, we demonstrated by subcellular fractionation and indirect immunofluorescence that the CAAX proteases (Ste24p and Rce1p) and the methyltransferase (Ste14p) are localized to the membrane of ER, presumably with their active sites facing the cytosol (Romano et al., 1998, Schmidt et al., manuscript submitted for publication). Those findings, together with the results presented here that show dual roles for Ste24p, lead to our current view of the intracellular trafficking and processing of **a**-factor (refer to Fig. 1). The newly synthesized **a-**factor precursor (P0) is prenylated by the cytosolic Ram1p/Ram2p complex, permitting its association with the ER membrane. At the cytosolic face of the ER membrane, prenylated **a-**factor (P0*) completes COOH-terminal CAAX processing, including AAXing by Rce1p or Ste24p, followed by carboxyl methylation by Ste14p to form P1. Next, the first NH_2-terminal cleavage step is mediated by Ste24p to yield P2. P2 is subsequently shuttled to another compartment where Axl1p (or Ste23p) (Schmidt, W.K., and S. Michaelis, unpublished data) performs the final cleavage step to generate M, which is then exported by Ste6p.

This model raises two intriguing issues. First, since Ste24p, Rce1p, and Ste14p are localized to the same intracellular membrane and act sequentially in CAAX processing, these three components could form a complex, a notion that we are presently investigating. Second, how does a prenylated protein, and **a**-factor in particular, traffic from the ER to its final destination (the plasma membrane)? We propose that **a**-factor could diffuse through the cytosol, use a carrier, or hitchhike on the outside of vesicles using the classical secretory pathway (Schmidt et al. 1998).

Human Ste24p Functions in Yeast

The COOH-terminal CAAX protein motif and its processing enzymes are conserved evolutionarily among eukaryotes. Here we report the cloning of the first mammalian CAAX prenyl protease, human (Hs) *STE24*. We have shown elsewhere that *S. cerevisiae* (Sc) *STE24* defines a novel subfamily of zinc metalloproteases containing homologues from bacteria (*E. coli, H. influenza)* and other fungi (*S. pombe*) (Fujimura-Kamada et al., 1997). Additionally, ESTs that are similar to *STE24* exist in many multicellular organisms (*A. thaliana, C. elegans, D. melanogaster, M. musculus, H. sapiens*). Members of the Ste24p subfamily are characterized by multiple membrane spans, a zinc metalloprotease motif (HEXXH), several highly conserved regions designated I, II, and III, and a COOH-terminal dilysine motif potentially involved in ER retrieval. Hs Ste24p and Sc Ste24p are 36% identical and 51% similar; Hs Ste24p shares the common features of this subfamily. However, instead of the canonical dilysine motif for ER retrieval (lysines at positions −3 and −4, or −3 and −5 from the COOH terminus), Hs Ste24p possesses what may be a degenerate dilysine motif (lysines at positions −3 and −6). It will be interesting to determine if human Ste24p, like its *S. cerevisiae* counterpart, is localized to the ER membrane. We expect that this will be the case, since another CAAX processing component (Ste14p methyltransferase) localizes to the ER membrane in yeast and mammalian cells (Dai et al., 1998; Romano et al., 1998).

We show by complementation that Hs Ste24p can function in yeast (Fig. 9 *C*). Notably, Hs Ste24p complements the mating defect of the double mutant (*ste24 rce1*), suggesting that it is capable of mediating not only COOH-terminal AAXing, but also NH_2-terminal processing of **a**-factor (Fig. 9 *C*). What is the physiological substrate for Hs Ste24p; is there a human homologue for **a**-factor that undergoes similar processing steps? Clearly, no such human substrate has been discovered to date. A potential substrate is the prelamin A precursor, which undergoes a series of maturation steps that includes COOH-terminal CAAX processing, followed by a subsequent proteolytic cleavage located 14 residues NH_2-terminal to the prenyl cysteine (Weber et al., 1989). This latter cleavage may be analogous to the NH_2-terminal processing of **a**-factor, which occurs at sites that are 26 and 12 residues away from the prenyl cysteine (Chen et al., 1997*b*). The endoprotease that processes the prelamin A precursor has yet to be cloned, although an enzymatic activity has been detected in nuclear extracts (Kilic et al., 1997). In addition to a possible role in NH_2-terminal proteolytic cleavage similar to the P1→P2 cut of the **a**-factor precursor, Hs Ste24p is also likely to be involved in the processing of multiple CAAX proteins found in diverse human cell types. ESTs of Hs *STE24* have been identified in cDNA libraries derived from a variety of tissues (fetal brain, bone, prostate, fetal lung, pancreas tumor, human tonsillar cells enriched for germinal center B cells, retina, and heart) indicating a wide expression pattern. We anticipate that future studies will reveal the mammalian substrates of Ste24p, some of which may use a processing pathway similar to that of yeast **a**-factor.

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