Saccharomyces cerevisiae a-Factor Mutants Reveal Residues Critical for Processing, Activity, and Export

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Most fungi secrete pheromones that play important signaling roles in mating to stimulate cell and/or nuclear fusion. In Saccharomyces cerevisiae and other ascomycetes, the mating pheromones produced by the two mating types differ, in that one is an unmodified peptide, while the other is a prenylated and carboxymethylated peptide produced by the two mating types differ, in that one is an unmodified peptide, while the other is a prenylated and carboxymethylated peptide. These pheromones are synthesized as precursors terminating in a C-terminal CAAX motif which includes prenylation, proteolysis, and carboxymethylation (by Ram1p/Ram2p, Ste24p or Rce1p, and Ste14p, respectively); (ii) N-terminal processing, involving two sequential proteolytic cleavages (by Ste24p and Ax1lp); and (iii) nonclassical export (by Ste6p). Once exported, mature a-factor interacts with the Ste3p receptor on M4Tr cells to stimulate mating. The a-factor biogenesis machinery is well defined, as is the CAAX motif that directs C-terminal modification; however, very little is known about the sequence determinants within a-factor required for N-terminal processing, activity, and export. Here we generated a large collection of a-factor mutants and identified residues critical for the N-terminal processing steps mediated by Ste24p and Ax1lp. We also identified mutants that fail to support mating but do not affect biogenesis or export, suggesting a defective interaction with the Ste3p receptor. Mutants significantly impaired in export were also found, providing evidence that the Ste6p transporter recognizes sequence determinants as well as CAAX modifications. We also performed a phenotypic analysis of the entire set of isogenic a-factor biogenesis machinery mutants, which revealed information about the dependency of biogenesis steps upon one another, and demonstrated that export by Ste6p requires the completion of all processing events. Overall, this comprehensive analysis will provide a useful framework for the study of other fungal pheromones, as well as prenylated metazoan proteins involved in development and aging.

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FIG. 1. a-Factor biogenesis pathway. (A) Schematic of the a-factor biogenesis pathway, with intermediates and machinery shown for each step. See text for a detailed description of each step. The a-factor precursor encoded by MFA1 is shown (P0), with the N-terminal extension (consisting of two portions, shaded in gray and unshaded), the mature sequence (shaded in black), and the CAAX motif indicated. The P0 precursor is converted to mature, extracellular a-factor (M) through the indicated biosynthetic intermediates (steps 1 to 6). Farnesylation (of the C-terminal Cys) and carboxymethylation (-O-Me) are indicated. (B) Metabolic labeling analysis of a-factor in a WT strain (bottom panel) and in isogenic deletion mutants that block each step of the pathway. Cells were pulse labeled with [35S]Cys for 5 min and chased with excess unlabeled Cys for the indicated times. Intracellular (I) and extracellular (E) fractions were prepared, and a-factor species were immunoprecipitated and analyzed as described in Materials and Methods. The mobility of the precursor (P0), intermediates (P0*, P1, and P2), and mature (M) a-factor are marked. Note that P0 and P0* are rapidly converted to P1 and are only visible in mutant strains that result in their accumulation (ram1Δ and rce1Δ ste24Δ strains, respectively). For the ste14Δ mutant, all the species are unmethylated and are therefore labeled as P1', P2', and M'.
known about critical residues within α-factor that are recognized by this machinery. The exception to this is CAAX processing (Fig. 1A, steps 1 to 3), for which α-factor has been an important model molecule in defining the sequence specificities of the prenylation and CAAX endoproteolysis enzymes (14, 16, 20, 30, 66). By contrast, systematic studies have not been performed to analyze the sequence requirements for the N-terminal processing steps by Ste24p and Axl1p (Fig. 1A, steps 4 and 5). Similarly, the determinants for Ste6p recognition are poorly defined (Fig. 1A, step 6). It has been suggested that Ste6p recognition may be solely dependent on the prenyl and methyl CAAX modifications and not the specific residues within the mature peptide, but there is very little evidence to support this suggestion. Studies with synthetic peptides have demonstrated that the CAAX modifications are required for recognition by, or access to, the α-factor receptor (Ste3p) (40); however, little is known about the residues in the mature peptide that may also contribute to interaction with Ste3p.

In this study, we identified residues within α-factor critical for biogenesis and function through an extensive mutagenesis analysis. This comprehensive analysis revealed, for the first time, distinct sequence determinants for the N-terminal processing steps mediated by Ste24p and Axl1p. In addition, numerous mutations were identified throughout the mature portion of an α-factor that do not affect biogenesis or export but have a strong effect on activity and likely affect interaction with Ste3p. Interestingly, we also identified several mutants in which biogenesis is normal but export is strongly impaired, suggesting that Ste6p may recognize specific residues or structural features of α-factor in addition to the CAAX modifications.

We also performed an in-depth analysis of an isogenic set of biogenesis machinery mutants, which clearly indicated that a block at any step in C- or N-terminal processing prevents export of α-factor. Interestingly, whereas farnesylation (Fig. 1A, step 1) is required for all downstream steps (steps 2 to 6), N-terminal processing (steps 4 and 5) was found to proceed in the absence of C-terminal proteolysis and carboxymethylation (steps 2 and 3). However, these partially processed intermediates were not competent for export by Ste6p. Thus, only properly processed, mature α-factor is a substrate for the Ste6p transporter. Overall, the mutants generated in this study form the basis for a better understanding of the steps in α-factor biogenesis and the requirements of the machinery that mediate those steps and provide a foundation for dissecting the biogenesis of other lipid-modified fungal phenomes.

**MATERIALS AND METHODS**

**Plasmids and strains.** The starting plasmid for α-factor mutagenesis was pSM475 (20). This plasmid contains the MFA1 open reading frame with 624 bp of 5′ untranslated region and 833 bp of 3′ untranslated region cloned into pRS316 (CEN URA3) as an EcoRI-XbaI fragment; to facilitate subcloning, an HpaI site at –50 and an MluI site at +130 were introduced by site-directed mutagenesis. Degenerate oligonucleotide mutagenesis was used to mutagenize the MFA1 open reading frame in blocks of five or six codons (pSM91, Q2 to T7; oSM90, A8 to E12; oSM89, K13 to E17; oSM88, K18 to Y22; SM73, I23 to V27; oSM86, F28 to A32). Degenerate oligonucleotides contained 12 nucleotides flanking the 5′ and 3′ sides of the degenerate nucleotides. During oligonucleotide synthesis, at the sites of degeneracy the normal (wild-type) nucleotide was “spiked” with an equimolar mixture of all four nucleotides (at a ratio of 93% normal to 7% mixture). Mutagenesis was performed using a Mutagen Phagemid in vitro mutagenesis kit (Bio-Rad). Pools of mutant plasmids derived from each degenerate oligonucleotide were isolated from Escherichia coli and transformed into the α-factorless strain SM1458 (α-factor pl1 leu2 ura3 his4 can1 mfa1:LEU2 mfa2:Ura2) (20), and the transformants were screened by plate mating against halo tests (44, 46) to identify mutants defective in α-factor biogenesis or activity. Mutant plasmids were recovered from yeast and sequenced to determine the site of mutation. Sequencing of random plasmids from several pools indicated that the degenerate oligonucleotide procedure yielded single or multiple mutants in about 25% of plasmids. Deletion mutations were made by oligonucleotide-directed mutagenesis.

The strains used for this study are all derived from SM1058 (Table 1). The α-factor biogenesis machinery mutant strains used in the experiments shown in Fig. 1 and 2 are all isogenic to SM1058 and are described in Table 1. The wild-type (WT) strain used in the experiments shown in Fig. 3 is SM2331 (MATA trpl1 leu2 ura3 his4 can1 mfa1:Ura3 mfa2:Ura2) (20). The biogenesis machinery mutant strains shown in Fig. 3 are all derived from SM2331:SM3375 (ste24::LEU2), SM3689 (rec1::TRP1), and SM3691 (ste24::LEU2 rec1::TRP1) (63). An α-factor was expressed in these strains from pSM1605 (2α URA3 MEA1-CV4) (58) or pSM1795 (2α URA3 MEA1-CTLM) (38). For the analyses shown in Fig. 4 to 6, the α-factor mutant plasmids were transformed into an α-factorless strain, either SM1458 (see above) or SM1229 (MATA trpl1 leu2 ura3 his4 can1 mfa1::LEU2 mfa2::URA3) (44 (Table 2).

**Metabolic labeling to analyze α-factor biosynthetic intermediates.** Metabolic labeling experiments were performed essentially as described (20). Briefly, cells were pulse labeled for 5 min with [35S]Cys (or [35S]Met; see Fig. 3D) (PerkinElmer) and chased with excess unlabeled Cys and/or Met for the indicated times. For experiments with a single time point, samples were prepared after a 10-min chase. Intracellular and extracellular fractions were prepared, and α-factor was immunoprecipitated with anti-α-factor rabbit polyclonal antiserum 9-497. Immunoprecipitated α-factor species were resolved by 16% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using gel conditions previously described (20), and the α-factor species were visualized by phosphorimaging.

**Halo and mating assays.** Halo assays were performed essentially as described previously (44, 46). Briefly, cells were grown in the appropriate selective medium, concentrated by centrifugation, suspended in H2O, and spotted (5 μl) onto a lawn of cells supersensitive to α-factor (SM3375: MATα sst2 rne1 his6 met1 can1 cyh2) (50) spread on a yeast extract-peptone-dextrose (YPD) plate containing 0.04% Triton X-100. A zone of growth inhibition (the “halo”) results from α-factor-induced G1 arrest of the suppressive MATα cells. Plates were incubated at 30°C for 2 days and photographed.

For microtitre plate mating assays, appropriate strains of each mating type (MATα query strain and the tester strain SM1068 [MATα his1]) were grown overnight, diluted to an optical density of 0.1 OD600nm, and mixed in a 9:1 well plate (90 μl of MATα and 10 μl of MATα). Serial 10-fold dilutions were made in YPD medium, and the plate was incubated for 4 h at 30°C. The plate was centrifuged to pellet the cells, the supernatant was removed, the cells were resuspended in 100 μl of H2O, and a 10-μl aliquot from each well was spotted on mating-selective plates. Plates were incubated at 30°C for 3 days and photographed.

**Quantitative mating assays** were performed as previously described (2, 46). Briefly, the MATα (query strain) and MATα (SM1068) strains were grown overnight and diluted to an OD600 of 1.0. For each culture, 0.5 OD units was mixed and collected on a nitrocellulose filter that was placed on a YPD plate and incubated for 4 h at 30°C. The cells were then washed off the filter and dilutions were plated on mating-selective (synthetic medium with dextrose) and control

| Strain | Relevant genotype | Mating (%)* | Reference |
|--------|------------------|-------------|-----------|
| SM1058 | MATA trpl1 leu2 ura3 his4 can1 | 100 | 44 |
| SM1866 | raml1::URA3 | 0.0009 | 31 |
| SM3613 | rec1::TRP1 | 68 | 63 |
| SM3614 | rec1::TRP1 ste24::LEU2 | <0.0001 | 63 |
| SM1388 | ste14::TRP1 | <0.0001 | 32 |
| SM3103 | ste24::LEU2 | 24 | 28 |
| SM2679 | ad11::LEU2 | 2 | 1 |
| SM2744 | ad11::LEU2 ste23::LEU2 | 0.0004 | 1 |
| SM2721 | ste6Δ | 0.0002 | 6 |

* All strains are isogenic to SM1058.
* Mating is expressed as a percentage of WT mating (SM1058).

TABLE 1. Mating of α-factor biogenesis machinery mutant strains
TABLE 2. a-Factor mutant strains, plasmids, and quantitative mating data

| Mutation   | Strain\(^a\) | Plasmid\(^b\) | Mating (%)\(^c\) |
|------------|--------------|----------------|------------------|
| None (WT)  | SM2366       | pSM478         | 100              |
| TSN+A6V    | SM2307       | pSM559         | 97               |
| A8G        | SM2059       | pSM566         | 45               |
| A8T        | SM2054       | pSM558         | 63               |
| A9P        | SM2058       | pSM560         | 49               |
| ΔOPS       | SM2017\(^d\) | pSM543\(^d\)   | 100              |
| ΔOPSTAT    | SM2271\(^b\) | pSM606\(^d\)   | 100              |
| K13T       | SM2052       | pSM556         | 81               |
| S15R       | SM2055       | pSM562         | 72               |
| S16R       | SM2056       | pSM563         | 95               |
| S15R+E17Q  | SM2305       | pSM564         | 11               |
| E17K       | SM2053       | pSM555         | 2                |
| K18A       | SM2402       | pSM1038        | 100              |
| K19A       | SM2405       | pSM1041        | 100              |
| D20Y       | SM2310       | pSM1214        | 12               |
| N21I       | SM2311       | pSM1215        | 1                |
| Y22N       | SM2312       | pSM1216        | 0.1              |
| Y22D       | SM2313       | pSM1217        | 0.1              |
| ΔPEEK      | SM1938\(^e\) | pSM506\(^d\)   | 14               |
| ΔTSSIE     | SM1930\(^e\) | pSM488\(^d\)   | 5                |
| ΔNY        | SM1940\(^e\) | pSM506\(^d\)   | 0.0001           |
| I23M       | SM2314       | pSM572         | 38               |
| I23N       | SM2315       | pSM1219        | 0.1              |
| K25A       | SM2414       | pSM1044        | 100              |
| K25E       | SM2416       | pSM1046        | 100              |
| G26V       | SM2319       | pSM1222        | 0.01             |
| G26C       | SM2318       | pSM1221        | 25               |
| F26A       | SM3378       | pSM1226        | 23               |
| F28Y       | SM2320       | pSM1224        | 89               |
| F28N       | SM3379       | pSM1227        | 0.001            |
| W29C       | SM2324       | pSM1230        | 0.008            |
| W29R       | SM2323       | pSM1231        | 0.008            |
| D30E       | SM2325       | pSM1232        | 0.01             |
| P31A       | SM2326       | pSM1233        | 1                |
| P31Q       | SM2327       | pSM1234        | 0.1              |
| A32K       | SM2373       | pSM722         | 0.01             |
| A32S       | SM2328       | pSM716         | 92               |

\(^a\) The parental strain for all strains is SM1458 unless otherwise noted.
\(^b\) The parental strain is SM1229.
\(^c\) All plasmids are CEN URA3 unless otherwise noted.
\(^d\) CEN TRP1 plasmid.
\(^e\) Mating is expressed as a percentage of WT mating (SM1058).

(YPD) plates. The mating efficiency was calculated as the ratio of colonies on the mating-selective plate relative to the control plate and then expressed as a percentage of the amount of mating for the WT MATa strain (SM1058).

RESULTS

Phenotypes of mutants defective in the machinery required for a-factor processing and export. As a prelude to the study of mutations within a-factor, it was important to definitively establish the phenotypes of mutants defective in components of the a-factor biogenesis machinery. Genes encoding these components were originally isolated based on a-factor mating and halo bioassays; however, the severity of the biogenesis defects has not been systematically quantitated or compared in isogenic strains. Here, we have addressed this gap. To assess biogenesis, we performed metabolic labeling (Fig. 1B). We also carried out three bioassays for a-factor activity, providing an indirect measure of whether a-factor biogenesis has been completed: halo (Fig. 2A), microtiter plate mating (Fig. 2B), and quantitative filter mating (Fig. 2C). Notably, the last method provides a robust and sensitive assay extending through five logs of activity (from 100% mating for WT, down to 0.001% of WT mating for certain mutants) (Table 1). Below, we consider findings from these experiments.

In general, the results from all three bioassays (Fig. 2A, B, and C) show the same trend. As expected, deletions of genes known to act nonredundantly in a-factor biogenesis (ram1Δ, ste14Δ, and ste6Δ mutants) are completely defective for mating (<0.001% of WT, denoted by an asterisk in Fig. 2C) (Table 1). In contrast, for the gene pairs known to function redundantly (rcelΔ and ste24Δ; axl1Δ and ste23Δ) (1, 9, 10, 63), only the double mutants are completely defective for halo and mating. The single mutants (rcelΔ, ste24Δ, and axl1Δ) show residual activity (68%, 24%, and 2%, respectively) (Fig. 2C). By itself, the ste23Δ mutant has no phenotype because its contribution to cleavage is small compared to Axllp (data not shown; see references 1 and 35). An apparent inconsistency in these assays is that the ste6Δ mutant is completely defective for mating (Fig. 2B and C) but has a significant residual halo (Fig. 2A). In the ste6Δ mutant, mature bioactive a-factor is produced but is trapped inside the cell. We suspect that a small amount of lysis may occur as cells grow on the plate, allowing release of this intracellular a-factor and resulting in growth arrest of the MATa cells in the halo assay. However, because the lysed cells

FIG. 2. a-Factor halo and mating assays with isogenic biogenesis machinery mutant strains. (A) The WT strain and the indicated isogenic mutants were tested for their ability to form a halo on a lawn of MATa cells, as described in Materials and Methods. The diameter of the halo is proportional to the amount of a-factor secreted. (B) Microtiter plate mating assays were performed as described in Materials and Methods, with the dilutions indicated. (C) Quantitative mating assays were performed as described in Materials and Methods. The graph is derived from data presented in Table 1. Assays were performed in triplicate, with WT set to 100% and the mutants expressed as a percentage of WT mating efficiency. The asterisk (•) indicates mutants with a mating efficiency of <0.001%.
are dead and because extracellular a-factor cannot efficiently rescue the mating defect of cells unable to produce their own a-factor, no mating occurs (12, 44). In contrast to growth on plates as in Fig. 2A, when ste6Δ cells are grown in logarithmic phase in liquid culture where cell lysis is minimal, no a-factor is apparent in the culture supernatant (S. Michaelis, unpublished data).

We also examined the isogenic a-factor biogenesis mutants by metabolic labeling, which permits the visualization of biosynthetic intermediates (Fig. 1B) (20). The analysis shown here is the first time that a-factor biogenesis mutants have been tested together in a single experiment, permitting several important conclusions. First, compared to WT, it is striking that no a-factor (mature or precursor) is present in the extracellular (E) fraction of any of these mutants (Fig. 1B, bottom panel of each mutant analysis). This observation indicates that only properly processed mature a-factor is competent for export by Ste6p. Second, in general each processing step appears to be dependent upon completion of the previous step. For example, in the *ram1Δ* strain in which farnesylation is blocked, no subsequent steps in a-factor biogenesis occur. An important exception to this rule is the ste14Δ mutant. In this mutant, in which biosynthetic intermediates are not carboxymethylated (denoted P1, P2, and M in Fig. 1B), the N-terminal processing of a-factor nevertheless occurs, breaking the strict dependency rule stated above. It is notable, then, that whereas the first step of CAAX processing, farnesylation, is required for N-terminal processing, the last step, carboxymethylation, is not essential for N-terminal processing. Importantly, the non-methylated form of mature a-factor produced in the ste14Δ strain cannot be exported by Ste6p.

**CAAX processing is not required for N-terminal processing by Ste24p and Ax1p.** To further clarify the dependency (or lack thereof) of processing steps on one another, we wished to address whether the middle step of CAAX processing, endoproteolysis (step 2), is required for N-terminal processing of a-factor. However, this analysis is complicated because of the dual roles of Ste24p in the C- and N-terminal steps of a-factor biogenesis (10, 63). Thus, while an *rce1Δ ste24Δ* double mutant blocks CAAX endoproteolysis (step 2), N-terminal processing cannot proceed in any case, because Ste24p is also required for step 4 (Fig. 1A). To circumvent this issue, we generated a form of a-factor in which CAAX endoproteolysis was strictly Rce1p dependent, leaving Ste24p to function solely in N-terminal processing. This was accomplished by replacing the CAAX motif of WT a-factor (CVIA; top) can be performed by both Ste24p and Rce1p, while CAAX endoproteolysis of the CTLM variant (bottom) can be performed only by Rce1p (10) (as noted by the cross-through of Ste24p). The Met residues (M) in each a-factor variant are indicated in black type. (B) Halo assays showing that CTLM a-factor halo formation is dependent on Rce1p, unlike WT (CVIA) a-factor, which can be processed by Rce1p or Ste24p. (C) Metabolic labeling of WT (CVIA) a-factor and the CTLM mutant form was carried out as described in the legend of Fig. 1 at the 10-min chase point in the indicated strains. The bands in lane 7 marked with an arrowhead reflect aberrant forms of P2 and M that retain the AAX residues. (D) The experiment is as described for panel C, except that [35S]Met labeling was performed to label the Met residue in the CTLM CAAX motif. The arrowhead in lane 4 marks the aberrant P2 form that retains the AAX residues (TLM).
In a metabolic labeling to visualize biosynthetic intermediates (Fig. 3C), the intracellular processing patterns of WT (CVIA) a-factor and CTLM a-factor were found to be essentially indistinguishable, indicating that CAAX endoproteolysis, like carboxymethylation, is not essential for N-terminal processing. Strikingly, no mature a-factor is observed in the extracellular fraction of CTLM a-factor in rce1Δ (Fig. 3C, lane 7; compare this to the extracellular fraction of WT a-factor, lane 3) or by the halo assay (Fig. 3B). These results are consistent with the findings discussed above for the carboxymethyl transferase mutant, ste14Δ, which is not unexpected, as the unprotonylized CTLM a-factor cannot be methylated.

While the CTLM a-factor species produced in the rce1Δ strain are predicted to retain the AAX residues, a mobility shift was not apparent in the results shown in Fig. 3C, presumably because the contribution of prenylation (which significantly alters the gel mobility of proteins) obscures it. To directly determine whether or not CAAX endoproteolysis had occurred, we performed [35S]Met labeling of CTLM a-factor (Fig. 3D), which allows direct visualization of N-terminally processed species that retain the CAAX motif (since Met is the final residue of this CAAX variant and the only labeled residue that remains after P1 cut site, defining these two residues as important for efficient P1 → P2 processing by Ste24p). Having examined the processing machinery and biogenesis steps in detail, we next turned to a mutagenic analysis of a-factor itself. With the exception of the CAAX motif, which has been well studied (9, 14, 20), very few mutants that affect biogenesis or activity have been reported. Here, we randomly mutagenized MFA1, except for the CAAX motif, in blocks of five or six codons (15 to 18 nucleotides) and screened for mutants that do not produce mature a-factor, as assessed by mating and halo assays. Processing defects were determined by metabolic labeling, both for mutants compromised in activity and for several phenotypically silent mutants. In certain cases, deletions or specific point mutants were also generated and analyzed. This combination of approaches yielded a large collection of mutants defective in a-factor N-terminal processing, export, or activity. The results of this analysis are shown in Fig. 4 to 6, and mutants are discussed according to the biogenesis step that is affected.

The first of the N-terminal processing events which converts P1 a-factor to P2 (step 4) occurs between residues T7 and A8 and is carried out by the protease Ste24p (20, 28, 64). Three mutants were isolated that showed a striking accumulation of P1 a-factor and lack further processing: A8G, A8T, and A9P (Fig. 4A and B). In Fig. 4B, an intracellular species that runs slightly faster than mature (M) a-factor can be observed. This band has been previously characterized as an a-factor related peptide (afrp) and is a prenylated and methylated 7 mer derived from MFA1, whose production is independent of Ste24p and Axl1p and thus should not be confused with mature a-factor (19). It is variably pulled down in immunoprecipitations and as a result does not appear in all experiments.

The mutated residues A8 and A9 lie immediately C terminal to the P1 → P2 cut site, defining these two residues as important elements for Ste24p recognition. No mutants defective in a-factor production were recovered in the region N terminal to the processing site (Fig. 4A), suggesting that this region does not contain recognition determinants for Ste24p. For example, the TSN A6V double mutant that was recovered from the mutagenesis screen was phenotypically silent. Furthermore, the ∆QPS and ∆QPSAT deletions (Fig. 4B) had no effect on a-factor maturation or export.

These mutants were also subjected to quantitative mating assays (Fig. 4C and Table 2). The mutants with no biogenesis defect (Fig. 4B, lanes 2, 7, and 8) mated like WT, while the P1 → P2 processing mutants A8G, A8T, and A9P showed reduced...
mating. Notably, however, the mating defect was only partial, ranging from 45 to 60% of WT, similar to but slightly higher than that of the ste24Δ strain (~25%) (Fig. 2C). The reason for the high level of residual mating for these mutants is presumably the same as we propose for the ste24Δ strain (i.e., a low level of direct P1 → P2 processing by Axl1p) (see Discussion).

It should be noted that relatively few mutants were isolated that are defective in the Ste24p-dependent processing step. The random MFA1 mutants were screened primarily for plate-mating defects, and the high level of residual mating that occurs when the Ste24p processing site is blocked may explain why so few mutants were identified. To more fully define the site will require additional, focused site-directed mutagenesis of the region. However, our results suggest that residues immediately C terminal to the cut site are the most important, and the mutants we have isolated have provided valuable tools for blocking P1 → P2 processing of a-factor, independent of using a strain in which the Ste24p processing enzyme is absent.

Mutational analysis defines residues important for efficient P2 → M processing by Axl1p. Axl1p-dependent processing of a-factor occurs between residues N21 and Y22 (3, 20). Our random mutagenesis analysis revealed numerous mutations that prevent P2 → M processing in residues flanking both sides of this cleavage site (D20Y, N21L, Y22N, and Y22D) (Fig. 5A and B, lanes 11 to 14). Similar to results shown in Fig. 4B, the a-factor related peptide (afrp) species appears prominently in Fig. 5B but is distinct from M a-factor. Whereas no mutations C terminal to Y22 had an impact on Axl1p cleavage (see Fig. 6), mutations at E17, which is five residues N terminal to the Axl1p cut site, blocked processing. Indeed, two mutants altered at E17 were found (E17K and S15R+E17Q) (Fig. 5B, lanes 6 and 7); however, because the S15R single mutant is phenotypically silent, the effect of the double mutant is most likely due solely to the E17Q mutation. Interestingly, the K18A and K19A mutations are phenotypically silent (lanes 8 and 9), even though they are surrounded by mutations that block processing. We also generated several deletions (Fig. 5A and B, lanes 16 to 18). Not surprisingly, ΔNY, which removes the Axl1p cleavage site, blocked processing. Somewhat unexpectedly, even distant deletions within the N-terminal extension (ΔPEKE and ΔTSSE) also blocked Axl1p processing, suggesting that the structure and/or length of the N-terminal extension is important for this cleavage step.

It is notable that all of the mutants that affected Axl1p processing were severely defective in mating (Fig. 5C and Table 2), ranging from <0.1% to 10% of WT. This result is in contrast to the significant residual mating observed with the mutants defective for P1 → P2 (Ste24p) processing (Fig. 4C), suggesting that a block in P2 → M processing cannot be circumvented.

Mutations in the mature a-factor sequence reveal residues essential for export and activity. Random mutagenesis of the mature a-factor sequence (residues 22 to 32 of Mfa1p) was also carried out. Numerous mutations that resulted in mating defects were isolated throughout the sequence of the mature peptide (Fig. 6A). To determine the basis for the mating defect, we analyzed the mutants by metabolic labeling and SDS-PAGE (Fig. 6B). In all cases, intracellular mature a-factor was produced, indicating that all the processing steps occurred.

The mutants fell into two classes, based on the presence or absence of extracellular a-factor. The larger class of mutants exhibited extracellular mature a-factor at a WT level (Fig. 6B; note that this figure is a composite of multiple gels, each with its own WT control) but were significantly reduced in mating (40% to <0.1% of WT) (Fig. 6C and Table 2). For instance, the W29C a-factor mutant exhibited normal processing and export (Fig. 6B, lane 14) yet is completely defective for mating (<0.01% of WT) (Fig. 6C and Table 2), most likely because it is unable to productively interact with the Ste3p receptor. Other mutants in this class included I32M, I32N, F28A, F28N, W29R, D30E, and P31A (Fig. 6A, residues in black boxes; B, lanes 2, 3, 10, 12, 15, 16, and 17; and C). Complex (double and triple) mutations in a-factor at residues G26, V27, W29, D30, and A32 result in a similar phenotype and have been previously reported by another group (17). The present series of mutants greatly broadens the scope of residues that result in defective receptor interaction.

The smaller class of mutants were significantly reduced (G26C, P31Q, and A32K) (Fig. 6B, lanes 8, 18, and 20) or completely blocked (G26V) (Fig. 6B, lane 7) in export. This
The hallmark of all fungal genes encoding lipophilic pheromones is the C-terminal CAAX motif (8, 13, 14), which directs prenylation, endoproteolysis, and carboxymethylation. For *S. cerevisiae* α-factor, the best-studied member of this class of pheromones, mutagenesis of this motif has shown the importance of the CAAX modifications for pheromone biogenesis, stability, and bioactivity (16, 20, 55). Analysis of synthetic peptides also reflects the significance of the CAAX modifications for pheromone activity (40, 67). However, less is known about the importance of the rest of the precursor sequence in terms of directing maturation, export, and activity. Because of the considerable sequence divergence among the lipophilic pheromones, important residues cannot be predicted by sequence comparisons. The comprehensive mutational analysis of α-factor presented here has provided insights into substrate recognition determinants for the N-terminal processing steps of α-factor mediated by Ste24p and Axl1p and for recognition by the Ste3p receptor and the Ste6p transporter. A better understanding of how the biogenesis machinery recognizes its substrates will be valuable for understanding biogenesis of other fungal pheromones as well as mammalian CAAX proteins, like prelamin A, that undergo additional N-terminal processing similar to α-factor (4, 39, 68).

**Ste24p and the P1 → P2 processing step.** Ste24p is a multispansing endoplasmic reticulum membrane protein with a zinc metalloprotease activity. It is unusual among components of the α-factor processing machinery in that it plays two roles: an overlapping role with Rce1p in CAAX endoproteolysis (Fig. 1, step 2) (10, 63) and a unique role in the first N-terminal processing step (Fig. 1, step 4) (28). The sequence recognition determinants for CAAX endoproteolysis by Ste24p have been defined, but little is known about determinants for P1 → P2 processing. Here we report three mutants with alterations in the two residues immediately C terminal to the cut site that block this processing step: A8G, A8T, and A9P. We previously reported that A8G blocks P1 → P2 processing, both in vivo (28) and in vitro (58). These mutants indicate that Ste24p exhibits sequence specificity at the P1 → P2 processing site and that it is not simply acting as a molecular ruler and cleaving at a set distance N terminal to the farnesylcysteine (although farnesylation is clearly important for recognition, as Ste24p N-terminal processing does not occur in the absence of farnesylation [Fig. 1B]). It is interesting to note that the P1 → P2 processing site bears no sequence resemblance to the C-terminal CAAX endoproteolysis site (indeed, the latter must contain a farnesylcysteine), indicating that Ste24p is unusual in having the ability to recognize at least two very different sites. Yet in spite of this, Ste24p exhibits considerable sequence specificity, as demonstrated by the mutants identified here (Fig. 4). One possibility is that Ste24p has two active sites, one for each cleavage event, for which there is modest evidence (22), although additional work is needed to clarify this issue.

It is notable that when the sequences of all known lipophilic fungal pheromones are aligned, no consensus sequence around the N-terminal Ste24p cleavage site is apparent. Further de-
etailed mutagenesis of A8 and A9 (as well as other residues surrounding the cleavage site in α-factor) to define allowable residues may point toward a consensus sequence. Interestingly, our deletion analysis suggests that the first part of the N-terminal extension is dispensable, as ΔQPSTAT, which eliminates this extension, is still fully processed to mature α-factor. We observed similar results when we replaced this region with ubiquitin, which is cleaved off by ubiquitin-specific proteases to release P2 α-factor (10, 63). Clearly this N-terminal extension needs to be removed for Axl1p processing to occur efficiently (see below); however, the requirement for this N-terminal extension in the first place is unclear.

At first glance, it seems puzzling that there is considerable residual mating in the α-factor mutants that block P1 → P2 processing (Fig. 4C) and, indeed, in the ste24Δ mutant itself (Fig. 2). One possibility is that another protease can cleave P1 → P2 inefficiently, allowing a small amount of mature α-factor to be produced that is below the detection limit by metabolic labeling and SDS-PAGE but sufficient to support some mating. A more likely possibility is that a low level of processing by Axl1p can occur despite the absence of prior processing by Ste24p, producing mature α-factor directly from P1. Indeed, we have observed by metabolic labeling that overexpression of Axl1p in an ste24Δ mutant results in a small but detectable amount of mature α-factor (S. Michaelis, unpublished data). The AXLI gene is highly induced by α-factor (51), so in a mating assay when MATα cells are present, Axl1p may be induced sufficiently in the ste24Δ mutant to produce a low level of mature α-factor directly from P1, bypassing the requirement for P1 → P2 cleavage. This amount of mature α-factor may be enough to drive some residual degree of mating. In the metabolic labeling experiment with the ste24Δ strain (Fig. 1B), no MATα mating partner is present; therefore, Axl1p levels are low and no mature α-factor is observed.

Understanding the sequence requirements for the zinc metalloprotease Ste24p will be important for understanding other fungal pheromones. Interestingly, the human Ste24p ortholog, Zmpste24, can complement the ste24Δ mutant for α-factor production (58, 63). In addition, mutations in Zmpste24 lead to premature aging in mice and other laminopathies in humans because of its role in proteolytic processing of the nuclear scaffold protein prelamin A (39, 68). Thus, further insights into the sequence recognition requirements for Ste24p will have significance in terms of human health and disease.

Axl1p and the P2 → M processing step. A striking finding in this study was that we recovered many mutants defective in Axl1p cleavage (Fig. 5). These are the first mutants reported that block this processing step for α-factor or any fungal pheromone. The mutated residues flank the cleavage site, indicating that recognition involves residues both N terminal (D20 and N21) and C terminal (Y22) to the Axl1p cleavage site. Interestingly, point mutations at the acidic residue E17, some distance N terminal to the processing site, also impact cleavage, although mutations at the basic residues K18 or K19 do not affect processing. Deletions N terminal to the processing site (ΔPEK and ΔTSSE) also block processing. Together, these data suggest that the conformation and/or length of the N-terminal extension after Ste24p processing may also be important. Notably, no mutations C terminal to Y22 were found that block processing, suggesting that recognition is directed to the cut site itself and the N-terminal regions. It is notable that the lipophilic pheromones from several other fungi (Schizosaccharomyces pombe, Ustilago hordei, and Cryptococcus neoformans) also have an Asn residue that immediately precedes the predicted mature pheromone sequence (23, 26, 36), suggesting that Asn may form part of a consensus sequence for fungal pheromone processing.

Axl1p was discovered for its role in axin bud site selection in haploid cells and subsequently found to be required for α-factor maturation (1, 29). Axl1p is a zinc metalloprotease that shares homology with human insulin degrading enzyme. Interestingly, mutation of its protease domain does not affect its role in bud site selection but does block α-factor processing, indicating that these two functions are separable (1). Similar to the ste24Δ deletion, some residual mating (~2%) is also observed in an axl1Δ deletion. In this case, the residual mating is known to be due to the activity of Ste23p, an Axl1p homolog (1). The contribution of Ste23p to α-factor processing is very small compared to Axl1p such that the ste24Δ mutant on its own has no phenotype. Interestingly, several of the point mutants in α-factor that block Axl1p processing are mating defective to the same extent as the axl1Δ ste23Δ double mutant, suggesting that the point mutation blocks recognition by both proteases. Thus, the sequence recognition determinants of Axl1p and Ste23p are likely to be similar. Similar to other gene products with a key role in mating, Axl1p is strongly α-factor induced and haploid specific (51), while Ste23p is not regulated in a mating or mating-type specific manner (29).

Activity mutants. Our mutagenesis study has revealed that the amino acid sequence of mature α-factor is critical for recognition by or access to Ste3p. Numerous mutations (at residues I22, F28, W29, D30, and P31) were found that do not block maturation or export but have a strong mating defect (Fig. 6). Another study found complex (double and triple) point mutations in mature α-factor that also affect activity, some of which overlap with the residues identified here (17). Thus, there appears to be exquisite sequence specificity in recognition of α-factor by the Ste3p receptor, in addition to the importance of the prenyl and carboxymethyl modifications elegantly demonstrated with synthetic peptides (40). It is interesting to note that a very conservative change, namely D30E, results in a significant reduction in activity, while a major change like K25E (or K25A) has no functional impact. Thus, the determinants for receptor binding are likely to be rather complex.

The sequence specificity required for α-factor to interact with its receptor is perhaps not surprising in light of the fact that many basidiomyceetes produce multiple prenylated pheromones, in some cases with only small sequence differences, yet these can be specifically differentiated by particular receptors (13, 18, 27). In addition, alanine scanning with synthetic peptides of the Ustilago maydis pheromone α revealed particular residues critical for biological activity (62). Synthetic peptides derived from the α-factor mutants reported here could be important tools for looking at specificity of receptor binding, for instance, if used as a basis for suppressor screens to find compensatory mutations in Ste3p. Studies with α-factor and its receptor, Ste2p, have provided important insights into peptide ligand-receptor interaction (45). Similar studies with α-factor will be of great interest because of the relevance to lipophilic
signaling molecules; however, development of an a-factor-Ste3p binding assay has been a challenge because of the inherent complications resulting from a lipophilic peptide binding to a membrane receptor.

**Export mutants.** Export of a-factor is mediated by Ste6p, a member of the ATP binding cassette transporter superfamily whose substrates include lipophilic molecules and proteins. Of all the a-factor mutants examined from our screen, only one appeared to be completely export defective (G26V), while three others were significantly reduced in export (G26C, P31Q, and A32K), indicating that peptide sequence is an important factor in transport. Peptide length may also be a factor for Ste6p transport. For example, in machinery or a-factor mutants that block Axl1p processing (i.e., axl1Δ and a-factor point mutations) (Fig. 1B and 5B, respectively), no P2 a-factor is ever observed in the extracellular fraction. In addition, the ΔPEKΔ and ΔTSSE deletions that block P2 to M processing are also not exported, in spite of their reduced length.

The CAAX modifications are also critical for Ste6p-mediated export of a-factor: mature a-factor that has not undergone CAAX endoproteolysis (Fig. 3) or carboxymethylation (Fig. 1B) is not exported. The role of the farnesyl modification cannot be assessed in vivo because all subsequent processing depends on farnesylation of the a-factor precursor, and an in vitro transport assay for Ste6p-mediated a-factor transport has not been developed (34, 43).

The limited number of export-defective mutants recovered from our screen suggests that the sequence recognition determinants for export are not overly stringent. Several other observations support this conclusion. First, Ste6p ectopically expressed in S. pombe can transport M-factor, which is prenylated and carboxymethylated but has a different sequence than a-factor. Furthermore, while the basidiomycetes clearly encode many pheromones and receptors, there is no evidence for multiple transporters, suggesting that a single transporter can export pheromones with a variety of sequences; in fact, C. neoformans Ste6 was recently shown to function in both mating types (33). Our most severe export-defective mutant was G26V; interestingly, Becker and coworkers (15, 45, 71) have suggested that G26 allows mature a-factor to bend and that this conformation could be necessary for recognition by the Ste3p receptor. Taken together, it is likely that recognition for export by Ste6p may be more dependent on the conformation of the mature pheromone than on its specific sequence.

**Concluding comments.** This study presents for the first time a direct phenotypic comparison of mutants defective in components of the a-factor biogenesis machinery and mutations in a-factor itself that affect each post-CAAX processing step of its biogenesis. The machinery mutant analysis allowed us to examine the order and dependency of the processing steps on each other, revealing that CAAX endoproteolysis and carboxymethylation are not required for N-terminal processing to occur and that all steps must be carried out for export to proceed. In addition, our mutational analysis of the a-factor precursor revealed residues critical for N-terminal processing by Ste24p and Axl1p, for export by Ste6p, and for activity (i.e., interaction with Ste3p). These results provide the basis for elucidating the sequence determinants for other fungal pheromones. Studies on a-factor biogenesis have already been important for understanding mammalian prelamin A processing by Zmpste24 (4, 39, 68). Interestingly, recent experiments have suggested that prenylated signaling molecules may play a critical role for germ cell migration in zebrafish and Drosophila (54, 65). Thus, the relevance of understanding a-factor biogenesis may extend significantly beyond studies of fungal pheromones.

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