Roles of Prenyl Protein Proteases in Maturation of *Saccharomyces cerevisiae* a-Factor

Victor L. Boyartchuk[1] and Jasper Rine

Division of Genetics, Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

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ABSTRACT

In eukaryotes small secreted peptides are often proteolytically cleaved from larger precursors. In *Saccharomyces cerevisiae* multiple proteolytic processing steps are required for production of mature 12-amino-acid **a**-factor from its 36-amino-acid precursor. This study provides additional genetic data supporting a direct role for Afc1p in cleavage of the carboxyl-terminal tripeptide from the CAAX motif of the prenylated **a**-factor precursor. In addition, Afc1p had a second role in **a**-factor processing that was independent of, and in addition to, its role in the carboxyl-terminal processing *in vivo*. Using ubiquitin-**a**-factor fusions we confirmed that the pro-region of the **a**-factor precursor was not required for production of the mature pheromone. However, the pro-region of the **a**-factor precursor contributed quantitatively to **a**-factor production.

A recurring theme in the production of peptide hormones and pheromones, from preproopiomelanocortin of humans to the mating pheromones of yeast, is the synthesis of larger precursor proteins from which the active peptides are released by specific proteases. The yeast *Saccharomyces cerevisiae* uses peptide pheromones for intercellular signaling before mating. These pheromones indicate the proximity and direction of cells of the opposite mating type. The yeast mating pheromones, known as **a**-factor and α-factor, bind to serpentine receptors coupled to heterotrimeric G-proteins and initiate a MAP kinase-mediated mating response pathway (Sprague and Thorner 1992).

The **a**-factor and α-factor pheromones are synthesized as larger precursors whose maturation and secretion require rather different posttranslational processing steps and different routes from the ribosome to the exterior of the cell. Posttranslational glycosylation and proteolysis of the α-factor precursor occur sequentially as the prepropeptide progresses through the classic secretory pathway, culminating in three proteolytic cleavages that release mature pheromone from the precursor (Fuller *et al.* 1988). In contrast to α-factor, **a**-factor secretion is independent of the secretory pathway and is mediated by Ste6p, a member of the ABC family of transporters (Kuchler *et al.* 1989; McGrath and Varshavsky 1989). The **a**-factor is synthesized as a 36-residue precursor. Following synthesis, the first modification is the coupling of a prenyl lipid, farnesyl, to a cysteine residue four amino acids from the carboxyl terminus (Schafer *et al.* 1989; Schafer and Rine 1992). Prenylation is dependent on the presence of a carboxyl-terminal CAAX motif (Hancock *et al.* 1991; Moores *et al.* 1991) in which C is cysteine, AA represents two aliphatic amino acids, and X can be almost any other amino acid (C. E. Trueblood, V. L. Boyartchuk and J. Rine, unpublished results). The amino-terminal 21-residues of pro-**a**-factor and the 3-carboxyl-terminal residues of the CAAX motif of **a**-factor are trimmed by proteolytic processing steps that depend upon prior prenylation of the 36-amino-acid precursor.

To date at least four different proteases and three different cleavage sites appear to be involved in the maturation of **a**-factor. Cleavage between the 21-amino-acid amino-terminal extension and the first amino acid of the mature **a**-factor is mediated by two different metalloproteases encoded by the *AXL1* and *STE23* genes (Adames *et al.* 1995). Carboxyl-terminal trimming of the CAAX motif is achieved by the Afc1p and Rce1p proteases (Boyartchuk *et al.* 1997). Removing the first 7 amino-terminal amino acids from the **a**-factor precursor, an additional proteolytic cleavage was detected and reported to be catalyzed by the Afc1p protease (Fujimura *et al.* 1997). A fundamental puzzle is why a cell devotes four different proteases to produce a 12-amino-acid peptide from a 36-amino-acid precursor. One possible explanation is that cells cannot synthesize short peptides on ribosomes efficiently, and hence proteases are required to release a biologically active small peptide from a larger precursor. A second possibility is that the additional amino acid sequences contribute in some way to the production or release of active **a**-factor or

Corresponding author: Jasper Rine, 401 Barker Hall, Division of Genetics, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720. E-mail: jrine@uclink4.berkeley.edu

[1]*Present address:* Howard Hughes Medical Institute, Department of Genetics, Harvard Medical School, Boston, MA 02115.

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference
JRY3089	*MAT***a** *ste6::HIS3 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Kuchler *et al.* (1989)
JRY3443	*MAT*α *sst2-4 his3 trp1-1 ura3-1*	Boyartchuk *et al.* (1997)
JRY5314	*MAT***a** *his3-11,15 leu2-3,112 trp1-1 ura3-1*	Boyartchuk *et al.* (1997)
JRY5315	*MAT***a** *afc1*Δ*::HIS3 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Boyartchuk *et al.* (1997)
JRY5316	*MAT***a** *rce1*Δ*::TRP1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Boyartchuk *et al.* (1997)
JRY5317	*MAT***a** *afc1*Δ*::HIS3 rce1*Δ*::TRP1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Boyartchuk *et al.* (1997)
JRY5390	*mat***a**Δp *mfa1::hisG mfa2*Δ*::hisG ade2 leu2-3,112 lys2-801 ura3*	This study
JRY5459	*mat***a**Δp *afc1*Δ*::HIS3 mfa1::hisG mfa2*Δ*::hisG his3 leu2 trp1 ura3*	This study
JRY5460	*MAT***a** *mfa1::hisG mfa2*Δ*::hisG his3 leu2 trp1 ura3*	This study
JRY5461	*MAT***a** *afc1*Δ*::HIS3 mfa1::hisG mfa2*Δ*::hisG his3 leu2 trp1 ura3*	This study
JRY5462	*MAT***a** *rce1*Δ*::TRP1 mfa1::hisG mfa2*Δ*::hisG his3 leu2 trp1 ura3*	This study
JRY5463	*MAT***a** *afc1*Δ*::HIS3 rce1*Δ*::TRP1 mfa1::hisG mfa2*Δ*::hisG his3 leu2 trp1 ura3*	This study
JRY5464	*MAT***α** *axl*Δ*::URA3 ste23*Δ*::URA3 ade2-1 leu2-3,112 trp1-1 ura3-1 sst1*Δ *mfa2*Δ*::FUS1-lacZ his3*Δ*::FUS1-HIS3*	Adames *et al.* (1995)
JRY5465	*mat***a**Δ*p ste14*Δ*::TRP1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	This study

have some independent role on their own in the mating process. In this study we explored the role of the amino-terminal extension of the **a**-factor precursor in the production of functional **a**-factor and resolved just where Afc1p participates in **a**-factor maturation.

MATERIALS AND METHODS

Halo assays: The cells of *MAT***a** mating type were suspended in water to a fixed density. Levels of biologically active **a**-factor produced by yeast strains were assayed by spotting 3 μl of the suspension (~5×10^6 cells) onto the lawn of *MAT*α *sst2-4* cells (JRY3443). The radius of the resulting "halo" of growth inhibition is proportional to the amount of pheromone produced (Sprague 1991). Mutations in the yeast *SST2* gene render haploid yeast cells of either mating type supersensitive to the pheromone produced by cells of the opposite mating type (Chan and Otte 1982).

Strain construction: All modifications of yeast strains were performed using standard genetic methods. The strains containing deletions of either *AFC1* or *RCE1* and deleted for both **a**-factor structural genes, *MFA1* and *MFA2*, were created by crossing a *mat***a**Δp *mfa1*::*hisG mfa2*Δ::*hisG* strain (JRY5390) containing a MATα plasmid (pJR157) to a *MAT***a** *afc1*Δ::*HIS3* strain (JRY5315) and a *MAT***a** *rce1*Δ::*TRP1* (JRY5316) strain. A *mat***a**Δp *afc1*Δ::*HIS3 mfa1*::*hisG, mfa2*Δ::*hisG* strain (JRY5459) containing a *MAT*α (pJR157) plasmid was crossed to JRY5316 to obtain a *MAT***a** *afc1*Δ::*HIS3 rce1*Δ::*TRP1 mfa1*::*hisG mfa2*Δ::*hisG* strain (JRY5463). All strains used in this study are listed in Table 1.

Ubiquitin fusion constructs: All **a**-factor gene constructs were created using the polymerase chain reaction (PCR). The primers that were used for amplification contained restriction enzyme sites to facilitate subsequent subcloning steps (Table 2). A DNA fragment containing a full-length **a**-factor coding sequence (*MFA1*) was amplified with 5-MFA1B and 3-MFA1S primers using a plasmid containing the *MFA1* locus genomic fragment (pJR1099) as a template. All ubiquitin gene fragments were amplified using the same 5′ primer (5-Ub) incorporating a *Bam*HI site and the following 3′ primers for respective fusions: UbMfa1-3-UbMfa1; Ub(-7)-3-Ub(-7); and Ub(-21)-3-Ub(-21). A plasmid (pUb23) containing a ubiquitin gene fused to *LacZ* was used as a template. The **a**-factor fragments for the Ub(-21), Ub(-7)CTLM, and UbMfa1 fusions were generated using the 3-MFA1S 3′ primer containing a *Sal*I restriction site and the 3-MFA1E 3′ primer containing an *Eco*RI site for the Mfa1CTLM, M(-7), and Ub(-7)CTLM constructs. The following 5′ primers were used for amplification of portions of the **a**-factor coding sequence: UbMfa1-5-UbMfa1; M(-7) and M(-7)CTLM-5-M(-7); Ub(-7) and Ub(-7)CTLM-5-Ub(-7); and Ub(-21)-5-Ub(-21). The fragments containing the wild-type CAAX motif sequence (CVIA) were amplified from pJR1099, and CTLM variants from an *MFA1*-CTLM site-directed mutant pJR1913. To fuse ubiquitin to the **a**-factor fragments, ubiquitin and **a**-factor-derived PCR products were mixed and reamplified using 5-Ub and either 3-MFA1E or 3-MFA1S primers. The resulting PCR products were subcloned between *Bam*HI and either *Eco*RI or *Sal*I sites in YCplac111 *LEU2 CEN* vector (Gietz and Sugino 1988) containing a *TDH3* promoter fragment inserted in *Hin*dIII-*Bam*HI sites.

RESULTS

The amino-terminal 21-residue leader of the a-factor precursor was not required for biological activity: To test the role of the amino-terminal extension of the **a**-factor precursor in the production of mature **a**-factor, we created a series of gene fusions designed to produce **a**-factor precursors lacking a part or all of the 21-residue amino-terminal extension. Mature active **a**-factor protein, which begins with a tyrosine, cannot be produced simply from a truncated **a**-factor gene because the preceding methionine would be required for translation initiation. Therefore, we chose not to introduce methionine initiation codons at various positions within the **a**-factor gene. Instead, we exploited previous studies that established that the ubiquitin protease, which usually

TABLE 2

Oligonucleotides used in this study

Oligo	Sequence
5-MFA1B	5′-GTACAAAGGGATCCAATAGAAATGCAACC-3′
3-MFA1S	5′-CAATGGACAGTCGACAATTAACTGG-3′
5-Ub	5′-TCGCCTGCGGATCCTTCTTCTTCC-3′
3-UbMfa1	5′GTAGATGGTTGCATACCACCTCTTAGCCTTAGC-3′
3-Ub(-7)	5′TTTGGAGCGGCACCACCTCTTAGCCTTAG-3′
3-Ub(-21)	5′-CACCTTTGATAATGTAACCACCTCTTAGCCTTAGCAC-3′
3-MFA1E	5′-TCACTGTATACGGAATTCTCAGC-3′
5-UbMfa1	5′-GCTAAGAGGTGGTATGCAACCATCTACCGCTAC-3′
5-M(-7)	5′-GAAATGGATCCATCTACCGCTATGGCCGCT-3′
5-Ub(-7)	5′-CTAAGAGGTGCCGCTCCAAAAGAAAAG-3′
5-Ub(-21)	5′-GCTAAGAGGTGGTATGTACATTATGTACATTATCAAAGGTGTCTTC-3′

cleaves ubiquitin monomers from a polyubiquitin precursor, can precisely cleave an amino-terminal ubiquitin from any fusion protein (Bachmair *et al.* 1986). Thus, we designed a series of chimeric genes that would produce various fusion proteins in which an amino-terminal ubiquitin monomer was fused, in frame, at several different positions within the **a**-factor precursor (Figure 1).

The ability of each fusion protein to produce active **a**-factor was evaluated by halo assays in which production of active **a**-factor is measured by the extent of inhibition of growth of a lawn of α cells (Figure 2). Remarkably,

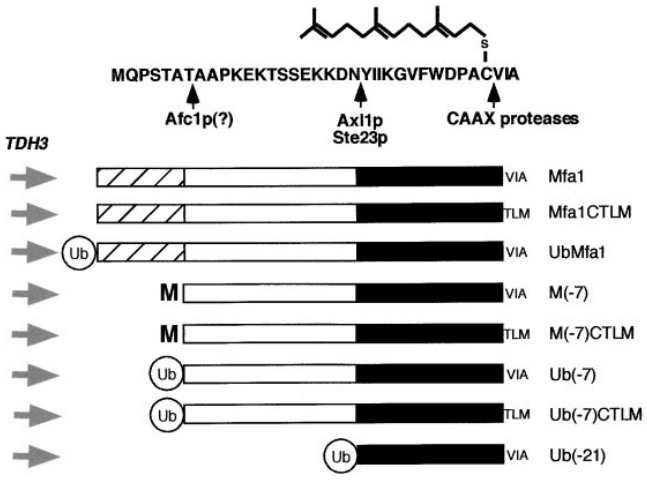

Figure 1.—Variations of yeast **a**-factor genes. The arrows at the amino acid sequence of Mfa1p indicate the sites of proteolytic cleavages. All precursors were expressed from a yeast constitutive promoter (*TDH3*) and were maintained in yeast on a YCplac111 *LEU2 CEN* plasmid. The first repeat of ubiquitin was fused, using PCR, to the amino-terminal processing intermediates [Ub(-7), Ub(-7)CTLM, and Ub(-21)] and to the complete **a**-factor coding sequence (UbMfa1). In addition, truncation constructs with methionine replacing the first 7 amino acids [M(-7) and M(-7)CTLM] were used. In this and subsequent figures the circles represent the ubiquitin moiety, the hatched boxes represent the first 7 amino acids of the proregion of **a**-factor, the open boxes represent the remainder of the proregion, the black boxes represent the mature **a**-factor sequence, and the three letters represent the particular CAAX motif suffix encoded by that construct. The ubiquitin repeat is 79 amino-acids long and is not drawn to scale.

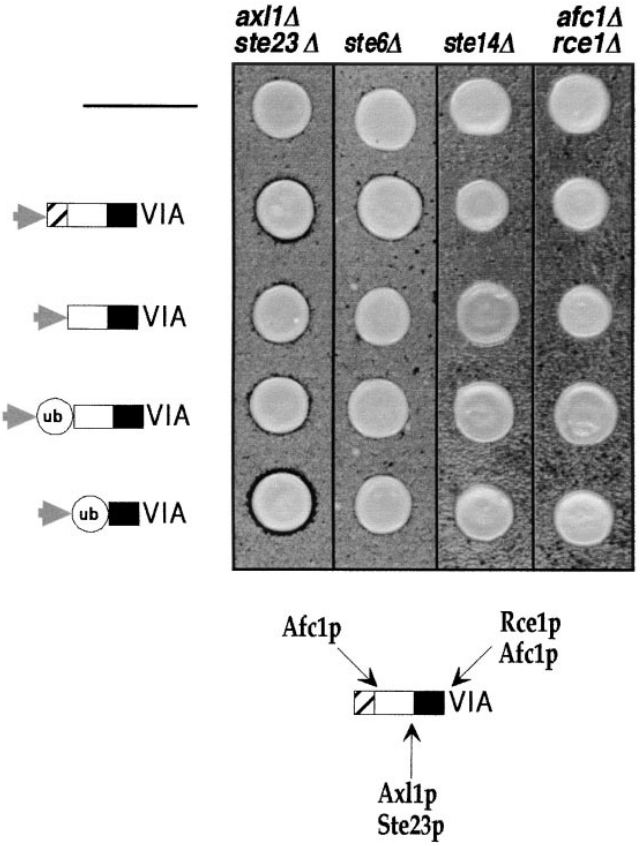

Figure 2.—Bypass of requirement for amino-terminal processing of **a**-factor. Production of mature **a**-factor required amino-terminal proteolysis by Axl1p and Ste23p. The exception was a chimera in which ubiquitin was fused to **a**-factor lacking any amino-terminal extension [Ub(-21)], which bypassed the requirement for all amino-terminal proteases. Processing of this precursor, as well as all other precursors, depended on the presence of genes required for carboxyl-terminal proteolysis (*AFC1* and *RCE1*), methylation (*STE14*), and export (*STE6*) of the pheromone.

a fusion protein that completely lacked the amino-terminal 21 amino acids (Ub-21) of the **a**-factor precursor produced active **a**-factor. Production of mature **a**-factor from this fusion was independent of the *AXL1*- and *STE23*-encoded proteases and was still dependent on the *STE14*-encoded carboxyl-methyltransferase (Ashby *et al.* 1993; Sapperstein *et al.* 1994; Figure 2). Moreover, these data confirmed published reports that the 21-residue leader is not essential for **a**-factor processing (Kuchler *et al.* 1992; Egner *et al.* 1995). The small halo still evident in a *ste6* mutant, carrying a construct designed to overexpress a wild-type form of **a**-factor, is likely to be caused by a small amount of cell lysis, resulting in release of mature **a**-factor into the surrounding medium.

Efficient a-factor production required the first seven amino-terminal amino acids: The amount of **a**-factor produced from the various ubiquitin fusions was clearly less than that produced from the wild-type **a**-factor gene. The ubiquitin moiety *per se* was not responsible for the decreased **a**-factor production because fusion of ubiquitin to the amino terminus of the complete **a**-factor sequence had no deleterious effect on **a**-factor production (Figure 3). Thus, we inferred that the amino-terminal extension of the **a**-factor precursor contributed quantitatively to **a**-factor maturation. To evaluate the contribution of the first seven amino acids to **a**-factor maturation, we used a mutant form of the precursor [M(-7)] in which translation began at a methionine added in front of the proposed Afc1p cleavage site after the seventh amino acid (Chen *et al.* 1997). To control for the possible effect of methionine on processing of **a**-factor precursor, we also fused ubiquitin to the proposed Afc1p processing intermediate in a separate construction [Ub(-7)]. Both variations of the **a**-factor processing intermediate produced similar, but lower than wild-type, levels of the pheromone (Figure 3). As expected, these **a**-factor variants still required amino-terminal cleavage by Axl1p and Ste23p, as well as the Ste6p transporter, the *STE14*-encoded carboxyl-methyltransferase, and the CAAX proteases (Figure 2). Thus, the first seven amino acids of the proregion of **a**-factor and/or a protein that recognized those amino acids were required for efficient production of **a**-factor. The smaller halos produced by these fusion proteins were unlikely to be due to defects in gene expression, because all of the fusion genes were expressed from the strong *TDH3* (GPD) promoter (Bitter and Egan 1984).

Afc1p was required for carboxyl-terminal processing of prenylated proteins: The yeast *AFC1* gene appears to encode a Zn-dependent metalloprotease that, together with the Rce1 protein, accounts for all of the carboxyl-terminal processing of prenylated CAAX proteins (Boyartchuk *et al.* 1997). Therefore, the decrease in **a**-factor levels in the *afc1*Δ strain is, at least in part, a result of a carboxyl-terminal proteolytic defect (Figure 3). However, a recent study has concluded that the

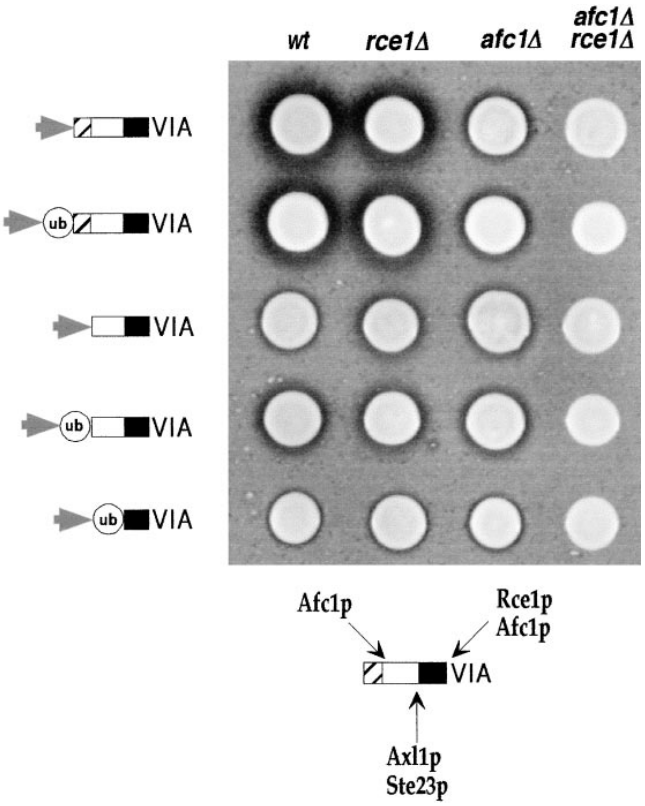

Figure 3.—Requirement for either Afc1p or Rce1p for carboxyl-terminal processing of **a**-factor. Plasmids carrying the various **a**-factor genes were introduced in *MAT***a** strains deleted for both copies of the **a**-factor structural genes, *MFA1* and *MFA2*. Measurements of the levels of mature **a**-factor were performed using a wild type (JRY5460) and protease-deletion strains JRY5462 (*rce1*Δ), JRY5461 (*afc1*Δ), and JRY5463 (*afc1*Δ *rce1*Δ). Production of **a**-factor required presence of at least one of the CAAX proteases.

principal role of Afc1p is amino-terminal proteolytic processing of **a**-factor (Fujimura *et al.* 1997). Thus, it was important to determine whether the *in vivo* defect in **a**-factor production in *afc1*Δ cells was due to a defect in amino-terminal processing, carboxyl-terminal processing, or both. The *in vivo* requirement for Afc1p in carboxyl-terminal proteolysis was tested using the ubiquitin-**a**-factor fusions. In these proteins, removal of the ubiquitin at the amino terminus is mediated by a ubiquitin protease. Therefore, the production of mature **a**-factor from such a fusion bypasses any requirement for amino-terminal proteolysis. Plasmids containing the Ub-**a**-factor fusions were transformed into strains lacking both the *MFA1* and *MFA2* **a**-factor genes. In addition, these strains carried deletions of *AFC1* or *RCE1* (or both) genes. The amount of biologically active mature **a**-factor made by the ubiquitin fusions in either the *afc1*Δ or *rce1*Δ strains was comparable to that of the wild-type strain (Figure 3). However, a strain lacking both *AFC1* and *RCE1* was completely deficient in the synthesis of active **a**-factor. Thus, either Afc1p or Rce1p were capable of carboxyl-terminal processing of **a**-factor *in*

vivo, even from precursors that completely lack the **a**-factor amino-terminal extension. These data provided the formal proof of an *in vivo* role for Afc1p in carboxyl-terminal processing of **a**-factor. Moreover Afc1p and Rce1p were of comparable effectiveness in processing of such precursors. Nevertheless, these data did not rule out the possibility that Afc1p also participates somehow in amino-terminal processing.

Substrate specificity of Afc1p and Rce1p: Genetic studies revealed that Afc1p and Rce1p displayed striking substrate specificity. The **a**-factor precursor gene, *MFA1*, was subjected to site-directed mutagenesis to alter the wild-type CVIA CAAX motif sequence to sequences found in other prenylated proteins. Specifically, in one **a**-factor variant the CVIA sequence was changed to CAMQ, which is found at the carboxyl terminus of the β-subunit of rabbit muscle glycogen phosphorylase kinase (Heilmeyer *et al.* 1992). The CTLM CAAX sequence of the *STE18*-encoded γ-subunit of a heterotrimeric G-protein (Whiteway *et al.* 1989) was used to create another **a**-factor variant.

The **a**-factor mutants as well as the wild-type *MFA1* gene were introduced into wild-type yeast and into strains lacking either Rce1p or Afc1p. The processing of the **a**-factor-CAMQ variant required Afc1p; Rce1p could not process this substrate (Figure 4). In contrast, the processing of the **a**-factor-CTLM variant required Rce1p; Afc1p could not process this substrate. This result was consistent with an earlier observation of the effect of *AFC1* and *RCE1* deletions on Ras2p activity. The decrease of Ras2p activity in *rce1*Δ strains indicated involvement of Rce1p in Ras2p processing, whereas the *afc1*Δ mutants had little if any effect on Ras activity (Boyartchuk *et al.* 1997). These data established that Rce1p and Afc1p processed some distinct sets of prenylated substrates and overlapped in the processing of others.

***AFC1* played a role in amino-terminal processing of the a-factor precursor:** The differences in substrate specificity of Afc1p and Rce1p allowed us to bypass the requirement for carboxyl-terminal processing by Afc1p in the production of active **a**-factor. The **a**-factor-CTLM variant was used to determine whether Afc1p indeed had a role in amino-terminal processing of **a**-factor. In wild-type cells, a full-length **a**-factor precursor terminating in CTLM made a robust halo, although somewhat smaller than that produced by the wild-type **a**-factor precursor. In the *afc1*Δ mutant, **a**-factor terminating in CTLM produced a much smaller halo (Figure 5). Because this form of **a**-factor was completely dependent on Rce1p for carboxyl-terminal processing, these data established that indeed Afc1p had a role in **a**-factor processing in addition to its function in carboxyl-terminal CAAX proteolysis.

If the decreased **a**-factor halo size produced by **a**-factor-CTLM in the *afc1*Δ mutant were due exclusively to a defect in amino-terminal proteolysis, then forms of

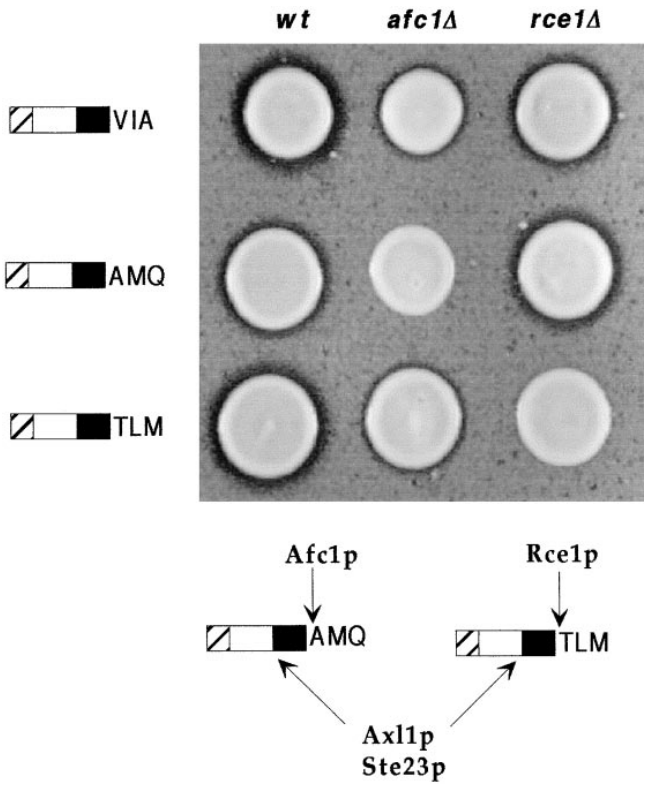

Figure 4.—Levels of pheromone produced by **a**-factor constructs with altered CAAX motif sequences. The CAAX motif sequence specificity of Afc1p was revealed by its inability to process CTLM variants of **a**-factor in the absence of Rce1p. The wild-type CVIA and mutant CAMQ *MFA1* sequences in the *rce1*Δ strain produced levels of **a**-factor identical to those of the wild-type (wt) strain. Deletion of *AFC1* revealed that Rce1p was unable to proteolyse the CAMQ mutant, whereas the CTLM variant was processed to the same extent as the wild-type CVIA **a**-factor. All **a**-factor variants shown on this panel were expressed from the *MFA1* promoter.

a-factor-CTLM that bypass the need for amino-terminal processing [pM(-7)CTLM and pUb(-7)CTLM; Figure 1] should produce halos similar in size to those produced by wild-type cells containing full-length **a**-factor terminating in CTLM. However, such **a**-factor precursors produced halos in both wild-type cells and in *afc1*Δ mutants that are smaller than the halo produced by the full-length Mfa1p-CTLM precursor (Figure 5). Together these data suggested that the role of Afc1p in **a**-factor processing was inseparable from the role of removing the first seven amino acids. That is, removal of the first seven amino acids by two independent means did not bypass the requirement for the amino-terminal role of Afc1p in **a**-factor production.

DISCUSSION

The results presented here established several important features of **a**-factor processing and revealed the relative roles of two prenyl-protein proteases in **a**-factor production. First, active **a**-factor could be produced

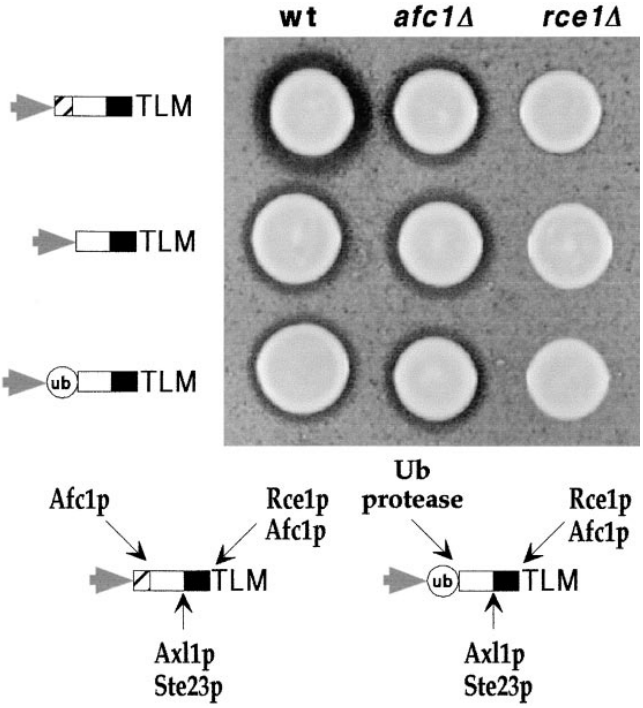

Figure 5.—Requirement for Afc1p for efficient production of **a**-factor. Removal of the first 7 amino acids of **a**-factor precursor by alternative means did not bypass the requirement for Afc1p for efficient production of mature pheromone. All chimeric constructs required Rce1p activity for carboxyl-terminal processing of their CTLM termini. M(-7)CTLM lacked the 7-amino-acid amino-terminal extension with translation initiating at a methionine introduced at the proposed Afc1p cleavage site. Ub(-7)CTLM used the ubiquitin protease to release a pro-**a**-factor precursor intermediate containing the remaining 14 amino acids of the amino-terminal extension. Neither of these precursors was able to produce wild-type (Mfa1CTLM) levels of **a**-factor. All **a**-factor constructs in this experiment were expressed from the *TDH3* promoter.

from precursor forms that entirely lacked the 21 amino-terminal residues found in the **a**-factor precursor, confirming previously reported observations (Kuchler *et al.* 1992; Egner *et al.* 1995). Such forms of the precursor bypassed the requirement for the Axl1p and Ste23p amino-terminal proteases but still depended upon both Afc1p and Rce1p CAAX proteases, the *STE14*-encoded carboxyl-methyl transferase, and the *STE6*-encoded transporter for release of active **a**-factor. Thus, the amino-terminal extension of **a**-factor plays no essential role in **a**-factor processing or function. Second, the amino-terminal extension on **a**-factor contributed quantitatively to production of mature **a**-factor. Third, one of the prenyl-protein proteases, Afc1p, was required for the enhancement provided by the amino-terminal extension. Fourth, the enhancement of **a**-factor production was independent of amino-terminal or carboxyl-terminal proteolysis by Afc1p.

A recent publication reported the identification of Afc1p, also known as Ste24p, as an amino-terminal protease involved in **a**-factor maturation (Fujimura *et al.* 1997). However, our data established unequivocally that Afc1p had a role in the carboxyl-terminal processing of **a**-factor and suggested strongly that this role was direct proteolysis of the CAAX motif. Moreover, we confirmed another role for Afc1p in **a**-factor precursor processing and provided evidence for the proteolysis-independence of this role.

Our data indicated that the first seven amino acids of the **a**-factor precursor enhanced production of **a**-factor. As described above, Afc1p contributed in some way to processing of full-length **a**-factor precursor, yet Afc1p had no role in producing active **a**-factor from **a**-factor-CTLM precursors lacking the first seven amino acids. Our data do not exclude the possibility that Afc1p itself mediates the amino-terminal cleavage, but given that the prenyl lipid is required for carboxyl-terminal cleavage by Afc1p and Rce1p, it will be rather interesting if Afc1p actually performs both CAAX proteolysis and amino-terminal proteolysis. In the absence of a direct biochemical activity linking amino-terminal proteolysis to Afc1p, the possibility of still another protease playing a role in **a**-factor processing cannot be excluded. In principle, efficient processing of the **a**-factor precursor might require recognition of the first seven amino acids by a protein that is itself processed in an Afc1p-dependent manner.

What is the role of Afc1p in facilitating **a**-factor processing? As described above, Afc1p may have a role in amino-terminal proteolysis in addition to its role in carboxyl-terminal proteolysis. However, the presence or absence of Afc1p did not affect processing of precursors that had the first seven amino acids removed by alternative means. Conversely, efficient **a**-factor production depended on Afc1p function even for the **a**-factor substrates that could not be carboxyl-terminally processed by Afc1p (Figure 5). These data could be accommodated by models in which Afc1p itself recognizes the prenylated **a**-factor precursor, presents the precursor to other processing enzymes, and also participates in carboxyl-terminal processing. In the absence of Afc1p, the other enzymes would still process the **a**-factor precursor, but would do so less efficiently. Substrates lacking the first seven amino acids cannot benefit from the Afc1p-dependent enhancement of processing. Given that Afc1p, Rce1p, Axl1p, Ste23p, Ste14p, and Ste6p, are all integral membrane proteins, it is tempting to speculate that these enzymes may be part of a large membrane complex that is specialized for processing of **a**-factor. Moreover, human homologs of all of these proteins are present in GenBank, raising the possibility that secreted prenyl peptides may play an important role in human biology.

Finally, this study demonstrated that a 30-amino-acid peptide can be synthesized on yeast ribosomes. The systematic gene identification efforts use 100-amino-acid open reading frames as the minimum definition

of a predicted gene. These data, as well as the sequence of *MFA1* and *MFA2* themselves, emphasize that the current gene identification criteria may ignore some very interesting genes.

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