To the Graduate Council:

I am submitting herewith a dissertation written by Stevan Marcus entitled "Biosynthesis and Target Cell Interactions of the *Saccharomyces cerevisiae* **a**-Factor Mating Pheromone." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

Jeffrey M. Becker, Major Professor

We have read this dissertation
and recommend its acceptance:

Mary Ann Handel

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Vice Provost
and Dean of The Graduate School

BIOSYNTHESIS AND TARGET CELL INTERACTIONS OF THE *SACCHAROMYCES CEREVISIAE* **a**-FACTOR MATING PHEROMONE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Stevan Marcus

May 1991

DEDICATION

This work is dedicated to my parents, Hili and Manley Marcus, whose support and encouragement have been greatly appreciated, to the memory of my aunt, Grete Giering, and to my loving wife, Karen, whose patience has finally been rewarded.

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I most of all want to thank my loving wife, Karen, who has tolerated my nonsense and kept me in line for the past three years–I love you. I also thank the members of my committee, Dr. Mary Ann Handel, Dr. W. Stuart Riggsby, and Dr. Robert Villafane, for their help and encouragement. Special thanks also go to Dr. Fred Naider, without whose support and encouragement this work would not have been possible. Thanks also to Munira Basrai, who has been a dear and faithful friend (you're next!); John Norman (thanks for your friendship and encouragement); to the other charter member of the "a-team", Guy "L" Caldwell (you're persistence will be greatly rewarded); to my buddy, Dave "D.J. Vetteman" Miller (it's been a lot of fun); to the man who makes a-factor, Chu-Biao Xue; and to all the other members of the Becker lab who have been such a pain in the neck–I mean joy–to work with: Greg Abel, Kumar "Segal" Alagramam, Effie Bargiota, Angus "Mohonk" Dawe, Lisa Kahn, Hui-Fen Lu, Mark Mason, Angie McKinney, and Jackie "James Earl" Ray Perry. Special thanks go to Guy, David, and Greg for help on the SAR work, and to Hui-Fen for her diligent preparation of cell extracts.

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Finally, I am eternally grateful to my mentor and dear friend, Dr. Jeffrey M. Becker. In the fall of 1983, he took me into his lab as a part-time undergraduate technician. At the time, I was a mediocre student, at best. My performance in school improved markedly during the following two years, and I ended up graduating with honors. Dr. Becker then hired me as a research assistant, and during the following two years I learned a great deal about many things, including a lot about myself. Dr. Becker encouraged me to enter graduate school, and then continued to help me develop both as a scientist and person.

Thanks, Jeff, for all that you have done for me and, most of all, for believing in me. Without your support, guidance, and patience, none of this would have been possible.

ABSTRACT

The **a**-factor mating pheromone of *Saccharomyces cerevisiae* is an isoprenylated, carboxy methyl esterified dodecapeptide (NH_2-YIIKGVFWDPAC[farnesyl]-$COOCH_3$] which is post-translationally modified in a manner analogous to that of the *ras* proto-oncogene product, Ras. The objectives of this research were to 1) detail steps involved in **a**-factor maturation and 2) gain an understanding of the interactions of **a**-factor and its *MAT*α target cell. A comprehensive overview of **a**-factor-related research leading to this work is presented in Part I of this dissertation.

Part II of this dissertation summarizes efforts made toward the characterization of the **a**-factor post-translational processing pathway. An *in vitro* maturation assay was developed which demonstrated that the order of **a**-factor -CAAX post-translational processing steps is as follows: 1) isoprenylation of the cysteine sulfur, 2) proteolysis of -AAX, and 3) carboxy terminal methyl esterification. Additional experiments demonstrated that the *RAM* gene product is required for farnesyl transferase activity and that the activity is dependent on a divalent cation.

Part III of this dissertation discusses a detailed analysis of the role of the C-terminal cysteine modifications in the biological activity of **a**-factor. Both the farnesyl and carboxy methyl ester modifications of **a**-factor were found not to be absolutely essential for pheromone activity, and could be replaced by other moieties to produce analogs with varying degrees of biological activity. However, replacement of both the farnesyl and carboxy methyl ester groups by hydrogen atoms resulted in a pheromone with negligible activity. Mating restoration assays demonstrated that *MATa* cells do not have to actively produce **a**-factor in order to mate.

Part IV of this dissertation outlines studies on an α cell specific **a**-factor degrading activity, which was found to be cell-associated, endoproteolytic, and not required for

response to pheromone. **a**-Factor degradation was not energy-dependent nor did it require pheromone internalization or interaction with its receptor. Evidence is also presented which demonstrates that **a**-factor degradation most likely plays a role in the recovery of α cells from the pheromone response.

Finally, Part V of this dissertation summarizes and interconnects Parts II - IV and suggests directions for future studies on **a**-factor.

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PART I

GENERAL INTRODUCTION

CHAPTER I

A HISTORY OF **a**-FACTOR-RELATED RESEARCH LEADING TO THIS DISSERTATION

Saccharomyces cerevisiae **a** and α haploids can conjugate to form **a**/α diploids. The process of conjugation is dependent upon the reciprocal action of mating pheromones termed **a**-factor, which is produced by **a** cells, and α-factor, which is secreted by α cells (details of the conjugation process are discussed in Parts III and IV of this dissertation). While the structure of the α-factor mating pheromone was determined well over a decade ago (13, 25), the structure of the **a**-factor pheromone remained elusive until fairly recently. Early reports by the research group of Wolfgang Duntze (6) suggested that **a**-factor had an apparent molecular weight of >600,000, which would have made it much larger than α-factor, a tridecapeptide of molecular weight 1684 (25). A later report by Duntze's group (4) indicated that **a**-factor was an undecapeptide consisting of the following amino acid composition: N-Tyr (Asx_1, Gly_1, Ala_1, Val_1, Ile_2, Phe_1, Lys_1, Trp_1, Pro_1). Subsequent cloning and sequencing of the **a**-factor structural genes, *MFa1* and *MFa2*, showed that the above amino acid composition represented part of the C-terminal portion of the primary product of the *MFa1* gene (7, fig. 1). Quite interestingly, the **a**-factor structural genes did not encode primary products containing hydrophobic signal sequences or sites for N-linked glycosylation, characteristic of most proteins processed via the classical yeast secretory pathway (22). Furthermore, studies by other investigators (23) showed that yeast secretory mutants, which could not secrete α-factor, were not defective in **a**-factor production, suggesting that **a**-factor might be processed via a novel secretory pathway. Elucidation of the steps involved in **a**-factor maturation and secretion, it was hoped, would allow for characterization of this novel secretory pathway.

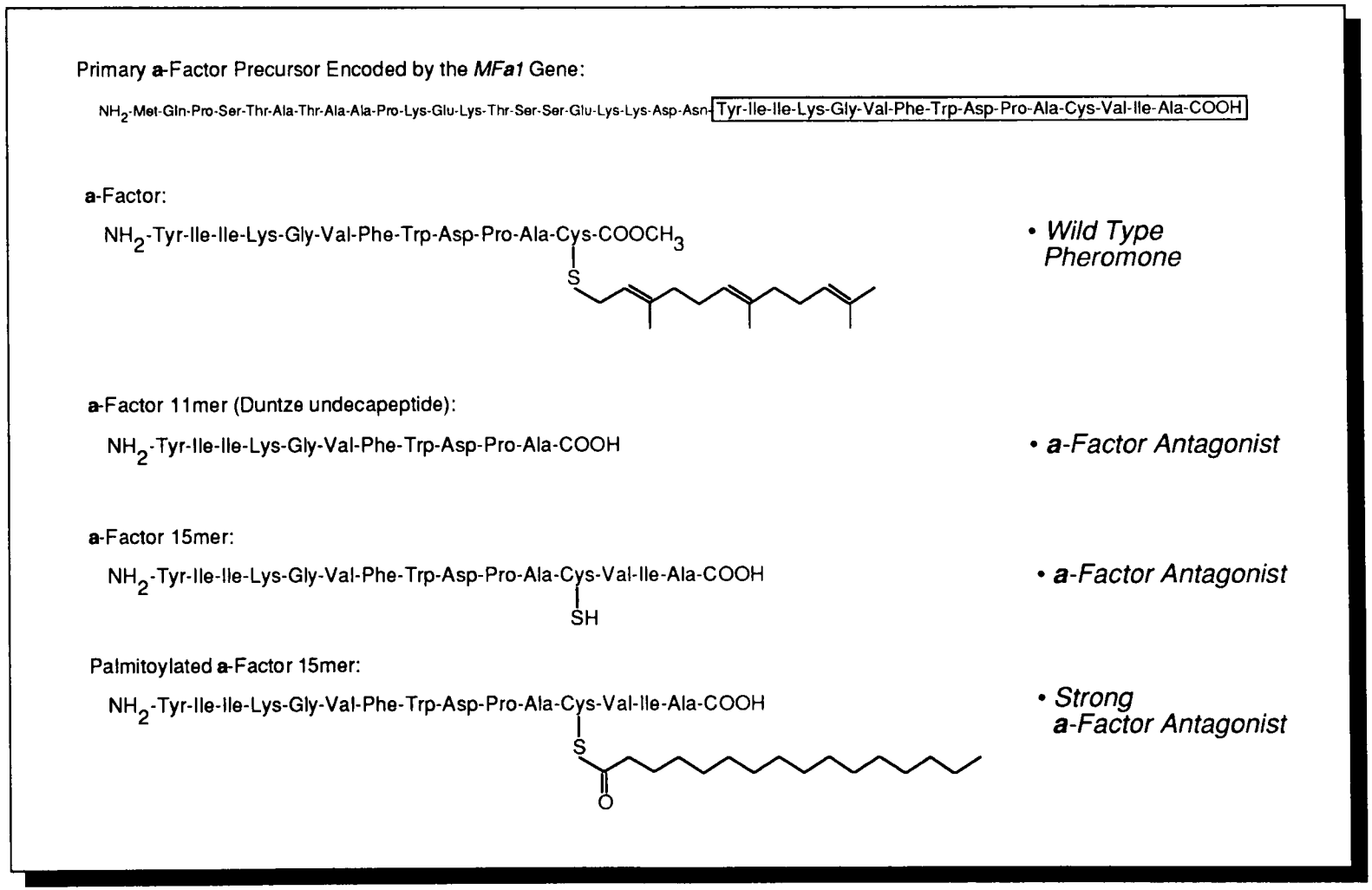

Fig. 1. Representative **a**-factor analogs synthesized prior to this dissertation research.

We synthesized the undecapeptide, YIIKGVFWDPA (fig. 1), corresponding to the sequence predicted by the Duntze group (4), and found it to be biologically inactive. It was, however, a weak **a**-factor antagonist (3). We synthesized a pentadecapeptide, YIIKGVFWDPACVIA, consisting of the Duntze sequence plus three additional C-terminal amino acids encoded by the *MFa1* gene (fig. 1). This peptide was found to be biologically inactive, but was, like the undecapeptide, also an **a**-factor antagonist (3). About this time, a paper of major significance (20) was published on the isolation of *S. cerevisiae* mutants which were defective in both **a**-factor secretion and normal localization and function of RAS, the yeast homolog of mammalian Ras. The gene cloned via complementation of this mutant phenotype was termed *RAM*, for RAS and **a**-factor maturation. An earlier report (14) had suggested that *S. cerevisiae* RAS proteins were post-translationally modified via palmitoylation. Interestingly, the primary **a**-factor and RAS precursors each contained the C-terminal amino acid sequence, -CAAX, (C is cysteine, A is an aliphatic amino acid, and X is any amino acid). Speculation began to arise that **a**-factor, like RAS, might be fatty acylated, that the -CAAX sequence might be the target of this modification, and that the *RAM* gene might encode the acyl transferase responsible for both **a**-factor and RAS modifications. Clearly, RAS and **a**-factor shared common post-translational processing events, and since defective *ras* genes were present in many human tumors (2), the study of **a**-factor took on even greater significance.

Believing that **a**-factor might be fatty acylated, we synthesized a palmitoylated pentadecapeptide, YIIKGVFWDPAC(palmitoyl)VIA (fig. 1). While this lipopeptide was still not biologically active, it was a much stronger **a**-factor antagonist than either the unmodified pentadecapeptide or the undecapeptide, suggesting that **a**-factor may indeed be a lipopeptide (3). Shortly thereafter, Duntze's group published yet another amino acid sequence for **a**-factor–YIIKGVFWDPAC (5). They also provided evidence that the C-terminal cysteine was S-alkylated and that the C-terminal carboxyl might be blocked. They

subsequently reported that the C-terminal cysteine was modified by both an S-farnesyl group and a carboxy methyl ester (1). Strikingly, other researchers later determined that, while only some Ras proteins are palmitoylated, all Ras proteins are isoprenylated (16). As a result of these and other studies (21, 24), it was now believed possible that the *RAM* gene might encode the farnesyl transferase responsible for isoprenylation of both **a**-factor and RAS. Other groups showed that many Ras proteins are also subject to carboxy terminal methyl esterification (12, 15), but the importance of this modification to the normal function of RAS and **a**-factor was not known.

Studies from several laboratories identified two genes, in addition to *RAM* and the **a**-factor structural genes, *MFa1* and *MFa2*, which were involved in **a**-factor maturation and secretion–*STE6* and *STE14* (fig. 2) (for review, see reference 11). The product encoded by the *STE6* gene displayed a high degree of homology to mammalian multiple-drug resistance transporters and bacterial peptide permeases (17, 19). As a result, it was believed that STE6 was responsible for transporting **a**-factor out of **a** cells; *ste6* mutants were, in fact, shown to produce mature **a**-factor intracellularly (17). While the *RAM* gene was thought to possibly encode a farnesyl transferase, and STE6 was believed to be an **a**-factor transporter, the function of the *STE14* gene remained elusive. One of the goals of this dissertation research was to characterize the order of **a**-factor maturation events and to attempt to assign definitive functions to one or more of the genes known to be involved in the maturation process. An **a**-factor maturation assay was successfully developed, and the *RAM* gene was shown to be required for farnesylation of **a**-factor. The work is summarized in Part II of this dissertation.

Shortly after publication of the structure of **a**-factor by Duntze's group, our research group succeeded in chemically synthesizing the pheromone (26). While other investigators had shown that farnesylation of **a**-factor was required for its secretion, it was unclear what role, if any, the modification played in the biological activity of the pheromone, nor was it

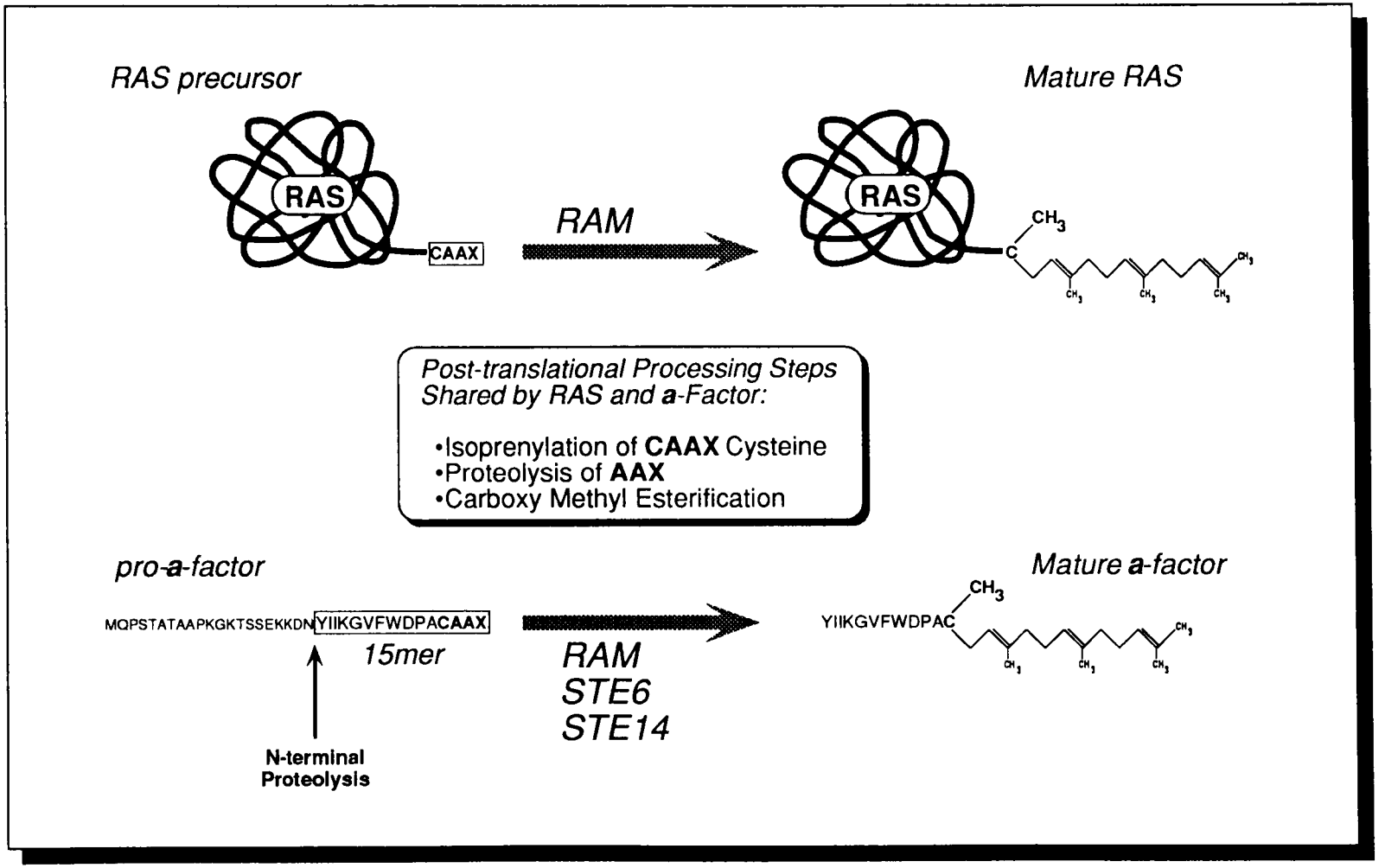

Fig. 2. Post-translational processing steps known to be shared by *S. cerevisiae* RAS and **a**-factor when this dissertation research was initiated. Genes which were known to encode products required for processing are indicated directly above (RAS) and below (**a**-factor) the arrows. The order of the post-translational processing steps was not known.

known whether the C-terminal carboxy methyl ester was required for activity. Duntze's group reported that the farnesyl group was dispensable, whereas the carboxy methyl ester was absolutely required for biological activity (1). However, their results were not conclusive. Because of the unusual structural characteristics of **a**-factor, the pheromone was an exceptional model for the study of lipopeptide hormone-target cell interactions. As a first step in studying these interactions, a variety of Cys^{12} analogs were synthesized for the purpose of fully clarifying the roles of farnesylation and carboxy methyl esterification to the biological activity of **a**-factor. The comparative analysis of the biological activity of these **a**-factor analogs, which represents the first structure-activity relationship study of this pheromone, is summarized in Part III of this dissertation.

In addition to their value in the study of structure-activity relationships, the availability of relatively large quantities of synthetic **a**-factor and various **a**-factor analogs placed us in the unique position of being able to investigate another aspect of **a**-factor-α-cell interactions–that of a potential α cell specific **a**-factor degrading activity. Studies done a number of years ago by Ciejek and Thorner (10) showed that **a** cells degrade α-factor as a mechanism of desensitization from the pheromone response. Later, it was shown that mutants defective in the protease responsible for α-factor degradation were supersensitive to the pheromone (8, 9). While there was some speculation that α cells might similarly degrade **a**-factor, there was no direct evidence to support such speculation. However, using radiolabeled **a**-factor, we were able to demonstrate that **a**-factor is indeed degraded by an α cell specific endopeptidase, which we have called **a**-factorase. Like its **a** cell specific counterpart, **a**-factorase appears to play a role in desensitization of cells from the pheromone response. In addition, **a**-factorase has several characteristics which are similar to those of mammalian neutral endopeptidase, a protein thought to be involved in common acute lymphocytic leukemia (18). The identification and characterization of **a**-factorase is described in Part IV of this dissertation.

Finally, Part V of this dissertation summarizes the work discussed in Parts II-IV and discusses some results in greater detail. In addition, an attempt is made to connect the more interesting and important aspects of Parts II-IV of the dissertation and to discuss possible directions for future research.

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PART II

TOTAL *IN VITRO* MATURATION OF THE *SACCHAROMYCES CEREVISIAE* a-FACTOR LIPOPEPTIDE MATING PHEROMONE*

* Part II appeared in modified form as **Marcus, S., G.A. Caldwell, C.-B. Xue, F. Naider, and J.M. Becker.** 1990. Total *In Vitro* Maturation of the *Saccharomyces cerevisiae* a-Factor Lipopeptide Mating Pheromone. Biochem. Biophys. Res. Comm. **172:**1310-1316.

CHAPTER I

INTRODUCTION

Sexual conjugation between *Saccharomyces cerevisiae* **a** and α haploid cells is dependent upon the reciprocal action of peptide pheromones termed **a**-factor, which is secreted by **a** cells, and α-factor, which is secreted by α cells. α-Factor maturation and secretion occurs via the yeast secretory pathway (6). In contrast, **a**-factor, an isoprenylated peptide (1), is processed by an alternative pathway which contains post-translational processing events shared by the *ras* proto-oncogene product (7, 8, 16, 17). Essential for proper **a**-factor and Ras maturation is a C-terminal amino acid sequence, -CAAX (C is cysteine, A is aliphatic amino acids, and X is any amino acid), contained in the primary precursors of each of these peptides. The -CAAX processing steps include isoprenylation of the cysteine sulfur, proteolysis of the -AAX sequence, and carboxy methyl esterification (7, 8). Studies have shown that isoprenylation is essential for normal Ras function (7, 13). Isoprenylation is, likewise, important for **a**-factor bioactivity, as removal of the isoprenyl moiety results in a marked decrease in activity (11). Vorburger et al. (19) demonstrated that rabbit reticulocyte lysates were capable of catalyzing the isoprenylation of nuclear lamins. In addition, *S. cerevisiae* cell extracts were recently used to show that the product of the *RAM/DPR1* gene (9, 13) is a component of the activity which catalyzes the isoprenylation of both *S. cerevisiae* Ras and **a**-factor (17). In this report, we present the first *in vitro* assay which demonstrates total maturation to **a**-factor and detects intermediates in this pathway. This assay may prove useful in screening for potential anti-Ras-related cancer therapeutic agents capable of blocking any of the steps involved in the post-translational modification of the **a**-factor and Ras -CAAX sequences.

CHAPTER II

MATERIALS AND METHODS

Preparation of yeast extracts and strains used

S. cerevisiae cultures grown at 30 °C to mid-log phase in YEPD (1% yeast extract, 2% peptone, and 2% dextrose) were harvested by centrifugation, washed once with sterile distilled H_2O, and resuspended to 0.3 g/ml (wet weight/volume) in ice-cold lysis buffer (7.5 mM KH_2PO_4, 42.5 mM K_2HPO_4, 2 mM EDTA, 20 mM b-mercaptoethanol, and 20 mM $MgCl_2$, pH 7.6). Cells were lysed using glass beads (0.45-0.50 mm, B. Braun, Germany) by vortexing for 60 sec intervals, with placement in ice for 2 min between each interval. Unbroken cells were removed by centrifugation for 10 min. at 1000x*g*, and the supernatant was centrifuged for 45 min at 30,000x*g*. The resulting supernatants (cell extracts) were diluted to 5 mg protein/ml, after estimation of protein concentration (Bio-Rad Protein Assay Concentrate), and stored at -70 °C until use. Cell extracts were prepared from the following strains: X2180-1A (*MATa SUC2 mal mel gal2 CUP1*) (Yeast Genetics Stock Center [YGSC]), SM1267 (*MATa ram^{ts}his3 ura3 trp1 ade8 can1*) (from Susan Michaelis), and SM1287 (isogenic to SM1267, but transformed with the episomal plasmid pYPG1, which contains the *RAM/DPR1* gene, thus allowing for overexpression of this gene) (also a gift from Susan Michaelis).

Synthesis of **a**-factor precursors

The various **a**-factor analogs used in this study are shown in Fig. 1. The **a**-Factor (NH_2-YIIKGVFWDPAC[S-farnesyl]-OCH_3) and **a**-factor pentadecapeptide (NH_2-YIIKGVFWDPA-CVIA-COOH) were synthesized as previously reported (2, 20, 21). All other analogs were synthesized using either solution-phase peptide synthesis or a combination of solid-phase and solution-phase peptide synthesis. All peptides were

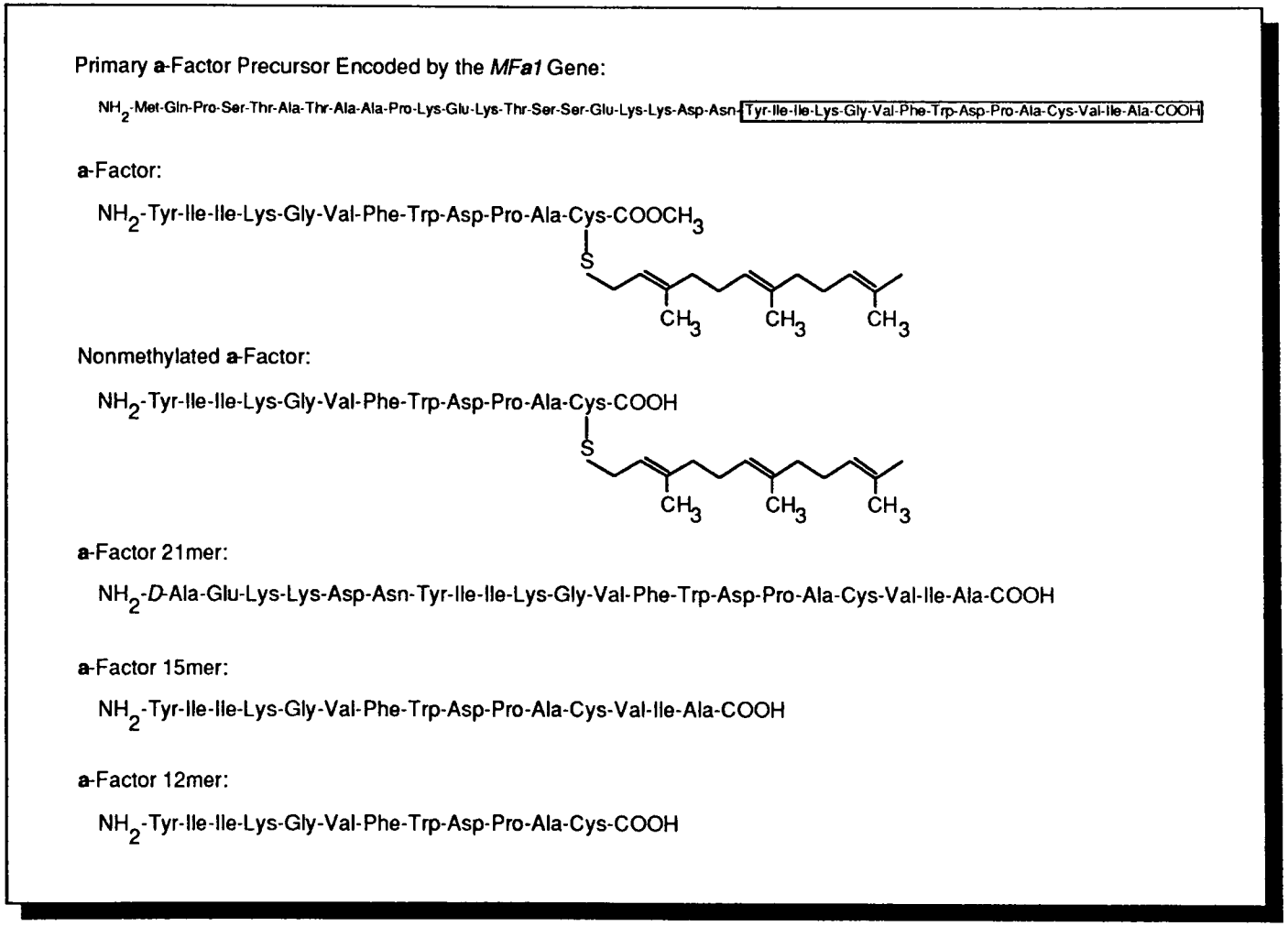


Fig. 1. Structures of a-factor and various a-factor analogs used in this study.

purified to greater than 98% homogeneity using reversed phase HPLC and were characterized using amino acid analysis, 400 MHz ^{1}H-NMR spectroscopy and Fast Atom Bombardment mass spectrometry. Details of the synthesis and characterization of these compounds will be published separately.

a-Factor maturation assay

The standard **a**-factor maturation assay was a modification of the assay described by Reiss et al. (14a) and consisted of the following components: 50 µl assay buffer (50 mM Tris-Cl, pH 7.5, 50 µM $ZnCl_2$, 20 mM KCl, 1 mM DTT) containing 1 mg/ml **a**-factor precursor, 2 µl [^{3}H]farnesyl pyrophosphate ([^{3}H]FPP) solution (from New England Nuclear, 10 Ci/ml, 0.0125 µM/ml), and 10 µl cell extract (50 µg protein). Reaction mixtures were incubated at 30 °C for 5 hr, then stopped by adding 62 µl of glacial acetic acid and placing in dry ice. Assays were analyzed by HPLC (Beckman Instruments, System Gold computer software) using the following methodology: column: DuPont Zorbax Protein Plus C_3 Reversed Phase; flow rate: 1.5 ml/min; gradient: 15% acetonitrile (ACN) from 0 to 10 min, 15 to 80% ACN from 10 to 75 min (1%/min). One min fractions were collected, then counted by liquid scintillation (Beckman LS 7000).

CHAPTER III

RESULTS

S. cerevisiae Cell Extracts Isoprenylate **a**-Factor Precursors

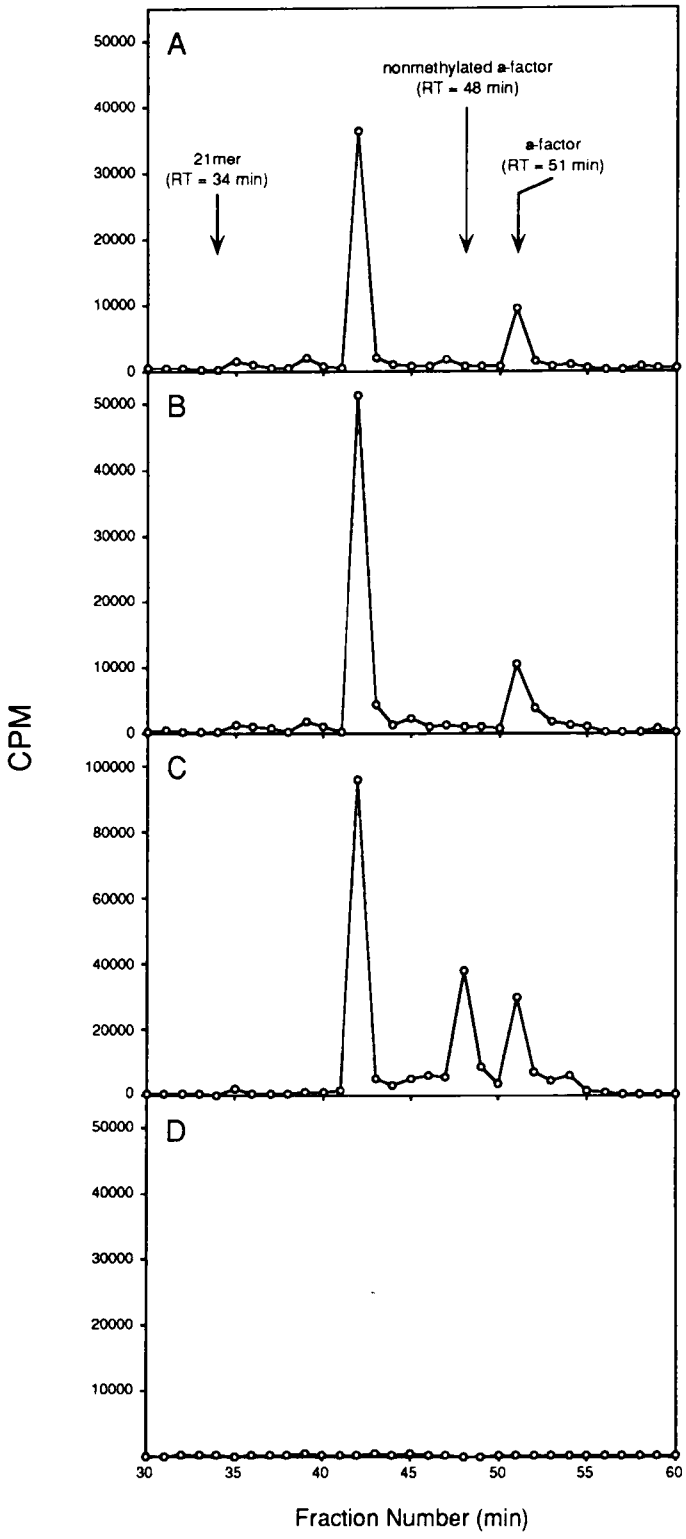
Because several post-translational modification steps are shared by Ras proteins and the *S. cerevisiae* **a**-factor, we set out to develop an assay whereby these processes could be studied *in vitro*. We synthesized potential **a**-factor precursors, as well as standards necessary for the identification by HPLC of **a**-factor and potential biosynthetic intermediates (Fig. 1). We found that *S. cerevisiae* cell extracts were capable of catalyzing isoprenylation of the both the **a**-factor 21mer and 15mer peptide precursors (Fig. 2A and 2B, respectively), producing a major product (retention time [RT] = 42 min) which we believe, based on similar results obtained by Schafer et al. (17), to be farnesylated 15mer. The additional steps of proteolysis, producing farnesylated, nonmethylated **a**-factor (detectable by using cell extract prepared from the *RAM/DPR1* overexpressing strain, Fig. 2C, RT = 48 min), and carboxy methyl esterification, producing mature **a**-factor (Fig. 2A, B, and C; RT = 51 min), were also detected.

The fact that 21mer maturation apparently produced the same products as 15mer maturation also suggested that proteolysis of the N-terminal precursor sequence was detectable in this assay. The omission of the **a**-factor precursor peptide resulted in no **a**-factor-related products, including the 42 min peak (Fig. 2D). This indicates that the 42 min peak is not a degradation product of [^{3}H]farnesyl pyrophosphate ([^{3}H]FPP).

Methylation of an **a**-Factor Precursor by *S. cerevisiae* Cell Extracts

To provide additional evidence that the 42 min RT peak was a farnesylated **a**-factor precursor, we added nonradiolabeled S-adenosyl-*L*-methionine (SAM) to the assay. We expected that if the 42 min peak was indeed a farnesylated **a**-factor precursor, addition

Figure 2. *In vitro* maturation of **a**-factor. **a**-Factor maturation assays were performed, then analyzed by HPLC and liquid scintillation, as described in the Materials and Methods section. A) Maturation of **a**-factor 21mer by X2180-1A (wild type) cell extract. B) Maturation of **a**-factor 15mer by X2180-1A cell extract. C) Maturation of **a**-factor 21mer by SM1287 (*RAM/DPR1* overexpressing) cell extract. D) Assay without **a**-factor precursor. The retention times (RT) indicated in panel A were determined using synthetic peptide standards.


A
nonmethylated a-factor
(RT = 48 min)
21mer
(RT = 34 min)
a-factor
(RT = 51 min)
B
C
D
CPM
Fraction Number (min)

of SAM would favor further processing of this product to mature **a**-factor. As shown in Fig. 3A, addition of SAM did allow for the conversion of the 42 min RT peak to mature **a**-factor. Since the same 42 min RT peak is obtained when the **a**-factor 15mer precursor is used in this assay, we believe that this peak represents farnesylated 15mer. Schafer et al. (17) reported farnesylation of a 15mer in their assay, but did not observe additional processing to **a**-factor.

To confirm that we were indeed detecting total maturation of **a**-factor, we used S-[methyl-^{3}H]adenosyl-*L*-methionine ([^{3}H]SAM) and nonradiolabeled FPP in our **a**-factor maturation assay. Only a single radiolabeled product (RT = 51 min) was produced under these assay conditions (Fig. 3B). Thus, this peak is farnesylated, carboxy methyl esterified, and coelutes with synthetic **a**-factor, strongly suggesting the formation of the mature pheromone. Since the **a**-factor 12mer (Figure 1) is not processed (not shown), and since only mature **a**-factor, but not the isoprenylated precursors (RT's = 42 and 48 min, respectively), is methylated, we have the first direct biochemical evidence for the order of **a**-factor -CAAX maturation steps: 1) isoprenylation, 2) proteolysis of the -AAX sequence, producing nonmethylated, farnesylated **a**-factor, then 3) carboxy methyl esterification.

The *RAM* Gene Product is Required for Isoprenyl Transferase Activity

A recent report (17) provided evidence that the *RAM/DPR1* gene product is a component of the activity responsible for the isoprenylation of **a**-factor and Ras. We have obtained results which strongly support this conclusion. Extracts prepared from a strain which overexpresses *RAM/DPR1* (SM1287) had much greater isoprenyl transferase activity than extracts prepared from wild type (X2180-1A) (Fig. 4) cells. An accumulation of nonmethylated **a**-factor (RT = 48 min)) was also apparently detectable when extracts from the *RAM/DPR1* overexpressing strain were used. Extracts from *ram/dpr1* mutants, however, contained no detectable isoprenyl transferase activity under our assay conditions.

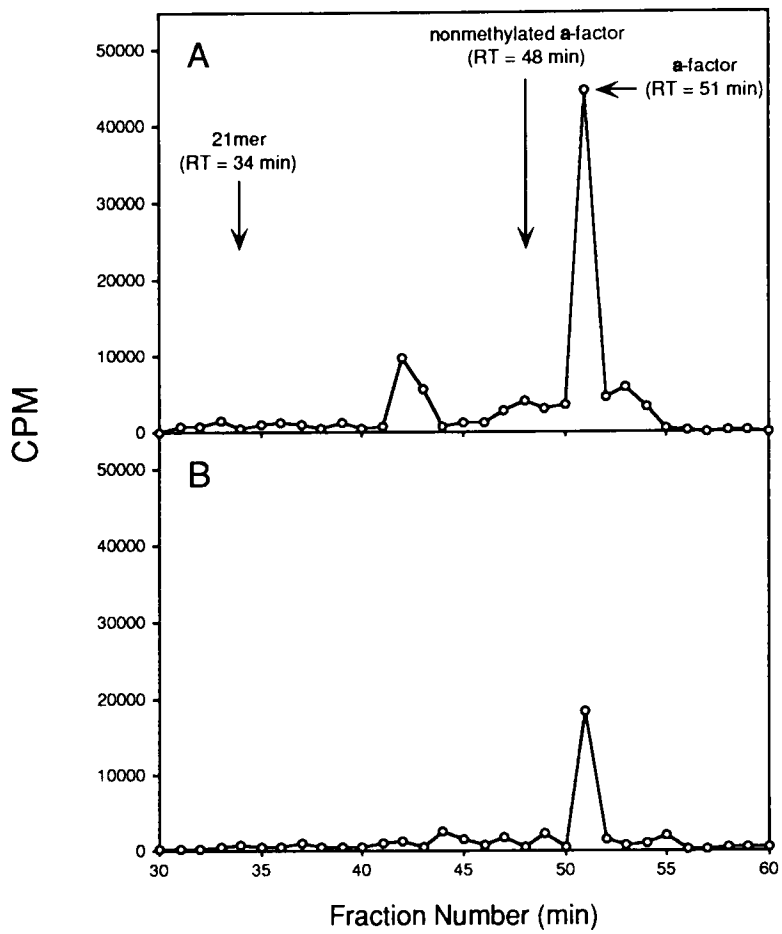


Figure 3. *In vitro* methylation of **a**-factor. **a**-Factor maturation assays were performed using **a**-factor 21mer and X2180-1A cell extract. A) Effect of addition of S-adenosyl-*L*-methionine (SAM) (to 160 µg/ml) to the standard maturation assay. B) **a**-Factor maturation assay using [^{3}H]SAM. Nonradiolabeled farnesyl pyrophosphate (to 320 µg/ml) was added to the reaction.

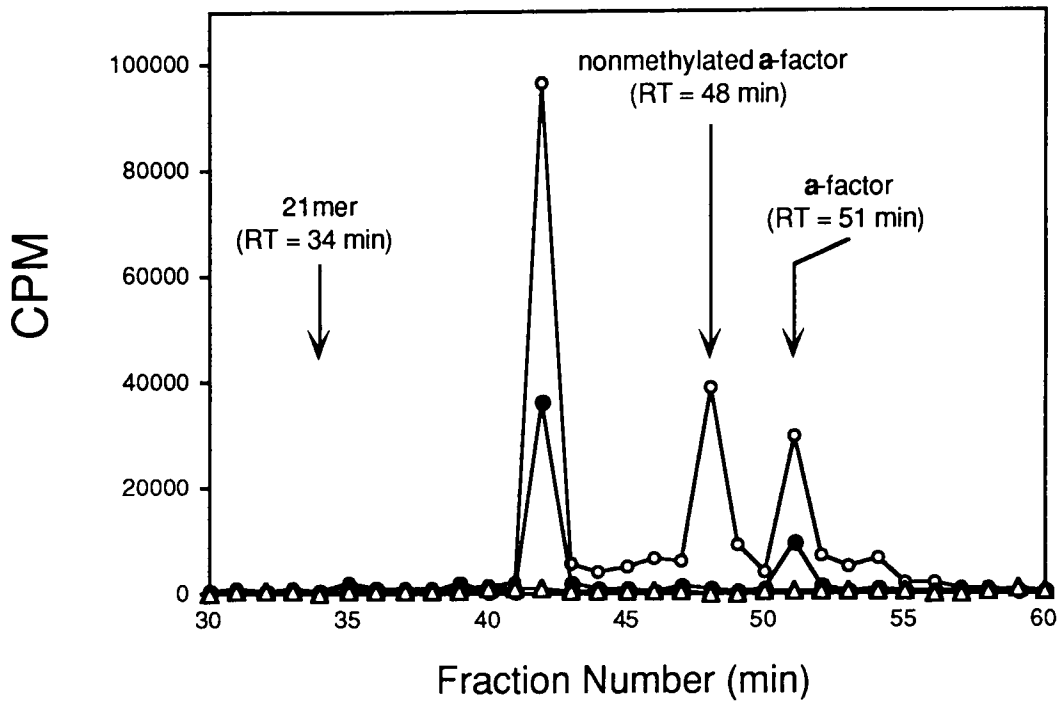


Figure 4. Evidence that the *S. cerevisiae RAM/DPR1* gene product is required for isoprenyl transferase activity. **a**-Factor maturation assays were performed using **a**-factor 21mer. Open circles represent maturation by SM1287 (*RAM/DPR1* overproducing) cell extract. Closed circles represent maturation by X2180-1A (wild type) cell extract. Extract from a *ram/dpr1* mutant (SM1267) lacked isoprenyl transferase activity (open triangles).

Isoprenyl Transferase Activity is Dependent on a Divalent Cation

Recent studies have shown that the isoprenylation of $p21^{ras}$ by a mammalian farnesyl transferase is dependent on a divalent cation (14a). However, it was not determined whether the cation was required for isoprenyl transferase activity *per se*, or required for the Ras protein to maintain a conformation necessary for it to be a suitable target for the transferase, or both. To investigate whether *S. cerevisiae* isoprenyl transferase activity requires a divalent cation, **a**-factor maturation assays were carried out in the presence of EDTA, an effective chelator of divalent cations. As shown in figure 5, EDTA completely inhibited isoprenyl transferase activty. Preliminary studies by our laboratory group suggest that **a**-factor does not maintain preferred conformational state, and we are aware of no peptides comprised of fewer than 15 amino acid residues which require a divalent cation for maintaining a preferred conformation. Thus, our results provide the first evidence that the *S. cerevisiae* isoprenyl transferase, and in all likelihood its mammalian counterpart as well, requires a divalent cation for activity.

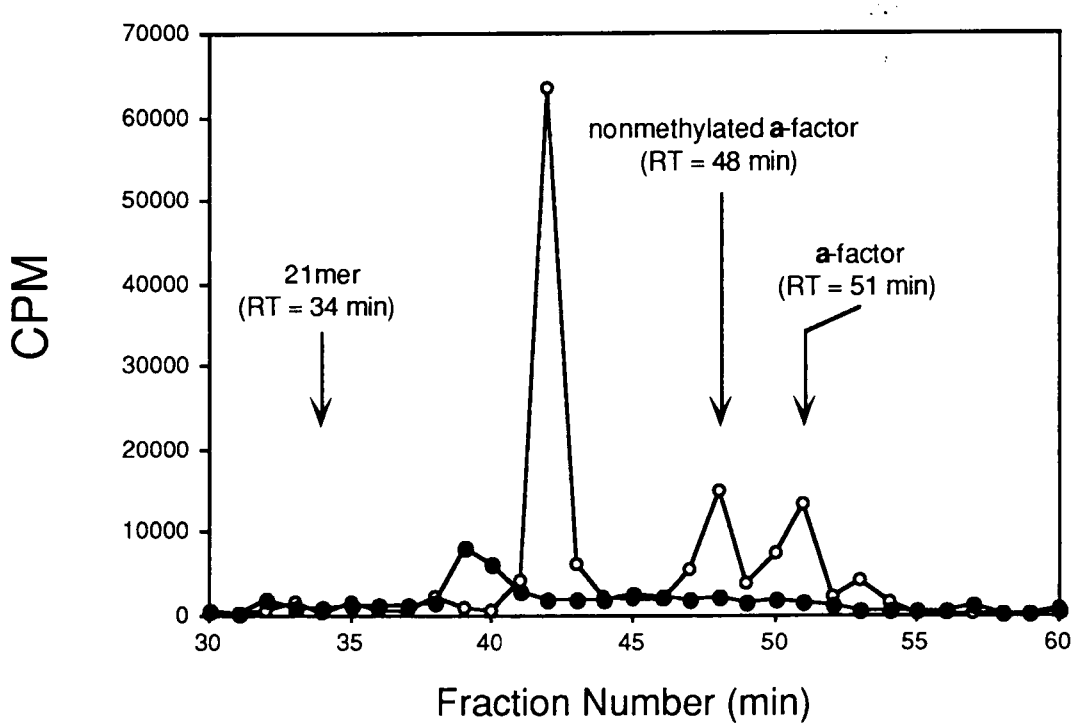


Figure 5. Isoprenylation of **a**-factor is dependent on a divalent cation. **a**-Factor maturation assays were performed, then analyzed by HPLC and liquid scintillation, as described in the Materials and Methods section using **a**-factor 21mer in the absence (open circles) and presence (closed circles) of 50 mM EDTA. The product eluting at 39-40 min (closed circles, 50 mM EDTA expt) was shown to be a degradation product of [^{3}H]FPP.

CHAPTER IV

DISCUSSION

Isoprenylation is clearly an important post-translational modification common to a number of eukaryotic proteins (7, 8). Furthermore, this modification has been shown to be essential for normal Ras function (13) and for maximal **a**-factor activity (11). Studies suggest that mutations which prevent isoprenylation of activated Ras significantly decrease or abolish its ability to transform cells. In addition, some evidence suggests that doses of cholesterol-reducing drugs that do not adversely affect cell growth, might be useful in inhibiting the transforming potential of activated Ras proteins (5, 16). Thus, isoprenylation is now considered a possible target for anti-Ras-related cancer therapeutic agents. The significance of carboxy methyl esterification to Ras function has not yet been established, although it has been speculated that this modification may have an important role in the activity of this protein (4).

In this report, we have presented the first assay which allows for the total maturation of **a**-factor *in vitro* and for the detection of intermediates of the maturation pathway. We have demonstrated that the order of **a**-factor -CAAX processing steps is as follows: 1) isoprenylation of the -CAAX cysteine, 2) proteolysis of -AAX, then 3) carboxy terminal methyl esterification. In addition, our results indicate that processing of the **a**-factor -CAAX sequence is not dependent on precursor sequence N-terminal of the **a**-factor peptide itself, since a precursor (15mer) lacking this N-terminal sequence was processed in our assay. It was further demonstrated that the *RAM* gene product is required for isoprenylation of **a**-factor, since *ram* mutants were totally devoid of detectable isoprenyl transferase activity. This result supports evidence obtained by Schafer et al. (17) which suggested that RAM was actually a component of an *S. cerevisiae* isoprenyl transferase.

The specific catalytic mechanisms of isoprenylation have not yet been elucidated. Reiss et al. (14a) have recently partially purified a mammalian farnesyl transferase, but have not yet structurally characterized the enzyme nor cloned the gene(s) which encode(s) it. They have shown that the transferase binds to CAAX tetrapeptides and that such peptides also act as inhibitors of the farnesylation of mammalian $p21^{ras}$ (14a, 14b). Reiss et al. (14b) have suggested that some CAAX peptides may themselves be substrates for the farnesyl transferase, although the smallest confirmed substrate was a -CAAX-containing heptapeptide.

The assay described in this paper may prove useful for the screening of inhibitors which might block any one of the steps common to **a**-factor and Ras maturation, thus, allowing for the identification of drugs potentially effective against Ras-related cancers. Additional aspects of **a**-factor maturation are discussed in greater detail in Part V of this dissertation.

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PART III

SIGNIFICANCE OF C-TERMINAL CYSTEINE MODIFICATIONS TO THE BIOLOGICAL ACTIVITY OF THE *SACCHAROMYCES CEREVISIAE* **a**-FACTOR MATING PHEROMONE*

* Part III appeared in modified form as **Marcus, S., G.A. Caldwell, D. Miller, C.-B. Xue, F. Naider, and J.M. Becker.** 1991. Significance of C-terminal cysteine modifications to the biological activity of the *Saccharomyces cerevisiae* **a**-factor mating pheromone. Mol. Cell. Biol. **11**:*in press.*

CHAPTER I

INTRODUCTION

Numerous proteins are now known to be modified post-translationally by addition of prenyl groups. Prenyl, also called isoprenyl, groups are comprised of anywhere from one (prenyl) to three (farnesyl) or more isoprene ($-CH_2C(CH_3)=CH_2-$) subunits. The consensus sequence for prenylation appears to be the C-terminal "-CAAX box", where C is cysteine, A is an aliphatic amino acid, and X is any amino acid (18, 19). The modification involves addition of the prenyl group to the cysteine sulfur followed by proteolysis of the C-terminal -AAX (38, 54). Examples of prenylated proteins include yeast and mammalian RAS (6, 53, 54), the γ subunit of yeast and mammalian G-proteins (16, 37), nuclear lamins (60), and various fungal mating pheromones (2, 30, 50, 51). In some cases, but not all, the carboxyl group of the C-terminal cysteine is also methyl esterified (2, 11, 13). Recent studies (14, 36, 49) suggest that other mammalian proteins are prenylated, indicating that this type of post-translational modification may be common to many proteins in eukaryotic organisms.

It is now recognized that prenylation is required for proper localization and function of mammalian and yeast RAS (47, 54), and maximal biological activity of several fungal mating pheromones (2, 58, 62). In addition, prenylation of the *S. cerevisiae* **a**-factor seems to be required for secretion of this peptide mating pheromone (47, 53). The significance of the second modification, the carboxy terminal methyl ester, found on some prenylated proteins is not as well understood. It is known, however, that this modification is important for the biological activity of some fungal pheromones (2, 17, 62). The purpose of this work was to further study the significance of prenylation and methyl esterification to the biological activity of the *S. cerevisiae* **a**-factor.

Conjugation between *S. cerevisiae* **a** (*MATa*) and α (*MATα*) haploid cells to form **a**/α diploids requires the reciprocal action of **a**-factor, produced by *MATa* cells, and α-factor, a peptide pheromone secreted by *MATα* cells (12, 24, 25). These pheromones induce in their target cells similar responses which are required for mating: growth arrest occurs at G_1 of the cell cycle (5, 23), cell surface agglutinins are produced (15), transcription of several genes is induced (1, 32, 59), and the cells undergo a morphological alteration termed "shmooing" (35, 56). α-Factor, a tridecapeptide, is processed and secreted via the well-characterized yeast secretory pathway comprised of the endoplasmic reticulum, Golgi, and secretory vesicles. Considerable studies on α-factor structure-activity relationships have been published (46). On the other hand, structure-activity relationship studies on **a**-factor, which is apparently secreted by a novel secretory pathway which has not been fully characterized (4, 27, 33, 38, 40, 42, 53, 54), have only recently been initiated. Anderegg et al. (2) found that replacement of the farnesyl group of **a**-factor by a methyl group (S-methyl **a**-factor) caused only a moderate decrease in biological activity. These workers concluded that the farnesyl group of **a**-factor was not required for biological activity. We have previously shown that replacement of the farnesyl group of **a**-factor by a hydrogen atom (nonfarnesylated **a**-factor) results in an approximately 800-fold decrease in biological activity (62).

While **a**-factor in the absence of *MATa* cells is capable of inducing a biological response in *MATα* cells, it had been suggested that *MATa* cells must actively produce **a**-factor in order to mate (43). In those studies, *MATa* cells having deletions of the **a**-factor structural genes (*mfa1 mfa2* mutants) were not capable of mating with wild type ($SST2^+$) *MATα* cells, even in the presence of exogenous **a**-factor. This result led the authors to hypothesize that the **a**-factor found extracellularly is not sufficient for mating, but rather that a *MATa* cell-bound form of **a**-factor might be required for mating. Recent studies by Jackson and Hartwell further demonstrated the importance of both **a**- and α-factor

production in a process they termed courtship (28, 29), a process by which opposite mating types select the mating partner which produces the most pheromone.

To further study **a**-factor structure-activity relationships, we synthesized several **a**-factor analogs and examined the role of C-terminal cysteine modifications in the biological activity of this lipopeptide mating pheromone. In this report, we show by use of four different and sensitive biological assays that removal of either the farnesyl group or the carboxy terminal methyl ester of **a**-factor leads to a similar reduction in the bioactivity of the modified pheromones. In addition, we show that the Cys-farnesyl group is not specifically required for high biological activity, since replacement of this group with any of several different hydrophobic hydrocarbon moieties resulted in analogs with biological activity close to that of **a**-factor. We further show that addition of exogenous **a**-factor allows *MATa mfa1 mfa2* mutants to mate with *sst2* or wild type ($SST2^+$) $MAT\alpha$ cells, demonstrating that it is not absolutely essential for *MATa* cells to actively produce **a**-factor in order to mate.

CHAPTER II

MATERIALS AND METHODS

Strains

Halo and shmoo assays were carried out using wild-type *S. cerevisiae* X2180-1B (*MATα*) (Yeast Genetics Stock Center [YGSC]) or a mutant supersensitive to **a**-factor, RC757 (*MATα sst2-1 rme1 his6 met1 can1 cyh2*) from Russell Chan (7, 8). *FUS1-lacZ* assays were carried out using *S. cerevisiae* strain LM23-116az (*MATα FUS1::lacZ(URA3) leu2 lys5 met1 ura3-52 ste2::TRP1*) from Lorraine Marsh. Mating experiments were done with *S. cerevisiae* strains RC757 (above), CR11 (*MATα cry1-11 thr4 his6 lys1*) from Wolfgang Duntze (55), SM1058 (*MAT**a** trp1 his4 leu2 ura3 can1*) and SM1229 (isogenic to SM1058, but *mfa1Δ::LEU2 mfa2Δ::URA3*) from Susan Michaelis (43), and JPY200 (*MAT**a** gal2 his3Δ200 leu2-3,-112 lys2-801 trp1-1$_{am}$ ura3-52*) and JPY201 (isogenic to JPY200, but *ste6Δ-1::HIS3*) from John McGrath (42).

Synthesis and Characterization of **a**-Factor and Cys^{12} Analogs

The **a**-factor (NH_2-YIIKGVFWDPAC[S-farnesyl]-$COOCH_3$) and **a**-factor analogs used in this study were synthesized by Chu-Biao Xue (College of Staten Island, City University of New York) using either solution-phase peptide synthesis or a combination of solid-phase and solution-phase peptide synthesis. Structures of all analogs are shown in fig. 1. Detailed protocols for the synthesis and characterization of **a**-factor, nonfarnesylated **a**-factor, and nonmethylated **a**-factor have been published previously (61, 62). Details of the synthesis and characterization of all other analogs will be published separately. All peptides were purified to greater than 98% homogeneity using reversed phase HPLC and were characterized using amino acid analysis, 400 MHz H_1-NMR spectroscopy, and Fast

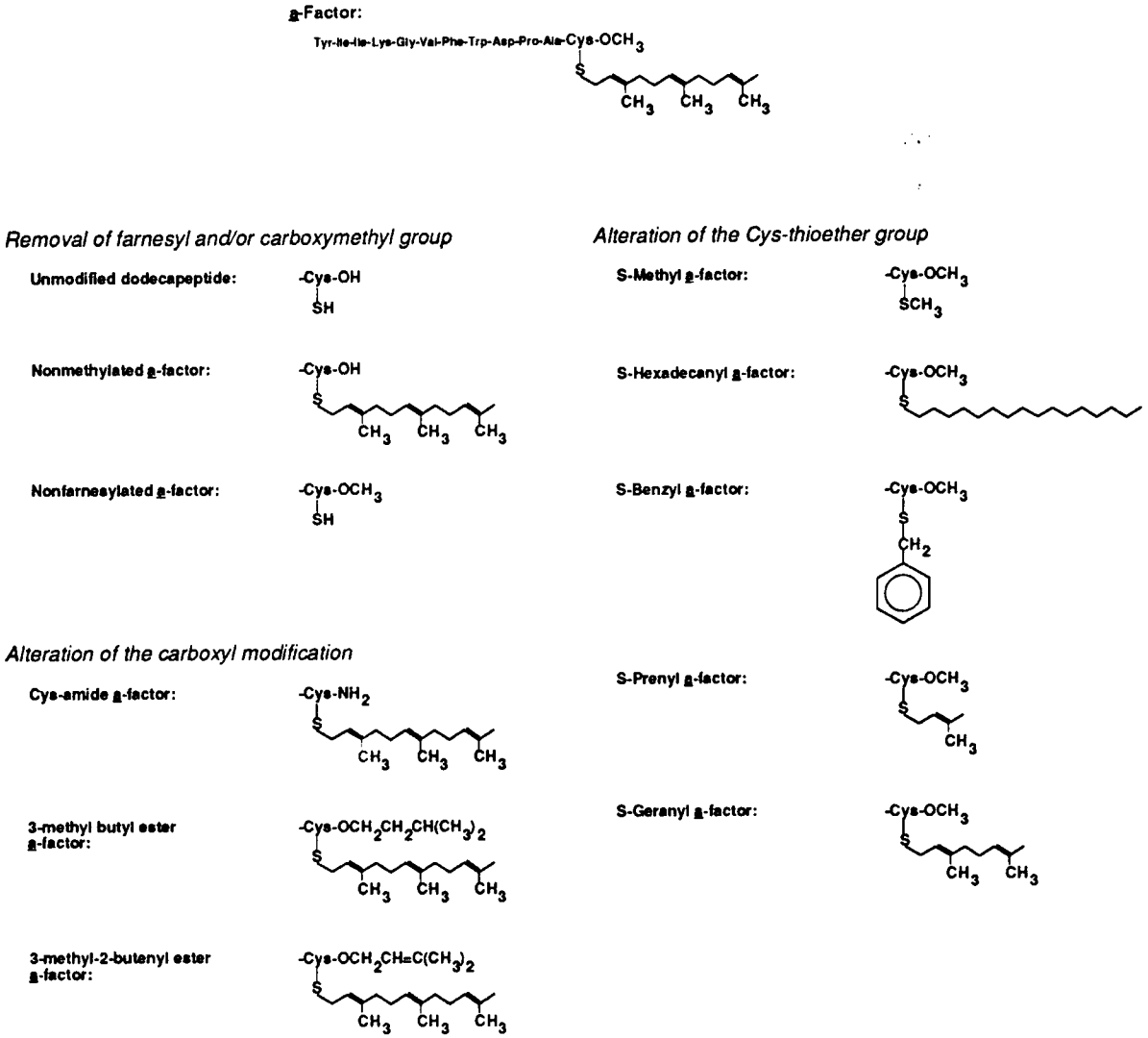

Fig. 1. Structures of *Saccharomyces cerevisiae* a-factor and various Cys^{12} analogs used in this study. All analogs are denoted only by the Cys^{12} modification in this figure. All of the analogs maintained the full peptide backbone of YIIKGVFWDPAC.

Atom Bombardment mass spectroscopy. **a**-Factor and **a**-factor analogs were dissolved directly at 1-5 mg/ml in methanol for use as stock solutions in all biological assays.

Shmoo Assays

RC757 or X2180-1B were grown at 30 °C with shaking to early log phase in YEPD, pH 4.5, with BSA (yeast extract, 1%; peptone, 2%; dextrose, 2%; and 0.2 mg/ml BSA [bovine serum albumin] in 0.05 M citrate, pH 4.5). The cells were harvested by centrifugation at 1000*g*, washed twice with sterile distilled water, and resuspended to 4 x 10^6 cells/ml in YEPD + BSA. Dilution series (1:2) of **a**-factor or **a**-factor analogs were prepared in siliconized borosilicate glass tubes with YEPD + BSA as the diluent. Five hundred µl of cell suspension was added to 500 µl of each of the **a**-factor or **a**-factor analog dilutions, and the resulting cell suspensions were incubated for 3.5 hrs at 30 °C with shaking. At the completion of the incubation period, the cells were placed on ice, and 10 µl portions of each suspension were placed in a hemocytometer and observed microscopically to quantitate the total number of cells, % shmoos (elongated, pear-shaped cells [35, 56]), and % unbudded cells. The endpoint of activity in this assay was defined as the concentration of pheromone or pheromone analog which caused approximately 50% of treated cells to shmoo. At higher concentrations of pheromone, 100% of treated cells were shmoos (fig. 2). The profiles of the response curves to all analogs were highly similar so that the amount of pheromone causing 10% shmoos, the 50% shmoo endpoint, or the minimum amount of pheromone for 100% response all gave nearly identical values for comparison of biological activity.

Halo Assay

YEPD, pH 4.5, (YEPD, pH 4.5 with 2% Bitek agar, Difco) plates were overlayed with 4 ml of RC757 cells (2.5 x 10^5 cells/ml) in noble agar (0.825%). Five microliter portions

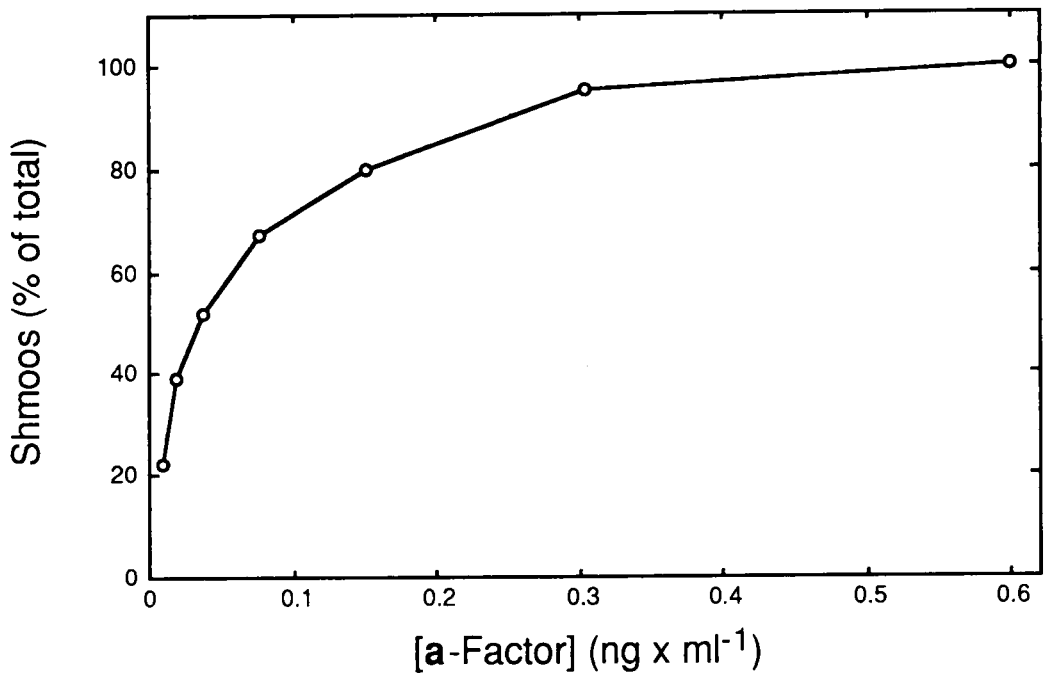

Fig. 2. Shmoo assay of synthetic **a**-factor. RC757 cells were incubated in YEPD for 3.5 hr in the presence of various concentrations of **a**-factor as described in Materials and Methods. At the completion of the incubation period, the cells were observed microscopically to quantitate the total number of cells and % shmoos.

of **a**-factor solutions at various concentrations in 0.2 mg BSA/ml water were then spotted onto the overlay. The plates were incubated at 30 °C for 24 to 36 hrs and then observed for clear zones (halos), an indication of the arrest of cell growth, at the various sample locations (fig. 3).

FUS1-lacZ Induction Assay

S. cerevisiae LM23-116az was grown overnight in YEPD at 30 °C. Cells were diluted to a concentration of 5 x 10^6 cells/ml and grown to 1-2 x 10^7 cells/ml at 30 °C. Cells were concentrated by centrifugation, then resuspended at 10^8 cells/ml. Induction was performed by adding 0.5 ml from dilutions of **a**-factor, **a**-factor analogs, α-factor, or water to 4.5 ml concentrated cells. These mixtures were vortexed and placed at 30 °C with shaking for 2 hrs. After this time, cells were harvested in a tabletop centrifuge and each pellet resuspended in 0.5 ml of Z buffer (16.1g $Na_2HPO_4 \cdot 7\ H_2O$, 5.5g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.246 g $MgSO_4 \cdot H_2O$, 2.7 ml β-mercaptoethanol per 1000 ml). For each sample, the OD_{600} was recorded and 1:5 and 1:10 dilutions of cells:Z buffer in a total volume of 1.0 ml were prepared. Permeabilization of yeast was performed by the addition of 75 µl 0.1% SDS and 60 µl chloroform followed by vortexing for 15 seconds. After equilibration to 30 °C, mixtures were assayed for β-galactosidase activity. Assays were initiated by the addition of 0.2 ml O-nitrophenyl-β-D-galactopyranoside (4 mg/ml in 0.1 M KPO_4, pH 7) to each sample. Upon visualization of a medium-yellow color (10 min to 4 hr), reactions were stopped by adding 0.5 ml 1M Na_2CO_3. Cells from all assay mixtures were pelleted and the supernatant fractions read at OD_{420} and OD_{550} and units of β-galactosidase were calculated (21, 44, 48). The endpoint of activity in this assay was defined as the concentration of pheromone required to induce a response in β-galactosidase units that was two-fold greater than the basal level observed in either untreated LM23-116az cells or LM23-116az cells treated with α-factor (1 µg/ml). Endpoints were calculated as the average

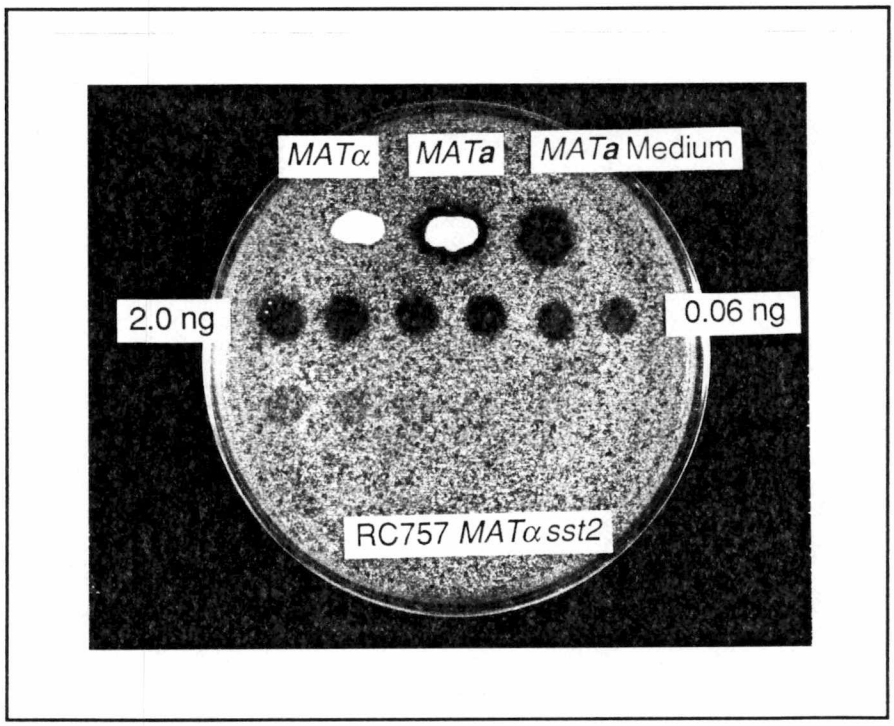

Fig. 3. Halo assay of synthetic **a**-factor. A lawn of RC757 *MATα* cells was prepared as described in Materials and Methods. A 1:2 dilution series of synthetic **a**-factor was prepared in 0.5 mg/ml BSA, then 5 µl of each dilution was spotted on the lawn. Pheromone bioactivity resulted in the formation of clear zones, or halos, at the areas where samples were spotted, an indication of growth arrest. X2180-1A (*MAT**a***) and X2180-1B (*MATα*) were patched and 5 µl of concentrated *MAT**a*** culture medium spotted onto the lawn, as indicated, all as controls. As indicated, the endpoint of **a**-factor activity was 0.06 ng.

value from three separate trials, with each trial being performed in duplicate. In all *FUS1-lacZ* assays performed, the level of β-galactosidase induction correlated to the concentration of pheromone used, such that a gradual increase in β-galactosidase units resulted from a corresponding increase in the concentration of pheromone tested (fig. 4).

Mating Restoration Assay

The mating restoration assay used in this study was a modification of the procedure developed by Horecka and Sprague (26). For dose response assays, stationary phase suspensions of *S. cerevisiae* strains RC757 and SM1229 were prepared in YEPD at 10^7 cells/ml. Equal volumes of the two suspensions were mixed together, then this mixed cell suspension was diluted 1:5 with sterile distilled water. One and one-half ml of the diluted mixed suspension was plated onto SD medium (Yeast Nitrogen Base without amino acids [Difco], 2% dextrose, and 2% Bitek agar [Difco]) for selection of diploids. The plates were incubated at 30 °C for about 5 hr prior to spotting of **a**-factor. **a**-Factor or **a**-factor analog dilutions were prepared using 2 mg/ml BSA as the diluent, then each dilution was spotted onto the mixed lawn of cells. The plates were incubated at 30 °C for two days, at the end of which time constellations of diploid colonies were apparent at the positions where **a**-factor was spotted onto the mixed lawn (fig. 5). The endpoint of **a**-factor activity was defined as the lowest amount of **a**-factor which produced a constellation of diploid colonies.

Mating efficiency assays were performed by a modification of the dose response assay discussed above and the mating assay used by Michaelis and Herskowitz (43). *MATα* cell suspensions were prepared at 10^8 cells/ml in YEPD. *MAT**a*** cell suspensions were prepared in YEPD at concentrations of 10^5, 10^6, 10^7, and 10^8 cells/ml. Equal volumes of *MAT**a*** and *MATα* suspensions were mixed together, then the mixed cell suspensions were diluted 1:5 with sterile distilled water. The remainder of the assay was the same as that used for the dose response assays. The efficiency of mating was calculated as the ratio of the number of

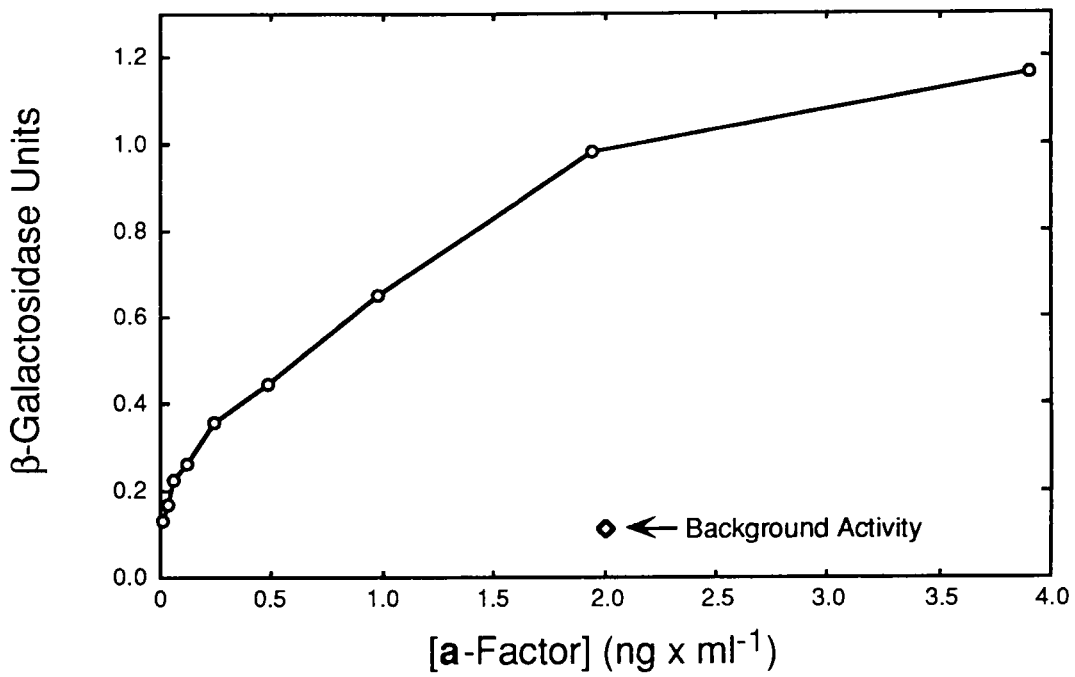

Fig. 4. *FUS1-lacZ* induction assay of synthetic **a**-factor. The *FUS1-lacZ* assay was performed using various concentrations of **a**-factor as described in Materials and Methods. Units of β-galctosidase were calculated as previously described (21, 44, 48).

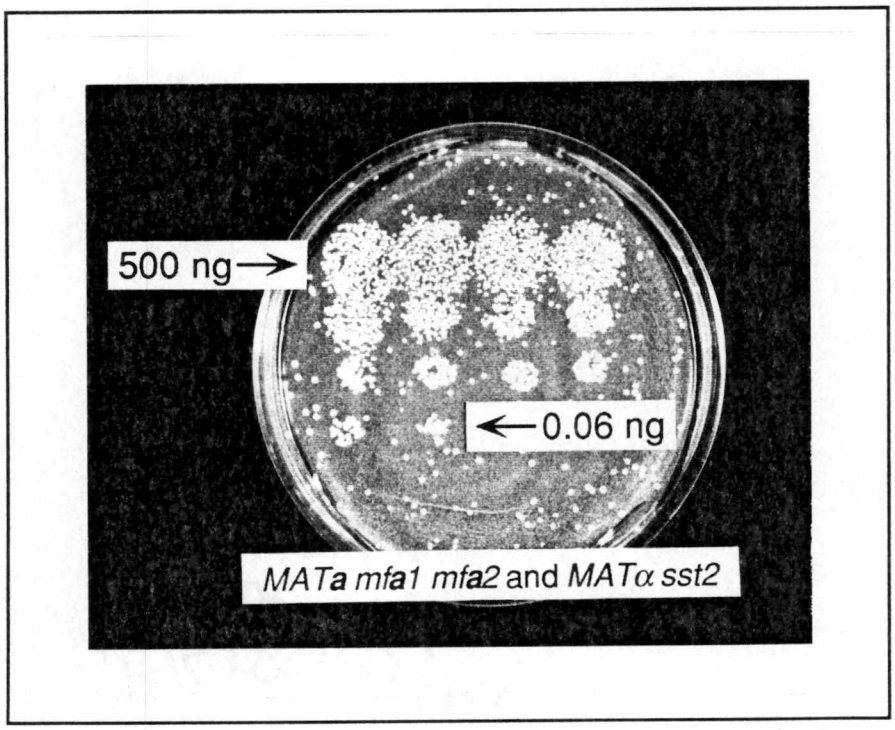

Fig. 5. Mating assay of synthetic **a**-factor. A mixed lawn of RC757 and SM1229 was prepared on minimal medium for selection of diploids as described in Materials and Methods. A 1:2 dilution series of synthetic **a**-factor was prepared in 0.5 mg/ml BSA, then 5 µl of each dilution was spotted onto the mixed lawn of cells. The plates were incubated at 30° for 2-3 days, at the end of which time diploid patches were apparent at the positions where **a**-factor was spotted onto the mixed lawn. As indicated, the endpoint of **a**-factor activity was 0.06 ng.

diploid colonies formed at the affected area (constellation of diploid colonies) to the total number of *MATa* cells calculated to be present in that area (as calculated from the total number of *MATa* cells plated). For example, if the diameter of a constellation of diploids was 10 mm and contained 400 colonies, then the diploid density was 5.1 diploids/mm^2. If a total of 1.5 x 10^6 *MATa* cells were plated on an 85 mm diameter agar plate, the density of *MATa* cells was 264.3 cells/mm^2. The mating efficiency was then calculated as the observed colony density in the diploid constellation (5.1 diploids/mm^2)/density of *MATa* cells (264.3 potential diploids/mm^2), which results in a value of 1.9 x 10^{-2}. In some cases, a dissecting microscope was used to visualize accurately the individual colonies in a small area.

CHAPTER III

RESULTS

Biological Significance of the Carboxy Terminus Methyl Ester and Farnesyl Groups of **a**-Factor

For our analyses of the biological activity of **a**-factor and Cys^{12} analogs, we employed four different assays: 1) the halo assay, an agar diffusion assay which measures pheromone response based on growth arrest of α cells (fig. 3); 2) the shmoo assay, an assay which measures pheromone response based on morphological alteration (shmooing) and growth arrest of α cells; 3) the *FUS1-lacZ* induction assay, an assay which measures pheromone response based on induction of a *FUS1-lacZ* reporter gene (41, 57); and 4) the mating restoration assay, which is discussed in more detail below. To address the significance of the farnesyl and methyl ester modifications to the biological activity of **a**-factor, we tested **a**-factor, unmodified **a**-factor dodecapeptide, nonfarnesylated **a**-factor, and nonmethylated **a**-factor (fig. 1). As shown in Table 1, using three different and sensitive bioassays our results clearly indicate that removal of either the farnesyl or methyl ester group leads to approximately a 100- to 1000-fold reduction in bioactivity. The unmodified **a**-factor dodecapeptide was virtually inactive, showing an endpoint of activity of 2.5 μg in the halo assay (40,000-fold less active than **a**-factor). The endpoint for **a**-factor response, as defined in Materials and Methods, was approximately the same in the shmoo assay (40 pg/ml) and the *FUS1-lacZ* assay (30 pg/ml). The rank order of activities of the various analogs was approximately the same for all assays.

Our results suggest that the farnesyl group and carboxy terminus methyl ester play near equal roles in the biological activity of **a**-factor. This conclusion is in contrast to that made by Anderegg et al. (2), who concluded that only the carboxy methyl ester was required for activity. It should be pointed out that in the previous study (2), S-methyl **a**-factor, and not

TABLE 1. Biological significance of the carboxy terminus methyl ester and farnesyl groups of **a**-factor.

	Endpoint of Activity[a]		
a-Factor/Analog	Halo Assay	Shmoo Assay[b]	*FUS1-lacZ* Assay
a-Factor	100	100	100
Nonmethylated **a**-factor	1.5	0.8	0.05
Nonfarnesylated **a**-factor	0.1	0.1	0.1
S-methyl **a**-factor	0.2	0.4	NT[c]
Unmodified **a**-factor peptide	0.0025	NT[c]	NT[c]

[a] All values are expressed as % activity relative to **a**-factor, with **a**-factor set at 100%. Endpoints of **a**-factor activity in each assay were as follows: halo assay, 0.06 ng; shmoo assay, 0.04 ng/ml; *FUS1-lacZ* assay, 0.03 ng/ml. Each data point represents an average of at least three determinations with a variation of not more than two-fold for each value reported.

[b] The tester strain used in this shmoo assay was RC757 (*sst2*).

[c] NT = not tested.

nonfarnesylated **a**-factor (S-H **a**-factor), was tested. For comparative purposes, we determined that S-methyl **a**-factor was about 2- to 4-fold more active than nonfarnesylated **a**-factor in the halo and shmoo assays (Table 1). In our assays, *sst2 MATα* cells were used and the assay medium was YEPD. Anderegg et al. (2) used *SST2*$^+$ *MATα* cells (X2180-1B) and YNB as the assay medium in their **a**-factor bioactivity assays. We tested the same analogs shown in Table 1 using the assay conditions of Anderegg et al. (2), and obtained a rank order of activity (Table 2) that was similar to the rank order obtained when *sst2 MATα* cells and YEPD were used (Table 1).

Consequences of Altering the Size and Hydrophobicity of the Cys-Thioether Group of **a**-Factor

To determine whether the farnesyl group of **a**-factor is specifically required for full bioactivity, the following **a**-factor analogs were synthesized: S-methyl **a**-factor, S-hexadecanyl **a**-factor, S-prenyl **a**-factor, S-geranyl **a**-factor, and S-benzyl **a**-factor (fig. 1). Replacement of the farnesyl moiety by a methyl group (S-methyl **a**-factor) produced an analog which was about two hundred to five hundred-fold less active than **a**-factor and 2- to 4-fold more active than nonfarnesylated **a**-factor (Tables 2 and 3). Replacement of the farnesyl group with a hexadecanyl group produced an analog that was 30- to 70-fold less active in the shmoo and halo assays and seven-fold less active in the *FUS1-lacZ* assay. When the sulfur group was modified with an S-benzyl moiety, the resulting analog exhibited activity that was 4- to 30-fold less active than **a**-factor in the shmoo and halo assays. The effect of alteration of the length of the Cys-isoprenyl group was determined by analysis of the activity of S-prenyl **a**-factor and S-geranyl **a**-factor. S-prenyl **a**-factor was found to be 15- to 70-fold less active than **a**-factor while S-geranyl **a**-factor was only 2- to 8-fold less active. Indeed, the S-geranyl **a**-factor is the most potent synthetic analog prepared to date.

TABLE 2. Activity of **a**-factor and various analogs in the shmoo assay using *SST2*$^+$ *MAT*α cells.

a-Factor/Analog	Endpoint of Activity[a]
a-Factor	100
Nonmethylated **a**-factor	6.5
Nonfarnesylated **a**-factor	1.6
S-methyl **a**-factor	6.5
Unmodified **a**-factor peptide	NA[b]

[a] The *SST2*$^+$ strain used in this shmoo assay was X2180-1B. The assay was carried out as described in Materials and Methods, except that YNB was used as the assay medium. All values are expressed as % activity relative to **a**-factor, with **a**-factor set at 100%. The endpoint of **a**-factor activity in this assay was 160 ng/ml. Each data point represents an average of at least three determinations with a variation of not more than two-fold for each value reported.

[b] NA = not active at 40 μg/ml.

TABLE 3. Biological activity of **a**-factor analogs with altered Cys-thioether groups.

	Endpoint of Activity[a]		
a-Factor/Analog	Halo Assay	Shmoo Assay[b]	*FUS1-lacZ* Assay
a-Factor	100	100	100
Nonfarnesylated **a**-factor	0.1	0.1	0.1
S-methyl **a**-factor	0.2	0.4	NT[c]
S-hexadecanyl **a**-factor	1.5	3.3	15
S-prenyl **a**-factor	1.5	6.7	NT[c]
S-geranyl **a**-factor	12	50	NT[c]
S-benzyl **a**-factor	3	25	NT[c]

[a] All values are expressed as % activity relative to **a**-factor, with **a**-factor set at 100%. Endpoints of **a**-factor activity in each assay were as follows: halo assay, 0.06 ng; shmoo assay, 0.04 ng/ml; *FUS1-lacZ* assay, 0.03 ng/ml. Each data point represents an average of at least three determinations with a variation of not more than two-fold for each value reported.

[b] The tester strain used in this shmoo assay was RC757 (*sst2*).

[c] NT = not tested.

Consequences of Altering the C-Terminal Carboxy Modification of **a**-Factor

Having determined the relative significance of the Cys-thioether modification of **a**-factor, we focused our attention on the C-terminal methyl ester modification. The following analogs were synthesized and analyzed: Cys-amide **a**-factor, 3-methyl butyl ester **a**-factor, and 3-methyl-2-butenyl **a**-factor (fig. 1). The Cys-amide **a**-factor was the most active of this series of analogs, being about 16-fold less active than **a**-factor (Table 4). 3-methyl-2-butenyl ester **a**-factor and 3-methyl butyl ester **a**-factor did not exhibit activities above that observed for nonmethylated **a**-factor, each being about 100-fold less active than **a**-factor.

MATa Cells Need Not Actively Produce **a**-Factor in Order to Mate with Either *sst2* or Wild Type *MATα* Cells

To investigate further the biological significance of extracellular **a**-factor, we tested **a**-factor and several Cys^{12} analogs in a recently developed mating restoration assay (26). In this assay, a mixed lawn of *MATa* cells defective in **a**-factor production (*mfa1 mfa2* mutants) and *MATα* cells were prepared on medium selective for growth of diploid cells. In the absence of exogenously added **a**-factor, *mfa1 mfa2* mutants could not mate with wild type *MATα* cells and mated with *sst2 MATα* at very low efficiency (10^{-6} to 10^{-5}) (Table 5). However, when **a**-factor was spotted on the lawn, constellations of diploid colonies grew at the locations where the pheromone was spotted (fig. 5). Although mating was not restored to a wild type level (Table 5), the results demonstrate that it is not absolutely essential for *MATa* cells to actively produce **a**-factor in order to mate with wild type *MATα* cells. The endpoint for mating restoration using wild type *MATα* cells was the same as the endpoint obtained in the halo assay for these same cells (2 μg).

As shown in Table 6, the proficiency with which various **a**-factor analogs restored mating correlated, for the most part, with activities found in the halo assay. However, the

TABLE 4. Biological activity of **a**-factor analogs with altered C-terminal carboxyl groups.

a-Factor/Analog	Endpoint of Activity[a]	
	Halo Assay	Shmoo Assay[b]
a-Factor	100	100
Nonmethylated **a**-factor	1.5	0.8
Cys-amide **a**-factor	6	6
3-methyl butyl ester **a**-factor	0.8	0.4
3-methyl-2-butenyl ester **a**-factor	1.5	0.8

[a] All values are expressed as % activity relative to **a**-factor, with **a**-factor set at 100%. Endpoints of **a**-factor activity in each assay were as follows: halo assay, 0.06 ng; shmoo assay, 0.04 ng/ml. Each data point represents an average of at least three determinations with a variation of not more than two-fold for each value reported.

[b] The tester strain used in this shmoo assay was RC757 (*sst2*).

TABLE 5. Exogenous **a**-factor restores mating of the *mfa1 mfa2* mutant to either wild type or *sst2* α cells.

MATα Strain[a]	*MATa* Strain[b]	Exogenous **a**-factor[c]	Mating Efficiency[d]
Wild type	Wild type	-	4.6×10^{-2}
Wild type	*mfa1 mfa2*	+	1.4×10^{-4}
Wild type	*mfa1 mfa2*	-	$<1.0 \times 10^{-8}$
sst2	Wild type	-	6.9×10^{-2}
sst2	*mfa1 mfa2*	+	4.2×10^{-3}
sst2	*mfa1 mfa2*	-	2.6×10^{-6}

[a] The α strains tested were RC757 (*sst2*) and CR11 (Wild type).

[b] The **a** cell strains tested were SM1058 (Wild type) and SM1229 (*mfa1 mfa2*).

[c] In assays testing the *SST2 MATα* strain, 4 μg of **a**-factor was spotted onto the lawn, as indicated by the + symbol. For assays testing the *sst2 MATα* strain, 0.125 μg of **a**-factor was spotted onto the lawn, as indicated by the + symbol.

[d] The mating restoration assay used for determination of mating efficiencies is described in the Materials and Methods section.

TABLE 6. Activity of **a**-factor and various analogs in the mating restoration assay.

a-Factor/Analog	Endpoint of Activity[a]
a-Factor	100
Nonmethylated **a**-factor	1.5
Nonfarnesylated **a**-factor	0.8
S-methyl **a**-factor	1.5
S-hexadecanyl **a**-factor	0.8
S-prenyl **a**-factor	25
S-geranyl **a**-factor	200
S-benzyl **a**-factor	25
Cys-amide **a**-factor	6
3-methyl butyl ester **a**-factor	0.4
3-methyl-2-butenyl ester **a**-factor	0.8

[a] The mating restoration assay is described in the Materials and Methods section using RC757 (*sst2*) as the *MAT*α strain. All values are expressed as % activity relative to **a**-factor, with **a**-factor set at 100%. The endpoint of **a**-factor activity in this mating restoration assay was 0.06 ng/ml. Each data point represents an average of at least three determinations with a variation of not more than two-fold for each value reported.

S-prenyl, S-geranyl, and S-benzyl **a**-factors exhibited activities higher than found in the halo assay.

One possible explanation for the inability of exogenous pheromones to restore mating of *mfa1 mfa2* mutants to a wild type level is that **a**-factor play an intracellular role that cannot be complemented by exogenous pheromone. We tested this hypothesis by examining the ability of a *ste6* mutant to mate with wild type *MAT*α cells in the mating restoration assay. The *STE6* gene product has a high degree of homology to mammalian multi-drug-resistance transporters (33, 42) and is thought to act as a transporter which secretes **a**-factor. No other role for *STE6* has been speculated, and it has been shown that *ste6* mutants produce mature **a**-factor intracellularly (33). We tested a *ste6*Δ mutant in the mating restoration assay and found, quite surprisingly, that exogenous **a**-factor did not restore mating at all (Table 7). This result indicates that the *STE6* gene product is required for mating even when **a**-factor is supplied exogenously.

TABLE 7. Exogenous **a**-factor does not restore mating of a *ste6Δ* mutant to either wild type or *sst2* α cells.

MATα Strain[a]	*MATa* Strain[b]	Exogenous **a**-factor[c]	Mating Efficiency[d]
Wild type	Wild type	-	3.7×10^{-2}
Wild type	*ste6Δ*	+	$<6.7 \times 10^{-8}$
Wild type	*ste6Δ*	-	$<6.7 \times 10^{-8}$
sst2	Wild type	-	4.8×10^{-2}
sst2	*ste6Δ*	+	9.0×10^{-5}
sst2	*ste6Δ*	-	9.0×10^{-5}

[a] The α strains tested were RC757 (*sst2*) and CR11 (Wild type).

[b] The **a** cell strains tested were JPY200 (Wild type) and JPY201 (*ste6Δ*).

[c] In assays testing the *SST2 MATα* strain, 4 μg of **a**-factor was spotted onto the lawn, as indicated by the + symbol. For assays testing the *sst2 MATα* strain, 0.125 μg of **a**-factor was spotted onto the lawn, as indicated by the + symbol.

[d] The mating restoration assay used for determination of mating efficiencies is described in the Materials and Methods section.

CHAPTER IV

DISCUSSION

Prenylation is now recognized as an important post-translational modification of numerous proteins in eukaryotic organisms. In this paper, we have presented results of a detailed study of structure-activity relationships for a prenylated yeast mating pheromone, the *S. cerevisiae* **a**-factor. Previous studies on the prenylated peptide pheromones, rhodotorucine *A* and tremerogen *A*-10, of the heterobasidiomycetous yeasts *Rhodosporidium toruloides* and *Tremella mesenterica*, respectively, demonstrated that hydrophobic modification of the C-terminal cysteines of each of these mating pheromones was required for biological activity (17, 58). Detailed studies on analogs of tremerogen *A*-10 demonstrated that decreasing the number of prenyl units from three to one resulted in a corresponding decrease in biological activity (17). In the present study, we have shown that absence of either the farnesyl group (NH_2-YIIKGVFWDPAC-$COOCH_3$) or carboxy terminus methyl ester (NH_2-YIIKGVFWDPAC(S-farnesyl)-COOH) of **a**-factor both result in a similar marked decrease, but not loss, of biological activity as determined by four sensitive bioassays. In addition, an analog lacking both the farnesyl group and methyl ester (NH_2-YIIKGVFWDPAC-COOH) was virtually inactive, being 40,000-fold less active than **a**-factor. These results support those shown in previous studies on the *S. cerevisiae* **a**-factor which utilized only the halo assay (62), and suggest that both the farnesyl group and carboxy terminus methyl ester of **a**-factor are important for high biological activity. This conclusion is in contrast to that of Anderegg et al. (2), who concluded that only the methyl ester modification of the **a**-factor peptide was required for high biological activity. However, Anderegg et al. (2) examined an S-methyl **a**-factor rather than S-H (nonfarnesylated) **a**-factor. Furthermore, they did not test doses of **a**-factor analogs greater

than five-fold higher than that of the dose of **a**-factor tested. This prevented them from detecting activity from analogs that were significantly less active than **a**-factor.

Additional **a**-factor analogs were studied to determine if S-alkyl modifications other than farnesyl would result in biologically active lipopeptides. We found that aliphatic, benzylic, and geranyl substituents resulted in pheromone analogs exhibiting from 0.2 to 50% of the activity of the natural mating factor (Table 3). As illustrated by the S-benzyl analog, an isoprenyl moiety is not required for high biological activity. The bioactivity of the pheromone increased as the length of the prenyl moiety was increased (S-farnesyl **a**-factor > S-geranyl **a**-factor > S-prenyl **a**-factor), and the S-hexadecanyl **a**-factor was more active than the S-methyl analog. Thus, within these two types of S-alkyl modifications, **a**-factor activity was found to increase as the S-alkyl group became more bulky and hydrophobic.

Replacement of the methyl ester by larger, more hydrophobic esters resulted in a marked decrease in activity (Table 4). In contrast, the Cys-amide **a**-factor (Fig. 1) had 6% of the activity of the native pheromone. Thus, the ester oxygen is not specifically required for interaction with the **a**-factor receptor and some hydrophobic groups are not well tolerated in the ester moiety. We are presently synthesizing additional analogs to investigate this latter point in more detail.

Some differences in the activity of **a**-factor analogs relative to **a**-factor were found, depending on the assay used for measuring activity. One must bear in mind that while each of the **a**-factor analogs contains the same peptide moiety, they differ in their overall physical characteristics, especially with regard to hydrophobicity, which influences peptide solubility in different media and the susceptibility to degradation by a recently identified *MAT*α cell specific **a**-factor protease (39). Furthermore, the conditions of each assay, as well as the specific physiological response measured, are different in each of the four assays. Thus, the threshold for response, as well as the stringency of response, in each

assay could be expected to vary. Indeed, previous studies have demonstrated that various pheromone responses require markedly different minimum doses of α-factor, from 10^{-11} M for agglutination to 10^{-8} M for shmooing (for review, see ref. 12). Each of the four assays used in this study measures the end result of a complex series of intracellular and/or intercellular events. While great strides have been made in characterizing specific aspects of the pheromone response pathway, it is, on the whole, still poorly understood. However, there is some evidence that the biochemical events subsequent to pheromone induction follow a branching pathway with physiological responses as the end result of different branches (9, 31). Therefore, some of the varying responses measured by different bioassays may reflect the different thresholds for different branches of the pathway.

While it is clear from our analyses of the biological activity of **a**-factor and various Cys^{12} analogs that hydrophobic modification of the C-terminal cysteine of this pheromone is required for high biological activity, it is equally evident that activity is dependent on more subtle factors than just the overall hydrophobicity of the C-terminus of **a**-factor. Characterization of the **a**-factor analogs by HPLC (data not shown) demonstrated that the S-hexadecanyl **a**-factor is the most hydrophobic analog and that both the 3-methyl butyl and 3-methyl-2-butenyl ester analogs are more hydrophobic than the **a**-factor. It is possible that optimal interaction of the pheromone with the receptor requires a long branched hydrocarbon on the cysteine sulfur and a smaller organic group on the cysteine carboxyl. Such a requirement would be similar to those models used to explain the interaction of aspartame-like sweeteners with their receptor (20). The possibility must also be considered that the requirement of hydrophobic modification of **a**-factor for biological activity reflects not only the specificity of the **a**-factor receptor of *MATα* cells, but also the ability of hydrophobically modified peptides to enter the plasma membranes of these cells . It is conceivable that the hydrophobic **a**-factor might enter the lipid bilayer of *MATα* cells and then diffuse through the membrane to bind its receptor. A similar process has been

proposed for the interaction of mammalian peptide hormones with the receptors of their target cells (3, 52).

An additional factor which might have an effect on the apparent activities of **a**-factor analogs is *MATα* cell degradation of **a**-factor. Previous studies on rhodotorucine *A*, the pheromone produced by mating type *A* cells of *Rhodosporidium toruloides*, have demonstrated that proteolysis of this farnesylated peptide by the mating type *a* target cell is a requirement for induction of the pheromone response (45). In contrast, *S. cerevisiae* ***MATa*** cells proteolyze α-factor as one mechanism of recovery from pheromone-induced growth arrest (10). Mutants which are unable to proteolyze this mating peptide (*sst1/bar1* mutants) still respond and are, in fact, supersensitive to α-factor (7, 8). These and other studies have demonstrated that hydrolysis of α-factor by ***MATa*** cells is not required for a pheromone response. We have recently identified a *MATα* cell specific **a**-factor degrading activity (39). As is the case for α-factor proteolysis by ***MATa*** cells, proteolysis of **a**-factor seems to be a mechanism of *MATα* cell desensitization from the pheromone response. The **a**-factor degrading protease appears to have substrate specificity dependent on both the peptide sequence and the cysteine modifying group. For example, S-hexadecanyl **a**-factor is not proteolyzed by *MATα* cells. We have not determined whether all the analogs tested in this study are proteolyzed by *MATα* cells, but the preferential degradation of **a**-factor analogs may influence their relative bioactivities.

Because of its lipophilic nature and results showing that ***MATa*** mutants defective in **a**-factor production were incapable of mating with wild type ($SST2^+$) *MATα* cells, even when **a**-factor was provided exogenously, some speculation had been raised that **a**-factor may be tethered to **a** cells and that its primary action on α cells occurs via cell-cell contact (25, 43). However, since **a**-factor is found in the culture supernatant of ***MATa*** cells, the pheromone is clearly released or secreted by these cells. Moreover, as demonstrated by numerous studies, the presence of ***MATa*** cells is not required for *MATα* cells to respond to

a-factor supplied as unpurified or partially purified preparations from spent medium of *MATa* cells. Our results demonstrate that synthetic **a**-factor can function to restore the ability of *MATa* mutants defective in **a**-factor production to mate with either *sst2* or wild type *MATα* cells (Table 5), although mating was not restored to a wild type level in either case. We found that the relative rank order of activities of the various **a**-factor analogs tested in this mating restoration assay was similar to the rank order of activities obtained in the halo assay (Table 6), which is also done in agar. The mating restoration experiments indicate that: 1) **a** cells do not have to actively produce **a**-factor in order to mate with α cells, although **a**-factor production might be required for mating at a wild type level, and 2) the cysteine modifications of **a**-factor are no more or less significant for inducing an α cell response to pheromone than for allowing **a** and α cells to conjugate. A possible explanation for the inability of concentrated *MATa* culture supernatant fractions to restore mating of *mfa1 mfa2* mutants to wild type *MATα* cells in an earlier study (43) is that the preparation did not contain a sufficient amount of **a**-factor to restore mating or contained a substance that inhibited mating.

As indicated above, exogenous **a**-factor could not restore mating efficiency of *mfa1 mfa2* mutants to a wild type level (Table 5). However, it is important to note that in previous studies (34), addition of exogenous α-factor also did not restore mating to wild type levels using *mfα1 mfα2 MATα* cells. One possible explanation for the inability of exogenous pheromones to restore mating of pheromone-defective *MATa* or *MATα* cells to a wild type level is that **a**- and α-factor play an intracellular role that cannot be complemented by exogenous pheromone. We tested this hypothesis by examining the ability of a *ste6* mutant to mate with wild type *MATα* cells in the mating restoration assay. The *STE6* gene product has a high degree of homology to mammalian multi-drug-resistance transporters (33, 42) and is thought to act as a transporter which secretes **a**-factor. No other role for *STE6* as been speculated, and it has been shown that *ste6* mutants

produce mature **a**-factor intracellularly (33). We tested a *ste6Δ* mutant in the mating restoration assay and found that exogenous **a**-factor did not restore mating at all (Table 7). A full interpretation of this result awaits better characterization of the *STE6* gene product, although our results may indicate roles, in addition to **a**-factor secretion, for this protein in the mating process. Perhaps the *STE6* gene product is necessary to correctly orient internally produced or exogenously supplied **a**-factor in order to facilitate mating.

An alternative explanation for our observation that exogenous **a**-factor does not restore mating efficiency of *mfa1 mfa2* mutants to a wild type level can be made by considering the results of Jackson and Hartwell (28, 29) on the courtship response of *S. cerevisiae* *MAT**a*** and *MATα* haploids. These investigators showed that both *MAT**a*** and *MATα* cells choose mating partners in response to a pheromone gradient, and that when given a choice of several different potential mating partners, the cell type(s) which produce the most pheromone are preferentially selected. Our results are, in fact, predictable based on Jackson and Hartwell's model for courtship (28, 29). Normal courtship occurs when *MAT**a*** and *MATα* cells are each producing normal levels of pheromone, which results in a recognizable gradient of respective pheromones toward which projections are directed (Fig. 6A). However, when the *MAT**a*** cell does not produce **a**-factor (*mfa1 mfa2* mutant), but the pheromone is instead provided exogenously, no **a**-factor gradient is established (Fig. 6B). Thus, while the *MATα* cell, which is producing α-factor, and therefore establishing a pheromone gradient, can effectively court the *mfa1 mfa2* mutant, the *mfa1 mfa2* mutant cannot court the *MATα* cell, even though the **a**-factor is provided exogenously. Instead, since there is no **a**-factor gradient, the *MATα* cell forms its projection randomly. We have recently determined that addition of exogenous **a**-factor to mixtures of wild type *MAT**a*** and *MATα* cells reduces their mating efficiency to a level very close to that of *mfa1 mfa2* mutants and wild type *MATα* cells in the presence of exogenous **a**-factor (data not shown). This result is consistent with the model shown in Figure 6. Thus, this model provides a

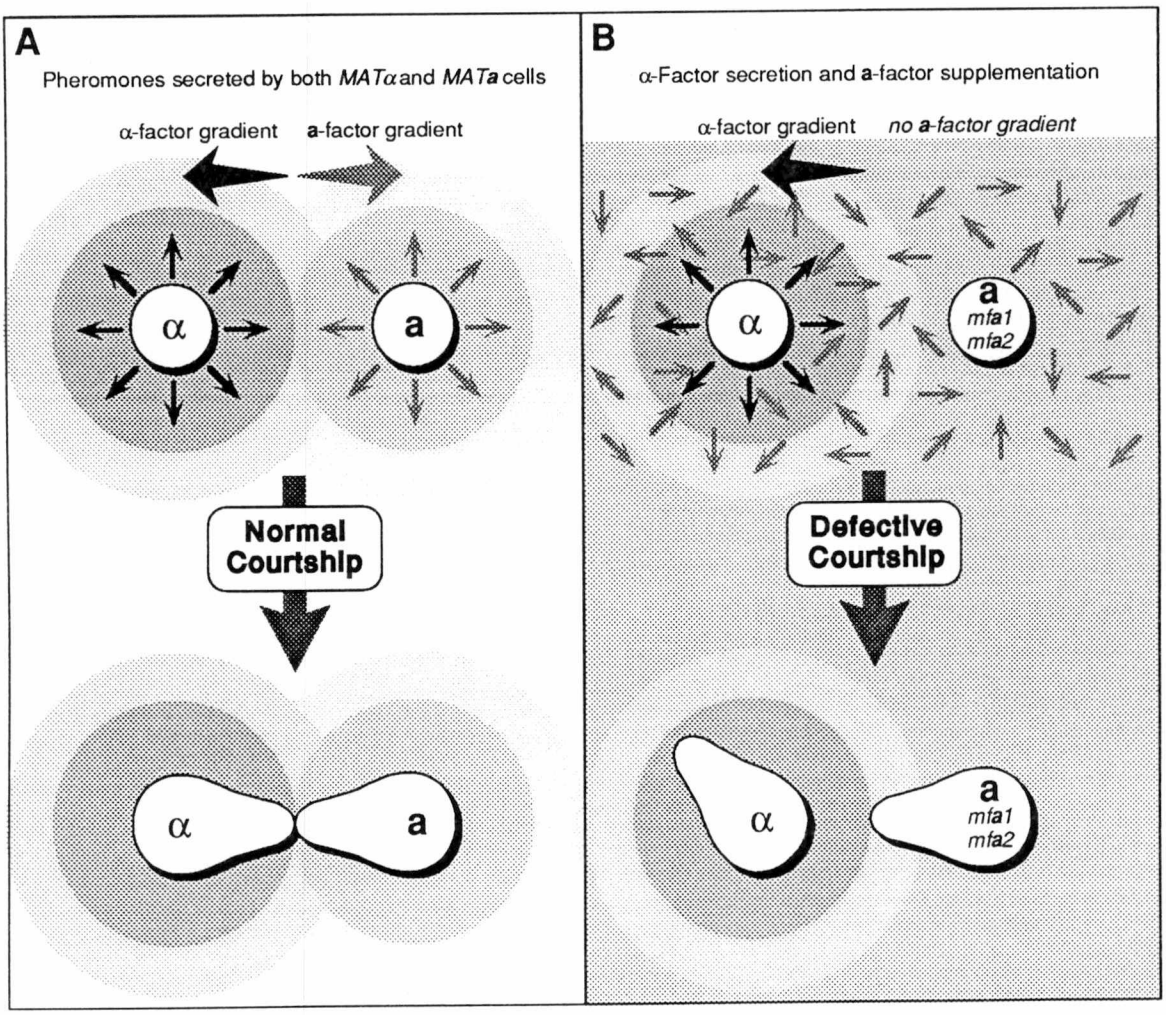

Fig. 6. Model to explain why exogenous **a**-factor does not restore mating of *mfa1 mfa2* mutants to a wild type level. Panel A. Under normal conditions, apposed *MATα* and *MATa* cells produce α-factor and **a**-factor, respectively, resulting in pheromone gradients which are highest in concentration at the surface of the respective secreting cells. Shmoo tip projections are formed in the direction of the gradients, resulting in successful courtship. Panel B. When an *mfa1 mfa2* mutant is in apposition to a wild type *MATα* cell, the *mfa1 mfa2* mutant is unable to court the *MATα* cell. Instead, since **a**-factor is supplied exogenously, the *MATα* cell forms its projection randomly.

likely explanation as to why exogenous **a**-factor does not restore mating efficiency of *mfa1 mfa2* mutants to a wild type level.

Our results also show that in the presence of exogenous **a**-factor, *sst2 MATα* mutants mate with *mfa1 mfa2* mutants at about a 30-fold greater frequency than do wild type (*SST2*$^+$) *MATα* cells. However, this result must be interpreted cautiously. We were not able to test the effect of higher doses of **a**-factor on the mating efficiency of wild type *MATα* cells to *mfa1 mfa2* mutants, due to the extremely limited solubility of the pheromone. Thus, it is possible that higher doses of **a**-factor might have increased the degree of mating restoration of wild type *MATα* cells to a level closer to that of *sst2 MATα* cells. In addition, we have observed that at the highest doses of **a**-factor used in our assays, *sst2 MATα* cells formed multiple shmoo projections. Thus, *sst2* mutants might mate to *mfa1 mfa2* mutants more efficiently than wild type *MATα* cells in the presence of exogenous **a**-factor because they form multiple projections.

The results reported herein reveal several important relationships between the structure of **a**-factor and its biological activity, and provide evidence which suggests that the pheromone works exogenously. Recently, studies have appeared which delineate the steps occurring during the post-translational processing of this farnesylated, carboxy methyl esterified peptide (27, 38, 40, 54). We are presently investigating in detail the specific interaction of the lipopeptide **a**-factor with its receptor, and are searching for antagonists and superactive analogs. It is evident that together with the α-factor, the **a**-factor represents a powerful paradigm for studying cell-cell communication and for learning about peptide hormone action. We anticipate that this system will provide further insights, some possibly unforeseen, into the role of prenylation and carboxy methyl esterification in the biochemistry of eukaryotic organisms.

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PART IV

DEGRADATION OF **a**-FACTOR BY A *SACCHAROMYCES CEREVISIAE* α MATING TYPE SPECIFIC ENDOPEPTIDASE: EVIDENCE FOR A ROLE IN RECOVERY OF CELLS FROM G_1 ARREST*

* Part III appeared in modified form as **Marcus, S., C.-B. Xue, F. Naider, J.M. Becker.** 1991. Degradation of **a**-factor by a *Saccharomyces cerevisiae* α mating type specific endopeptidase: evidence for a role in recovery of cells from G_1 arrest. Mol. Cell. Biol. **11:**1030-1039.

CHAPTER I

INTRODUCTION

Conjugation between *Saccharomyces cerevisiae* **a** and α haploid cells to form **a**/α diploids requires the reciprocal action of peptide pheromones termed **a**-factor (YIIKGVFWDPAC[S-farnesyl]-OCH_3, Fig. 1), produced by **a** cells, and α-factor (WHWLQLKPGQPMY), which is secreted by α cells (reviewed in 14, 24, 25, 40, and 42). These pheromones exert on their target cells similar effects which serve to prepare the cells for mating. Growth arrest occurs at G_1 of the cell cycle (7, 22), cell surface agglutinins are produced (20), transcription of several genes is induced (1, 28, 53), and the cells become pear-shaped, a morphological change termed "shmooing" (31, 51). Genetic analysis and nucleotide sequencing studies have led to the conclusion that the receptors which mediate the pheromone response pathway, **a**- and α-factor receptors, belong to the rhodopsin/adrenergic receptor/muscarinic acetylcholine receptor family (8) and interact with signal-transducing, membrane-associated GTP-binding protein(s) (G-proteins) (15, 37). Thus, study of the yeast pheromone response is providing important insights into signal transduction mechanisms common to many eukaryotic cells.

Desensitization, or the ability to adapt to or recover from a biological stimulus, is a characteristic found in organisms ranging from bacteria to mammals. Results from studies on the adrenergic and acetylcholine hormone-receptor families have demonstrated that several general mechanisms are utilized by eukaryotic cells to desensitize from hormone/pheromone action (29, 35, 46). These include receptor down regulation, structural modification of the receptor (e.g. phosphorylation), and destruction of the hormone/pheromone itself. In recent years, studies from several laboratories have revealed that *S. cerevisiae* **a** haploid cells utilize these same mechanisms to recover from mating pheromone-induced G_1 arrest. The α-factor receptor is subject to down-regulation after

binding of α-factor, although new receptors appear on the cell surface which are capable of binding pheromone (11, 26). Despite the appearance of new receptors at the cell surface, **a** cells remain unresponsive to α-factor. This characteristic indicates that there is an uncoupling of these new receptors from the pheromone response pathway. The α-factor receptor is also subject to phosphorylation of its C-terminal domain after binding of α-factor (42). Moreover, mutant receptors lacking this C-terminal domain appear to block recovery from pheromone-induced growth arrest, suggesting that modification of the C-terminal domain of the α-factor receptor plays a role in the process of desensitization to pheromone (27, 42).

Studies on the **a**-factor receptor similar to those mentioned above have only recently been initiated, but present evidence suggests that **a** and α cells share common signal transduction machinery (5, 41). These studies demonstrated that the **a**- and α-factor receptors (*STE3* and *STE2* gene products, respectively) are the only **a**- or α-specific gene products required for *S. cerevisiae* haploid cell response to mating pheromone. Furthermore, in both **a** and α cells, the *SST2* gene product has been shown to play a role in desensitization to pheromone, possibly by blocking a component of the pheromone response signaling pathway (9, 10, 16). The specific function of the *SST2* gene product has not yet been determined, although it has been speculated that it may play a role in modifying the mating pheromone receptors (16). *KSS1*, a recently characterized gene encoding a putative protein kinase, also plays a role in recovery of both **a** and α cells from the pheromone response (13). Overexpression of *KSS1* allows *sst2* mutants to overcome pheromone-induced growth arrest. Several other gene products are also common to the signal transduction pathways of both **a** and α cells, including G protein subunits and products required for cell and nuclear fusion (for reviews see references 14, 24, and 25).

Many mammalian hormones, neurotransmitters, and other bioactive molecules are known to be inactivated by enzymatic degradation (32), and, in many cases, such

inactivation plays a role in the recovery of target cells from biological stimuli. Examples include the hydrolysis of acetylcholine by acetylcholine esterase (35) and the proteolysis of enkephalin by enkephalinase (6). Most pertinent to the present study, it has been demonstrated that *S. cerevisiae* **a** cells produce a secreted endopeptidase (BAR protease) that cleaves α-factor, thus inactivating this peptide pheromone and aiding in the recovery of these cells from α-factor-induced growth arrest (12). Mutants that do not produce this protease, termed *bar1* (*sst1*) mutants, are supersensitive to α-factor (9, 10, 47). *MATα sst1* cells are not supersensitive to **a**-factor, indicating that *SST1* is an **a** cell specific gene.

The **a**-factor pheromone is an isoprenylated, methyl esterified lipopeptide whose structure has only recently been determined (2, Fig. 1). Like α-factor, **a**-factor is essential for mating in *S. cerevisiae* (36). Although it had been speculated that α cells degrade **a**-factor, such an activity had not previously been identified. In this paper, we report on direct biochemical and physiological evidence of an α cell specific **a**-factor degrading activity, which we call **a**-factorase.

CHAPTER II

MATERIALS AND METHODS

Materials

Yeast extract and Bacto peptone were purchased from Difco, Detroit, MI; 3,4-dichloroisocoumarin (3,4-DCI), phenylmethylsulfonylfluoride (PMSF), *L*-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), pepstatin-A, 1,10-phenanthroline, tosyl-*L*-arginyl methyl ester (TAME), ethylenediamine tetraacetic acid (EDTA), ethylene glycol-*bis* (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), potassium fluoride, sodium azide, and chloroquine from Sigma, St. Louis, MO; antipain from Boehringer Mannheim, Indianapolis, IN; Merck Silica gel (0.25 mm thickness) TLC plates from Bodman Chemicals, Stone Mountain, GA; and dextrose and solvents for HPLC and TLC from Mallinckrodt, St. Louis, MO.

Strains

The following *S. cerevisiae* strains were used in degradation, halo, and/or shmoo assays: X2180-1B (*MATα SUC2 mal mel gal2 CUP1*) (Yeast Genetics Stock Center [YGSC]), X2180-1A (*MAT**a** SUC2 mal mel gal2 CUP1*) (YGSC), and 2180**a**/α (from mating of X2180-1A and X2180-1B). XUM-20D (*MATα ssl1-7 cry1-11 ura3-52 leu2-3 trp1*) and CR11 (*MATα cry1-11 thr4 his6 lys1 gal12*) were the generous gift of Wolfgang Duntze. SY1187 (*MATα ste2-10::LEU2 leu2 ura3 met14 trp1*$_{am}$ *his6 ade1 ade2-1*$_{oc}$) and SY1372 (isogenic to SY1187, except *ste3Δ*) were the kind gift of George Sprague. SM1229 (*MAT**a** mfa1Δ::LEU2 mfa2Δ::URA3 trp1 his4 leu2 ura3 can1*) was the generous gift of Susan Michaelis.

Synthesis and Characterization of **a**-Factor and **a**-Factor Analogs

The various **a**-factor analogs used in this study are shown in Fig. 1. The **a**-Factor (NH_2-YIIKGVFWDPAC[S-farnesyl]-OCH_3) and **a**-factor pentadecapeptide (NH_2-YIIKGVFWDPA-CVIA-COOH) were synthesized as previously reported (4, 53). All other analogs were synthesized using either solution-phase peptide synthesis or a combination of solid-phase and solution-phase peptide synthesis. All peptides were purified to greater than 98% homogeneity using reversed phase HPLC and were characterized using amino acid analysis, 400 MHz H^1-NMR spectroscopy and Fast Atom Bombardment mass spectrometry. Details of the synthesis and characterization of these compounds will be published separately.

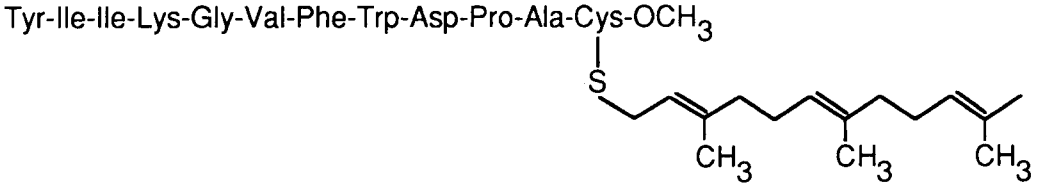
Radioiodination of **a**-Factor

a-Factor was radiolabeled with ^{125}I (Amersham) using the Iodo-Gen reagent (Pierce) according to the manufacturers instructions. Iodinated **a**-factor was purified by HPLC, dried by rotary evaporation, and dissolved in methanol. Subsequent HPLC analysis demonstrated that there was no noniodinated **a**-factor in the [^{125}I]**a**-factor preparation. [^{125}I]**a**-factor had biological activity comparable to that of **a**-factor. Specific activities of 1 to 5 x 10^5 CPM/μg were typically obtained.

Preparation of *S. cerevisiae* Particulate Fraction of Cell Lysate

S. cerevisiae X2180-1B was grown at 30 °C to mid-log phase in YEPD (1% yeast extract, 2% peptone, and 2% dextrose). Cells were harvested by centrifugation, washed once with sterile distilled H_2O, and resuspended to 0.3 g/ml (wet weight/volume) in lysis buffer (0.1 M K_2HPO_4, 2 mM EDTA, 20 mM β-mercaptoethanol, and 20 mM $MgCl_2$, pH 6.45). Cells were lysed using glass beads (0.45-0.50 mm, B. Braun, Germany) and vortexing for 60 sec intervals, being placed in ice for 2 min between each interval.

a-Factor:



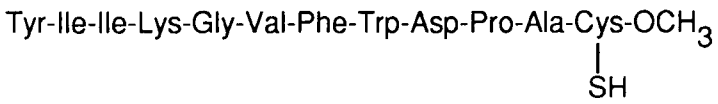
a-Factor Decapeptide:

Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro

a-Factor Pentadecapeptide:

Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys-Val-Ile-Ala-OH

Nonfarnesylated **a**-Factor:



S-Hexadecanyl **a**-Factor:

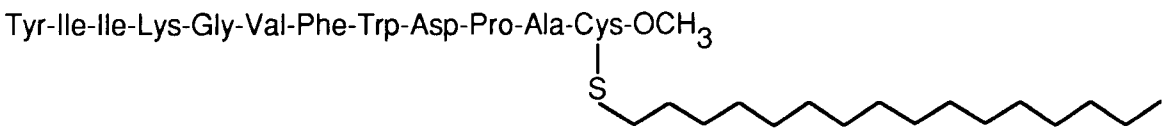


Fig 1. Structures of *Saccharomyces cerevisiae* **a**-factor and various analogs used in this study.

Unbroken cells were removed by centrifugation for 10 min. at 6000x*g*, and the supernatant was centrifuged for 45 min at 30,000x*g*. The resulting pellet was washed once with lysis buffer, then resuspended in a small volume of lysis buffer containing 50% glycerol. Protein estimation was carried out using Bio-Rad Protein Assay Concentrate. Extracts were stored at -20 °C.

Assay for **a**-Factor Degradation

S. cerevisiae strains were grown to late log phase in YEPD at 30 °C with shaking. Cells were harvested by centrifugation, washed once with YEPD, then resuspended at 1 x 10^8 cells/ml. Approximately 50,000 CPM of [^{125}I]**a**-factor was then added to 490 µl of cell suspension in siliconized 13 x 100 mm glass tubes. Particulate fractions of cell lysates were assayed by adding [^{125}I]**a**-factor stock to 50 µl of particulate fraction and 440 µl of YEPD. Proteolytic reactions were stopped at various times post **a**-factor addition by removing portions of cell suspension, pelleting cells by centrifugation, then spotting 10 µl of the culture medium onto TLC plates, followed by rapid air drying of the spotted reaction mixture. TLC plates were developed using n-BuOH:HOAc:H_2O (4:1:1, v:v:v) and exposed to Kodak X-OMAT AR film for 2 to 5 days. Where indicated, **a**-factor degradation was quantitated densiometrically by the use of a Visage 60 Image Analysis System (Bio Image, Ann Arbor, MI). Various physiological inhibitors were added to the proteolysis assay suspension from 100x stocks where indicated in the text. Also, where indicated, proteolysis assay media were analyzed by HPLC.

HPLC Analysis

All HPLC analyses used a Beckman instrument and System Gold computer software with the following methodology: column: DuPont Zorbax Protein Plus C_3 Reversed Phase; flow rate: 1.5 ml/min; gradient: 15% acetonitrile (ACN) from 0 to 10 min, 15 to 80% ACN

from 10 to 75 min (1%/min); detection: UV at 220 and 280 nm, γ emissions from 1 min fractions (Beckman Gamma 4000 Counter).

a-Factor Recovery Assays

S. cerevisiae strain X2180-1B was grown at 30 °C with shaking to early log phase in YEPD. The cells were harvested by centrifugation at 1000x*g*, washed twice with YEPD, and resuspended to 4.0 x 10^6 cells/ml in YEPD or YEPD with protease inhibitor (as indicated in text). Dilution series (1:2) of **a**-factor samples were prepared in borosilicate glass tubes with YEPD as the diluent. Five hundred microliters of cell suspension was added to 500 µl of each of the **a**-factor dilutions, and the resulting cell suspensions were incubated at 30 °C with shaking. Aliquots were removed hourly, placed on ice, and observed microscopically to quantitate the total number of cells, % shmoos, and % unbudded cells (100-200 cells counted/sample).

CHAPTER III

RESULTS

a-Factor is Degraded by α Cells, But Not by a Cells or a/α Diploids

In order to carry out various studies on the interaction of **a**-factor with α target cells, synthetic **a**-factor was radioiodinated. The procedure employed iodination of the single tyrosine residue at the N-terminal of synthetic **a**-factor (Fig. 1 and see Ref. 53 for synthesis of **a**-factor). Purified [^{125}I]**a**-factor had biological activity comparable to that of noniodinated **a**-factor, being only about 5-fold less active than the noniodinated pheromone as determined by the shmoo assay, a standard biological assay used to measure the activity of S. cerevisiae mating pheromones (data not shown). Although it had been speculated that α cells degrade **a**-factor in a manner analogous to the degradation of α-factor by **a** cells (48), direct evidence of an **a**-factor degrading activity had not yet been obtained. As shown in Fig. 2, **a**-factor is indeed degraded by α cells, but not by **a** cells or **a**/α diploid cells. Only a single degradation product was observed.

It was conceivable that the reason we did not detect [^{125}I]**a**-factor degradation by **a** cells was that these cells were in fact producing **a**-factor which competed with [^{125}I]**a**-factor for the degrading activity (see below and Table 2). To address this possibility, we determined that an **a** cell mutant (SM1229), which does not produce **a**-factor due to deletion of the **a**-factor structural genes, *MFa1 and MFa2*, did not degrade [^{125}I]**a**-factor (data not shown). This result supports the above observation that **a**-factor degradation is α cell specific.

A Cell-Associated Endopeptidase is Responsible for the Degradation of a-Factor

The α-factor degrading protease (BAR1) produced by **a** cells is a secreted enzyme (33). To determine if the **a**-factor degrading activity is also secreted, we analyzed the α cell culture medium and the particulate fraction of cell lysates in the **a**-factor degradation assay.

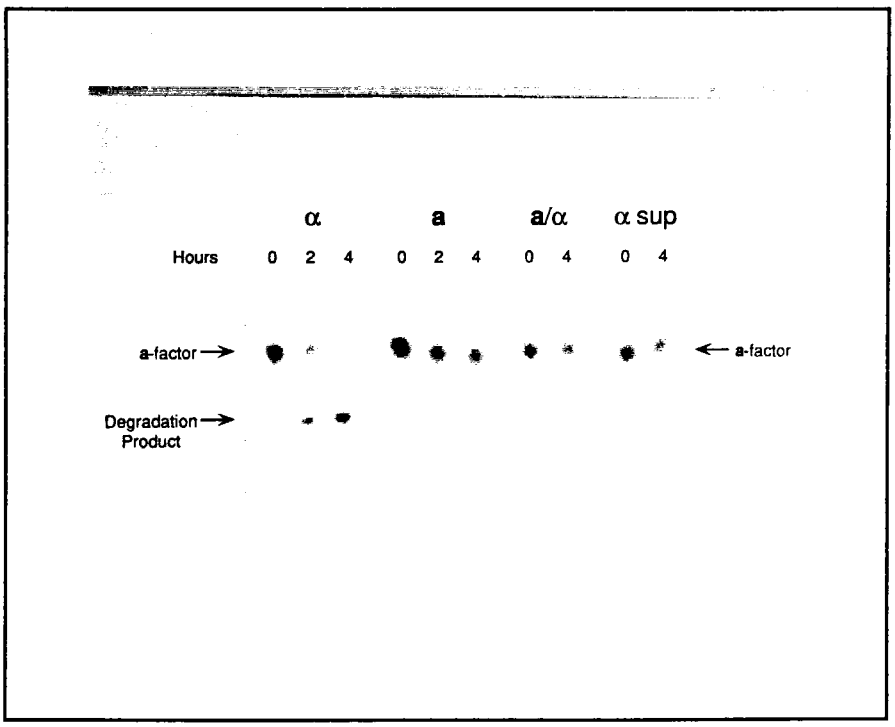


Fig 2. **a**-Factor is degraded by a cell-associated α mating type specific activity. Radioiodinated **a**-factor was incubated with *S. cerevisiae* X2180-1B (*MATα*) cells (α), X2180-1A (*MAT**a***) cells (**a**), X2180 **a**/α diploid cells (**a**/α), or cell free spent X2180-1B culture medium (α sup). **a**-Factor degradation assays were carried out as described in Experimental Procedures. At times indicated, cells were removed by centrifugation, then 10 µl of each supernatant was spotted onto TLC plates. Plates were developed using n-BuOH:HOAc:H_2O (4:1:1, v:v:v) and exposed to film for 2 days. The R_f of **a**-factor was 0.71, while the degradation product had an R_f of 0.60. No other spots were detected.

Cell-free spent α culture medium from a late log phase culture did not degrade [^{125}I]**a**-factor (Fig. 2). Medium from α cells treated with a concentration of **a**-factor (25 ng/ml) sufficient for inducing a biological response exhibited a similar lack of **a**-factor degrading activity (data not shown). However, the particulate fraction of cell lysates degraded [^{125}I]**a**-factor, producing the same ^{125}I-labeled breakdown product as whole cells (Fig. 3, B and C). These results indicate that the α cell specific **a**-factor degrading activity is cell-associated and not secreted. Preliminary experiments also suggest that pretreatment of wild type cells with **a**-factor results in an increased level of particulate-associated **a**-factor degrading activity (data not shown), indicating that the expression of the degrading activity is increased upon exposure of α cells to **a**-factor. The **a** cell specific BAR protease activity had previously been shown to increase substantially upon exposure of **a** cells to α-factor (35).

HPLC analysis of the α cell degradation of [^{125}I]**a**-factor showed that the primary iodinated breakdown product had a retention time (RT) of approximately 20 minutes (Fig. 3B). The additional minor degradation product(s) (RT = 3 min, excluded volume) detected by HPLC (Fig. 3, B and C) was attributed to a further hydrolysis of the peak at 20 min by cellular peptidases. Secondary proteolysis of α-factor by **a** cells was seen in another study (12). We had previously synthesized several different Cys^{12} **a**-factor analogs in our laboratory, including the unmodified **a**-factor dodecapeptide (NH_2-YIIKGVFWDPAC-COOH), nonmethylated **a**-factor (NH_2-YIIKGVFWDPAC[S-farnesyl]-COOH), nonfarnesylated **a**-factor (NH_2-YIIKGVFWDPAC-$COOCH_3$), and a C-terminal truncated **a**-factor decapeptide (NH_2-YIIKGVFWDP-COOH) (Fig. 1; 53 and unpublished data). In the HPLC system used for the analysis of **a**-factor degradation by α cells, these peptides had RT's as follows: NH_2-YIIKGVFWDPAC-COOH, 30 min; NH_2-YIIKGVFWD-PAC[S-farnesyl]-COOH, 44 min; NH_2-YIIKGVFWDPAC-$COOCH_3$, 32 min.; and NH_2-YIIKGVFWDP-COOH, 29 min. Since the primary ^{125}I breakdown product resulting

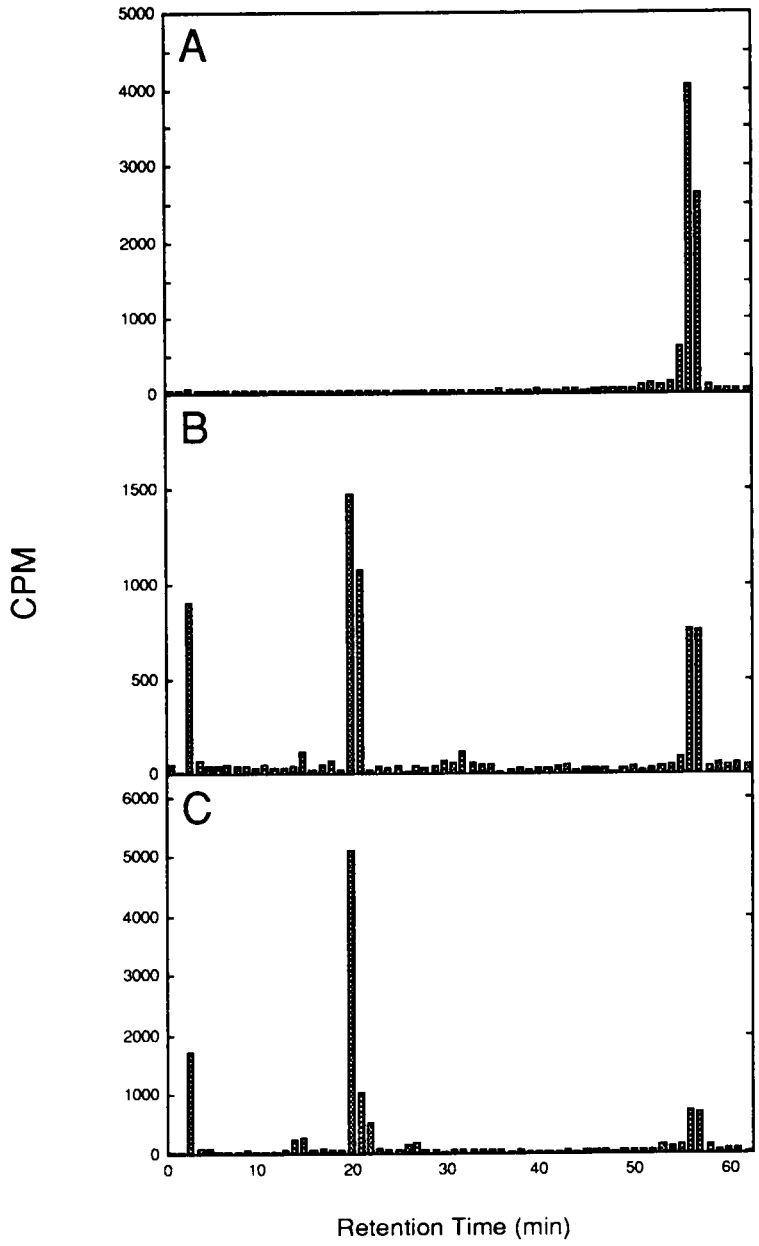


Fig 3. HPLC analysis of **a**-factor degradation by α cells and particulate fraction of cell lysate. Radioiodinated **a**-factor was incubated with *S. cerevisiae* α cells (X2180-1B) or particulate fraction of cell lysate, portions of the suspensions were subjected to centrifugation, and supernatants were analyzed by HPLC as described in Experimental Procedures. A) [^{125}I]**a**-factor. B) Degradation of [^{125}I]**a**-factor after incubation with α cells for 2 hr. C) Degradation of [^{125}I]**a**-factor after incubation with the particulate fraction of α cell lysate for 4 hr.

from α cell degradation of [^{125}I]**a**-factor had an RT of 20 min, it was less hydrophobic than any of the above mentioned **a**-factor analogs. Thus, the degradation of **a**-factor was not due to defarnesylation or demethylation, but rather to proteolysis at a bond to the N-terminal side of the Pro^{10}-Ala^{11} peptide bond. Furthermore, **a**-factor degradation to generate the primary product (RT = 20 min) was not the result of the action of an aminopeptidase, since iodotyrosine and diiodotyrosine, the only amino acid residues iodinated by the method used, have RT's of 3.2 and 4.6 min, respectively. Additional chromatographic comigration experiments using iodinated **a**-factor analogs suggest that **a**-factor may be cleaved at the Val^{6}-Phe^{7} peptide bond by an endopeptidase (data not shown). However, definitive determination of the site of cleavage of **a**-factor by α cells cannot be made without structural characterization of the actual breakdown products (i.e. amino acid analysis and mass spectrometry). Such analytical procedures require quantities of breakdown products which will be most effectively obtained using purified or partially purified enzyme.

Degradation of **a**-Factor Plays a Role in α Cell Recovery from the Pheromone Response

To further characterize the α cell specific **a**-factor degrading activity, degradation assays were carried out in the presence of various protease inhibitors. Phenylmethylsulfonyl-fluoride (PMSF), 1,10-phenanthroline, and tosyl-*L*-arginyl-methyl ester (TAME) were found to inhibit α cell degradation of [^{125}I]**a**-factor, whereas antipain, pepstatin, aprotinin, and several other inhibitors had no detectable effect on **a**-factor degradation (Table 1).

To investigate the functional role of α cell proteolysis of **a**-factor, we determined the effect of PMSF and TAME on both α cell growth and response to **a**-factor (1,10-phenanthroline was extremely toxic to cells and was not tested). If the proteolysis of **a**-factor is a mechanism of desensitization, we expected that α cells would exhibit a prolonged response to **a**-factor in the presence of these inhibitors. Conversely, if **a**-factor

TABLE 1. Effect of protease inhibitors on a-factor degradation.

Inhibitor	Protease Class Affected	Concentration in Degradation Assay[a]	a-Factor Degradation[b]
Antipain	serine/cysteine	1 mM	++++
Aprotinin	serine	0.01 mM	++++
3,4-DCI	serine	0.1 mM	++++
E-64	cysteine	0.01 mM	++++
EDTA	metallo	10 mM	++++
EGTA	metallo	10 mM	++++
Pepstatin	aspartic	0.01 mM	++++
PMSF	serine	2 mM	++
1,10-Phenanthroline	metallo	2 mM	-
TAME	serine	10 mM	+

[a] Degradation assays were performed as described in Experimental Procedures. Inhibitors were used at concentrations shown to be maximally effective for a number of proteases (Salvesen and Nagase, 1989).

[b] The degree of [^{125}I]a-factor degradation was determined by densiometric quantitation of the degradation products seen in autoradiograms with a Visage 60 Image Analysis System (Bio Image, Ann Arbor, MI).

++++ = 75-100% degradation of [^{125}I]a-factor after 6 hr

++ = 10-25% degradation of [^{125}I]a-factor after 6 hr

+ = 5-10% degradation of [^{125}I]a-factor after 6 hr

- = no degradation of [^{125}I]a-factor after 6 hr

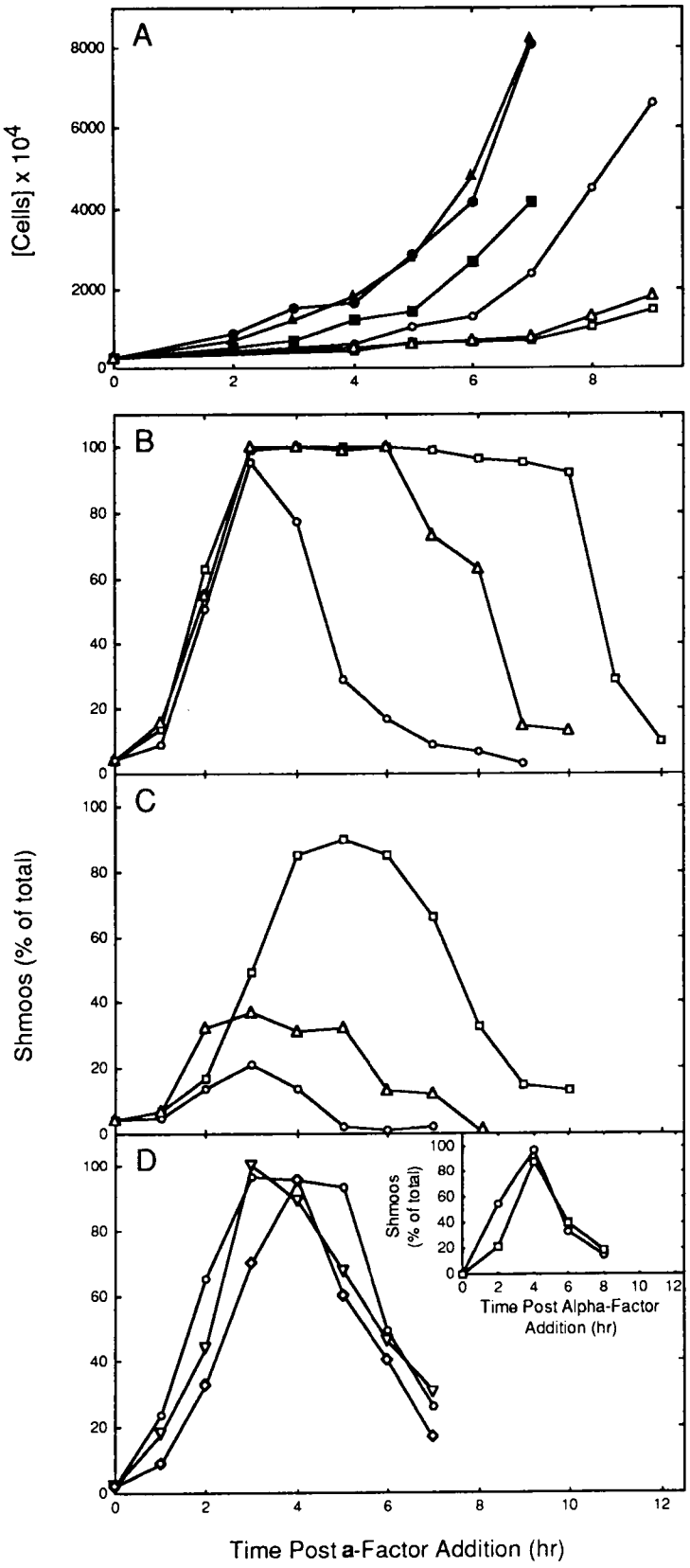
proteolysis was required for biological activity, we expected to see a significantly lowered response to **a**-factor in the presence of PMSF and TAME. As shown in Fig. 4, the former proved to be the case. PMSF (2 mM) had only a slight effect on cell growth, while TAME (1 mM) had no detectable effect. However, both inhibitors markedly prolonged the **a**-factor response of α cells. Two protease inhibitors, aprotinin (0.01 mM) and pepstatin A (0.01 mM), which did not inhibit the degradation of [I^{125}]**a**-factor (Table 1), were also found to neither prolong nor enhance the α cell response to **a**-factor (Fig. 4D). Ciejek and Thorner (12) previously showed that TAME prolongs the **a** cell response to α-factor. However, they also found, and we have verified (inset, Fig. 4D), that PMSF has no effect on the recovery of **a** cells from the α-factor response, indicating that this inhibitor does not affect part(s) of the desensitization process common to both **a** and α cells. These results provide physiological evidence that the degradation of **a**-factor plays a role in the desensitization of α cells from the **a**-factor pheromone response.

α Cell Degradation of **a**-Factor is Not Energy-Dependent and Does Not Require Receptor-Mediated Endocytosis of the Pheromone

To determine if **a**-factor degradation is energy-dependent, assays were carried out in the presence of energy inhibitors. Sodium azide (10 mM) and potassium fluoride (10 mM) did not inhibit **a**-factor proteolysis, either when added together or separately to the assay. Thus, **a**-factor degradation does not appear to be an energy-dependent process.

To determine if the degradation of **a**-factor is dependent in some way on the **a**-factor receptor (*STE3* gene product), we analyzed the ability of a *ste3* deletion mutant (SM1372) to proteolyze **a**-factor (Figure 5). This receptorless mutant was in fact able to degrade **a**-factor. This result indicates that (i) **a**-factor proteolysis cannot be attributed to a function of the **a**-factor receptor, (ii) degradation is not dependent on receptor-mediated endocytosis, and (iii) the expression of the endopeptidase responsible for **a**-factor proteolysis is not

Fig 4. PMSF and TAME enhance and prolong the α cell response to **a**-factor. Recovery assays were performed as described in Experimental Procedures using *S. cerevisiae* X2180-1B at a starting concentration of 2.5 x 10^6 cells/ml. A) Effect of PMSF and TAME on cell growth in the absence (closed symbols) and presence (open symbols) of **a**-factor (80 ng/ml). ●, and ○, growth in YEPD. ■ and □, growth in YEPD with 2 mM PMSF. ▲ and △, growth in YEPD with 1 mM TAME. B) Recovery of cells as measured by shmoos observed (% of total cells) in YEPD containing **a**-factor at a concentration 80 ng/ml. ○, recovery in absence of protease inhibitor. □, recovery in presence of 2 mM PMSF. △, recovery in presence of 1 mM TAME. C) Same as B, except concentration of **a**-factor was 40 ng/ml. D) Same as B, except different protease inhibitors tested. ○, recovery in absence of protease inhibitor. ▽, recovery in presence of 0.01 mM aprotinin. ◇, recovery in presence of 0.01 mM pepstatin A. Inset of D) X2180-1A (*MATa*) cells were monitored for recovery from α-factor (1 μg/ml YEPD) in the absence (○) or presence (□) of 2 mM PMSF.


A
B
C
D
[Cells] x 10^4
Shmoos (% of total)
Time Post a-Factor Addition (hr)
Shmoos (% of total)
Time Post Alpha-Factor Addition (hr)

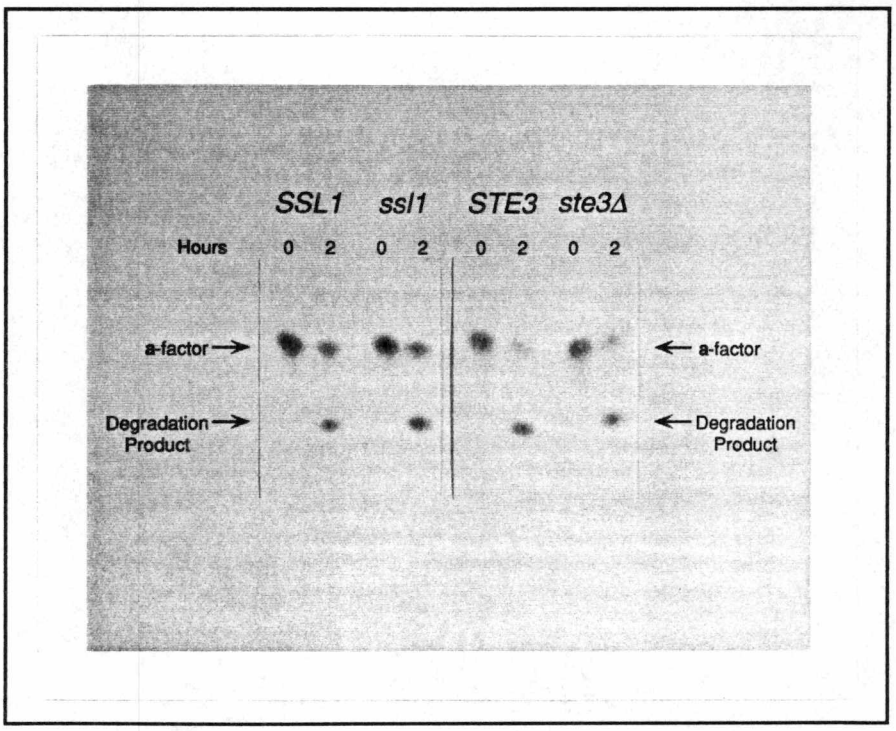


Fig 5. *ste3Δ* mutants and *ssl1* mutants degrade **a**-factor. Radioiodinated **a**-factor was incubated with *S. cerevisiae* strains CR11 (*SSL1*), XUM-20D (*ssl1*), SY1187 (*STE3*), or SY1372 (*ste3Δ*). **a**-Factor degradation assays were carried out as described in Experimental Procedures. At times indicated, cells were removed by centrifugation, then 10 µl of each supernatant was spotted onto TLC plates. Plates were developed using n-BuOH:HOAc:H_2O (4:1:1, v:v:v) and exposed to film for 2 days.

dependent on pheromone induction, since *ste3Δ* mutants are incapable of producing this response. However, as discussed above, pretreatment of wild type α cells with **a**-factor appears to result in an increased level of [^{125}I]**a**-factor degradation, suggesting that exposure of cells to pheromone induces enhanced expression of the **a**-factor degrading activity.

The **a**-Factor Degrading Protease Shows Substrate Specificity

Several nonradiolabeled **a**-factor analogs were tested for their ability to act as competitors of **a**-factor degradation. As shown in Table 2, incubation of [^{125}I]**a**-factor with α cells in the presence of a 100-fold molar excess of nonradiolabeled **a**-factor resulted in complete inhibition of proteolysis of the radiolabeled pheromone. In the presence of either a 100-fold excess of nonradiolabeled, nonfarnesylated **a**-factor or a 100-fold excess of nonradiolabeled pentadecapeptide (see fig. 1), degradation of [^{125}I]**a**-factor was measurably inhibited, but not abolished. A 100-fold excess of the truncated **a**-factor analog, NH_2-YIIKGVFWDP-COOH, did not inhibit the proteolysis of **a**-factor. Likewise, a 25-fold molar excess of bovine serum albumin (BSA) had no effect on **a**-factor degradation. In a separate experiment, **a** cells did not exhibit any detectable degradation of [^{125}I]S-hexadecanyl **a**-factor, which is biologically active, even in autoradiograms greatly overexposed (data not shown). These results suggest that the **a**-factor degrading activity exhibits substrate specificity.

ssl1 Mutants Degrade **a**-Factor

S. cerevisiae mutants supersensitive to **a**-factor were recently isolated by Steden et al. (48). One complementation group of mutants, *ssl1*, displayed an inability to remove the biological activity of **a**-factor added to cell suspensions, as compared to wild type α cells. In addition, *ssl1* was found to be an α cell specific mutation. These investigators suggested

TABLE 2. Competition of [^{125}I]**a**-factor degradation by nonradiolabeled **a**-factor, various structural analogs, and BSA.

Competitor	Molar Excess to [^{125}I]**a**-factor[a]	**a**-Factor Degradation[b]
a-factor	100-fold	-
nonfarnesylated **a**-factor	100-fold	+
a-factor pentadecapeptide	100-fold	+
a-factor decapeptide	100-fold	++++
BSA	25-fold	++++

[a] Structures of competitors are shown in Figure 1.

[b] Degradation assays were performed as described in Experimental Procedures.

++++ = 75-100% degradation of [^{125}I]**a**-factor after 6 hr

\+ = 5-10% degradation of [^{125}I]**a**-factor after 6 hr

\- = no degradation of [^{125}I]**a**-factor after 6 hr

that *SSL1* might encode the α cell equivalent of the **a** cell specific BAR protease. However, as shown in Fig. 5, we determined that the *ssl1-7* mutant is not defective in the ability to degrade **a**-factor. Steden et al. (50) had found that *ssl1* mutants revert to *SSL1*$^+$ at high frequency, although the *ssl1-7* mutation appeared to be somewhat stable (18). To ensure that we were not dealing with a revertant, we determined by use of the halo assay that the *ssl1-7* mutant (XUM-20D) was approximately 8-fold more sensitive to **a**-factor than its *SSL1*$^+$ parent strain (CR11). Thus, our results suggest that *SSL1* is apparently not the α cell equivalent of the **a** cell specific *BAR1* gene, but rather encodes a product whose mode of action is not yet known.

CHAPTER IV

DISCUSSION

Destruction of ligands by their target cells is thought to be an important mechanism for desensitization from pheromone/hormone responses in eukaryotic organisms. *Saccharomyces cerevisiae* **a** cells had been previously shown to secrete an endopeptidase that specifically cleaves the α-factor peptide, thus effectively inactivating this mating pheromone and assisting in the recovery of the cells from G_1 arrest (12, 33). We have reported here on the first biochemical and physiological evidence of an α cell specific **a**-factor degrading activity, which we call **a**-factorase. In contrast to the **a** cell specific BAR protease, which is secreted, **a**-factorase is a cell-associated enzyme. Our results suggest that **a**-factorase is an endopeptidase which most likely cleaves **a**-factor at the Val^6-Phe^7 peptide bond.

Mating type *a* cells of the heterobasidiomycetous yeast *Rhodosporidium toruloides* have also been shown to produce a cell-associated endopeptidase that cleaves rhodotorucine *A*, the farnesylated peptide pheromone produced by cells of the *A* mating type (38, 39). However, in contrast to the degradation of α-factor by *S. cerevisiae* **a** cells, proteolysis of rhodotorucine *A* is required for biological activity. Since **a**-factor, like rhodotorucine *A*, is a farnesylated peptide, and since **a**-factorase is cell-associated, we asked whether the α cell specific proteolysis of **a**-factor was required for biological activity or if it played a role in the recovery of α cells from the pheromone response. Several protease inhibitors were tested for their ability to inhibit **a**-factor proteolysis by α cells. Two inhibitors, PMSF and TAME, were found to significantly inhibit the proteolysis of **a**-factor and markedly prolong the α cell response to **a**-factor, but did not alter the onset of the response. These results suggest that the α cell proteolysis of **a**-factor plays a role in desensitization from the pheromone response in a manner analogous to the degradation of α factor by the **a** cell

specific BAR protease. Further evidence for the role of **a**-factor proteolysis in desensitization, but not in biological activity, was provided by showing that α cells are incapable of degrading [^{125}I]S-hexadecanyl **a**-factor, a structural analog of **a**-factor that is biologically active (unpublished results, Fig. 1). This result, however, must be interpreted cautiously, as S-hexadecanyl **a**-factor is about 30-fold less active than **a**-factor. Thus, if S-hexadecanyl **a**-factor is proteolyzed at levels not detectable by our assay, a correlation might be made between activity and proteolysis. Furthermore, while our results suggest that **a**-factor degradation is not required for biological activity, they do not rule out the possibility that **a**-factor degradation products might enhance the biological activity of the pheromone, or that the degradation products themselves might be biologically active. Previously, biologically-inactive, N-terminal fragments of α-factor were shown to prolong the time needed for **a** cells to recover from the action of the native pheromone (49). Conclusive determination of the physiological role of **a**-factorase will require the isolation of mutants defective in **a**-factorase activity and/or the cloning and subsequent disruption/deletion of the gene encoding this enzyme.

The observation that PMSF and TAME inhibited **a**-factor proteolysis suggests that **a**-factorase may be a serine protease. Furthermore, the enzyme may be metal-dependent/-activated, since α cells were incapable of proteolyzing **a**-factor in the presence of 1,10-phenanthroline. However, the lack of effects of other metal chelators (EDTA and EGTA) and serine protease inhibitors (aprotinin and 3,4-DCI) make it difficult to definitely conclude about the protease class of **a**-factorase. Such determination, as well as analysis of its substrate specificity, will require characterization of the purified enzyme.

Experiments showed that **a**-factor proteolysis is not energy-dependent, since the degradation of **a**-factor was not affected by energy inhibitors, and does not require receptor-mediated internalization of the ligand, as evidenced by the fact that a receptor deleted mutant (*ste3Δ*)was not defective in degradation. In addition, the **a**-factor receptor

itself does not contain the endopeptidase activity responsible for the degradation of **a**-factor, since the *ste3Δ* mutant retains **a**-factorase activity. These results also indicate that **a**-factor degradation is not dependent on either pheromone response, since *ste3Δ* mutants are incapable of producing this response, or receptor-mediated endocytosis. Although pheromone induction is not required for expression of **a**-factorase, preliminary experiments suggest that pretreatment of α cells with **a**-factor prior to the proteolysis assay results in an increased level of **a**-factor degradation. Thus, pheromone exposure may result in increased expression of **a**-factorase. The **a** cell specific BAR protease is under similar expressional control (34).

A report was published recently on the isolation of *Saccharomyces cerevisiae* mutants supersensitive to **a**-factor (48). Two complementation groups of mutants were isolated, *ssl1* and *ssl2*. *ssl2* was shown to be allelic to *sst2*, a mutation conferring pheromone supersensitivity to both **a** and α cells. However, *ssl1* was shown to be an α cell specific mutation in that *MATα ssl1* cells were supersensitive to **a**-factor, but *MATa ssl1* cells were not supersensitive to α-factor. In addition, *MATα ssl1* mutants were found to be incapable of inactivating externally added **a**-factor, in contrast to $SSL1^+$ cells. Contrary to the expectation that *ssl1* mutants might be incapable of degrading **a**-factor, we found that the *ssl1-7* mutant does degrade **a**-factor. Thus, our results suggest that the *SSL1* gene is not the α cell equivalent of the **a** cell specific *BAR1* gene. However, it is possible that in addition to proteolyzing **a**-factor, α cells may also produce a moiety which binds to the pheromone, rendering it inactive. Thus, *SSL1* may encode an **a**-factor blocking/binding product. Alternatively, *ssl1* mutants may be defective in receptor-mediated endocytosis of **a**-factor. If this is found to be the case, then it would suggest that **a** and α cells may have differences in specific recovery mechanisms of the pheromone response pathway. Further studies are clearly required to determine the function of the *SSL1* gene product.

The possibility has been proposed that **a**-factor, due to its modification by addition of a farnesyl lipid tail, may be "anchored" to **a** cells and that its primary effect on α cells may result from cell-cell contact, rather than the activity of secreted pheromone (25). However, it is also conceivable that secreted hydrophobic **a**-factor may enter the lipid bilayer of the plasma membrane of α cells and then "flow" through the membrane to reach its receptor. Such a process, proposed for neuropeptide-membrane interactions (3, 44, 45), would possibly make a membrane-bound degrading protease more effective than a secreted protease for serving a role in the process of desensitization. Preliminary experiments suggest that radiolabeled **a**-factor associates rapidly with the particulate fraction of cell lysates, suggesting that **a**-factor does readily associate with membrane lipids.

Mammalian hormones and neurotransmitters are also known to be degraded as a mechanism of inactivation (17, 32, 35). However, the proteases responsible specifically for the inactivation of most peptide hormones have not been identified. One of the best characterized peptide hormone degrading proteases is neutral endopeptidase (NEP), also called enkephalinase. NEP is a membrane-bound, zinc metalloendopeptidase that hydrolyzes a variety of bioactive peptides, including enkephalins (23), bradykinin (21), substance P (51), and the atrial natriuretic peptide (19). Moreover, the common acute lymphocytic leukemia antigen (CALLA), which is found on leukemic but not normal cells, has been shown to be identical to NEP (30). Thus, it is possible that the improper expression of peptide hormone degrading proteases can, in some cases, lead to carcinogenesis. It will be of great interest to purify **a**-factorase, clone the gene encoding this lipopeptide degrading endopeptidase to better understand its mode of action and regulation, and determine if related proteases are found in mammalian cells.

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PART V

SUMMARY

CHAPTER I

SUMMARY AND ADDITIONAL DISCUSSION OF PARTS II - IV: WHERE DO WE GO FROM HERE?

Study of the *S. cerevisiae* **a**-factor is providing important insights into cellular processes common to all eukaryotes and, in addition, is filling a significant gap that existed in our knowledge of the cell biology of this important model organism. The research discussed in this dissertation makes a significant contribution to our understanding of both **a**-factor biosynthesis and target cell interactions. Some results also have implications which extend to the broader scope of eukaryotic cell biology and cellular communication in general. In the following sections, the major results of this dissertation research, as well as related research from other laboratories, are summarized, and, in some cases, discussed in greater detail. Many questions about **a**-factor remain to be answered, and so directions for future research aimed at answering such questions are also discussed.

Characterization of **a**-Factor Biosynthesis and Secretion

By defining the steps of **a**-factor biosynthesis, it has been possible to characterize an extremely important, and previously unrecognized, post-translational processing pathway. The target sequence recognized by the processing enzymes of this novel pathway is the C-terminal amino acid sequence, -CAAX (C is cysteine, A is aliphatic amino acids, and X is any amino acid) (for reviews, see references 14 and 15). A number of eukaryotic proteins are now known to be processed by this novel pathway, including Ras and Ras-related proteins (5, 9, 34, 35, 38), G-protein subunits (12, 24), nuclear lamins (40), and other proteins of unknown function (11, 23, 25, 32). Research discussed in Part II of this dissertation has delineated that the order of -CAAX processing events is as follows: 1) isoprenylation of the cysteine sulfur, 2) proteolysis of the -AAX, and 3) methyl

esterification of the C-terminal carboxyl group. In addition, it was determined that the *RAM* gene product is required for isoprenylation of **a**-factor. Studies by other laboratories demonstrated that RAM is actually a component of the enzyme responsible for both **a**-factor and RAS isoprenylation (16, 35). RAM by itself, however, does not function as an isoprenyl transferase (35).

It is now believed that defective, or activated, *ras* genes may be responsible, at least in part, for 20 per cent or more of all human tumors, although the incidence of activated *ras* is as high as 40 per cent in colorectal tumors and 90 per cent in pancreatic tumors (1, 3, 4, 13). Because of the prominent role of activated *ras* genes in human tumors, drug therapies which inhibit Ras function would be extremely valuable. Genetic evidence already indicates that inhibition of isoprenylation of activated Ras results in suppression of the defective phenotype (31). Therefore, assays such as the one described in Part II of this dissertation could prove useful for the screening of isoprenyl transferase inhibitors; such inhibitors would be potential anti-Ras-related cancer therapeutic agents.

Prior to the completion of this dissertation research, research by other laboratories defined roles for two other genes known to be involved in **a**-factor biosynthesis–*STE6* and *STE14*. As discussed in Part I, it is now believed that the *STE6* gene encodes a transporter which secretes mature **a**-factor out of **a** cells (21, 29). *STE14*, on the other hand, encodes a product which is required for C-terminal methyl esterification of **a**-factor (18, 28), although it has not yet been determined whether STE14 is itself a methyl transferase nor whether STE14 is required for C-terminal methyl esterification of *S. cerevisiae* RAS. While still not complete, our overall picture of **a**-factor maturation and secretion is now fairly well detailed (fig. 1). The amino terminal segment of the primary **a**-factor precursor, pro-**a**-factor, is proteolyzed by an unidentified activity to produce the **a**-factor 15mer (Part II and reference 27). It is possible, if not probable, that N-terminal proteolysis occurs independently of farnesylation of the -CAAX cysteine. We do know that the 15mer can be processed to

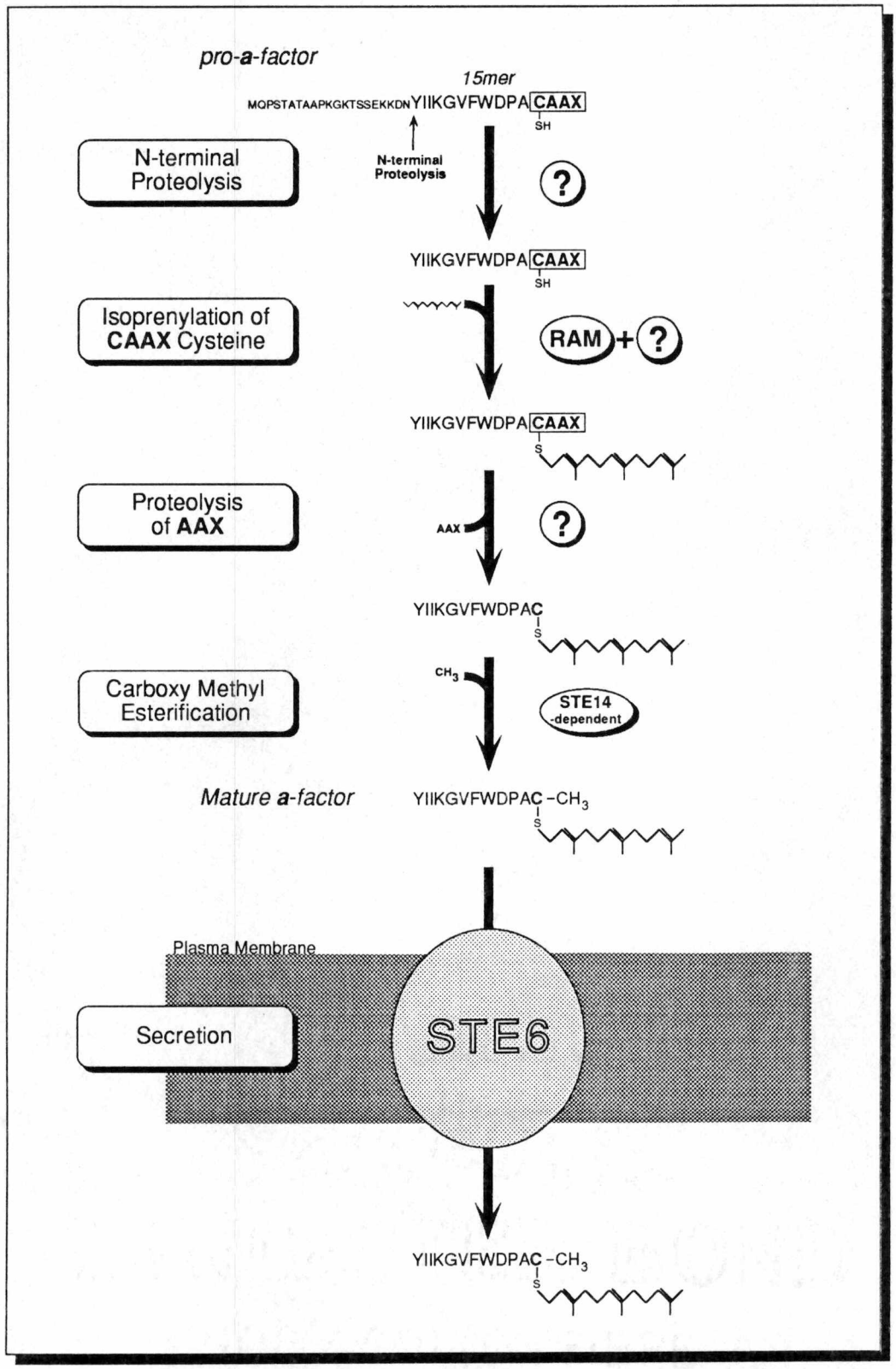

Fig. 1. Our current picture of a-factor maturation.

mature **a**-factor (Part II and reference 27). The -CAAX cysteine of either the 15mer, pro-**a**-factor, an N-terminal truncated pro-**a**-factor, or all three is farnesylated by an activity comprised of the *RAM* gene product and at least one other component (Part II and references 27 and 35). After farnesylation, the -AAX sequence is hydrolyzed, producing nonmethylated **a**-factor (Part II and reference 27). The protease(s) responsible for hydrolysis of -AAX have not yet been identified. Next, the C-terminal carboxyl of nonmethylated **a**-factor is methyl esterified (Part II and reference 27) by an activity whose expression is dependent on the *STE14* gene product (18). Finally, mature **a**-factor is pumped out of the **a** cell by the STE6 transporter (21, 29).

While, for the most part, the sequence of **a**-factor biosynthetic steps is now known, there are still some processing enzymes which have yet to be identified; these include the protease(s) responsible for N-terminal processing (to produce the 15mer), the other component(s) of the farnesyl transferase, and the protease(s) responsible for proteolysis of -AAX. The *STE6* gene product is not involved in post-translationally modifying **a**-factor, as *ste6Δ* mutants produce mature intracellular **a**-factor (21). While it is possible that RAM and/or STE14 are multifunctional, it is more likely that the genes encoding the other **a**-factor processing enzymes have not yet been identified. Since numerous screens have been carried out for the isolation of sterile mutants, it is likely that these unidentified genes are essential in *S. cerevisiae.* If this proves to be the case, then the remaining **a**-factor processing enzymes will have to be purified by traditional biochemical techniques–not a trivial task. For the time being, it would be a wiser investment of time to purify RAM and STE14 so as to fully characterize their functions and to investigate the possibility of multifunctionality. In particular, purification of STE14 will make it possible to determine whether the protein is indeed a methyl transferase and, if so, whether it is responsible for carboxy methyl esterification of *S. cerevisiae* RAS proteins.

Because of the importance of isoprenylation in Ras activity, a monumental effort is being made by numerous laboratories to fully purify isoprenyl transferase(s) and to clone the genes encoding them. The fact that the isoprenyl transferase responsible for farnesylation of **a**-factor is apparently comprised of at least two subunits, leads to the speculation that different subunit combinations might be responsible for target specificity by isoprenyl transferases. In addition, different subunits might be differentially localized in a cell.

As indicated above, it is believed that STE6 transports **a**-factor out of **a** cells. But what is the substrate specificity of STE6? Schafer et al. (34) showed that farnesylation of **a**-factor is required for its secretion. In addition, Marr et al. (28) showed that carboxy methyl esterification is not required for **a**-factor secretion, as *ste14* mutants secrete nonmethylated **a**-factor. However, *ste14* mutants were shown to secrete much less pheromone than wild type **a** cells. Is nonmethylated **a**-factor transported less efficiently than the wild type pheromone by the STE6 protein, or is nonmethylated **a**-factor more susceptible to intracellular degradation than wild type pheromone? Can STE6 transport other farnesylated peptides out of cells, or does it recognize both the farnesyl group and peptide sequence of **a**-factor? Why are RAS or other isoprenylated proteins not secreted? These are very important questions which remain to be answered, as understanding how STE6 functions may allow us to gain a better understanding of how multiple-drug resistance (MDR) proteins function. This, in turn, would make it possible to design better inhibitors of MDR proteins, which play a significant role in the treatment of many cancers (2). In our laboratory, Guy Caldwell is now randomly mutating the **a**-factor structural gene, *MFa1*, as part of a genetic approach to the study of **a**-factor structure-activity relationships. It will be most interesting to see whether non-CAAX-related modification of the **a**-factor amino acid sequence prevent its secretion. While not practical for our laboratory, it would also be quite interesting to express *STE6* in *Xenopus* oocytes. Radiolabeled **a**-factor could then be

injected into such oocytes to determine if they were capable of secreting the pheromone. Such an experiment would definitively demonstrate that STE6 is indeed an **a**-factor transporter.

Role of Cys^{12} Modifications in the Biological Activity of **a**-Factor

As discussed above, farnesylation is required for **a**-factor secretion, whereas carboxy methyl esterification is not. But what role do these same modifications play in the biological activity of the pheromone? The work presented in Part III of this dissertation answers this question. We can now say that farnesylation and carboxy methyl esterification play nearly equal roles in the biological activity of **a**-factor. But what is the specific nature of these structural requirements? Are the modifications required for binding to the **a**-factor receptor itself, or are they primarily required for association of the lipopeptide with the plasma membrane of the α target cell? If the latter is the case, what are the implications for the potential topology of the ligand-binding site of the **a**-factor receptor? The answers to questions such as these could provide important insights into the nature of lipopeptide hormone-receptor interactions in general.

We know from recent experiments that the hydrophobic **a**-factor, as might be expected, associates strongly with plasma membranes of both **a** and α cells. In addition, collaborative studies with Leaf Huang's laboratory show that **a**-factor also associates very strongly with artificial liposomes. Considering what we have now learned about the lipophilic **a**-factor, what models can be predicted for **a**-factor-receptor binding topology? Traditionally, peptide hormone-receptor interactions have, with few exceptions (33), been pictured as not being dependent on a direct association of the ligands with the plasma membrane, although membrane lipids are certainly involved in maintaining proper receptor conformation. If only the peptide portion of **a**-factor is required for efficient binding to the receptor in a membrane-independent manner, then we would not expect the farnesyl group to be

required for biological activity. Therefore, the model shown in figure 2A can, for the most part, be ruled out. But what about the model shown in figure 2B? In this model, **a**-factor-receptor binding still does not involve association of the pheromone with the plasma membrane, but does involve direct association of the modified C-terminal of **a**-factor with the ligand binding site. At least two experimental results would argue in favor of an alternative model. First, as indicated above, **a**-factor clearly associates quite strongly to plasma membranes. Consequently, one could hypothesize that, since **a**-factor associates so strongly with plasma membranes, that membrane association might be required for interaction of the pheromone with its receptor. The second experimental result supporting an alternative model for **a**-factor-receptor binding is the determination that **a**-factorase, the α cell specific **a**-factor degrading activity, is apparently a cell plasma membrane-associated enzyme (Part IV of this dissertation and data not shown). Since **a**-factorase is involved in α cell desensitization from the pheromone response, it once again seems reasonable to speculate that the enzyme would be membrane-associated because its target is membrane-associated. From these strictly empirical observations, it could be hypothesized that the interaction of **a**-factor with its receptor occurs after insertion of **a**-factor into the plasma membrane, as shown in fig. 2C. While this model seems most fitting for the interaction of **a**-factor with its receptor, it should also be noted that similar models have been described for the interaction of other peptide hormones, which are not hydrophobically modified post-translationally, with their receptors (33, 36). Determination of the **a**-factor-binding site and specific topology of the **a**-factor receptor in the plasma membrane should make it possible to define the mechanism of **a**-factor-receptor interaction. Such determinations are dependent, of course, on either X-ray crystallographic analysis of pure receptor crystals, both free and ligand-bound, or high resolution nuclear magnetic resonance (NMR) spectroscopy. Unfortunately, it is extremely difficult to isolate the quantities of receptor

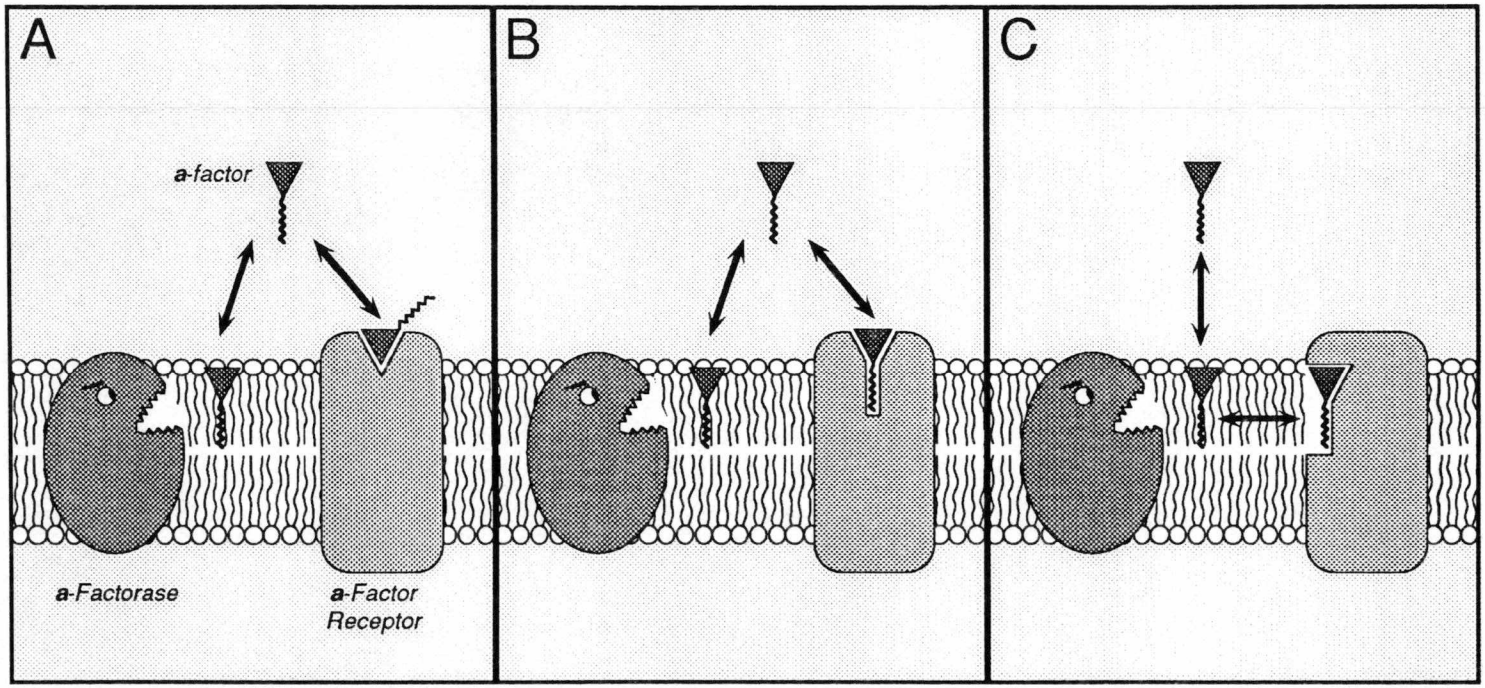

Fig. 2. Potential models for **a**-factor receptor ligand binding site topology. The active site of **a**-factorase, represented by the mouth, is purely speculative.

proteins required for those techniques. Consequently, it is unlikely that the **a**-factor receptor ligand binding site topology will be determined in the very near future.

While replacement of the farnesyl group of **a**-factor by a hydrogen atom significantly reduced the biological activity of the pheromone, replacement by other hydrocarbon moieties produced analogs with activities that were, in some cases, only slightly lower than **a**-factor. In the Discussion of Part III, several factors which might play roles in the observed biological activity of **a**-factor and its analogs were described. One potential factor which was not discussed was the contribution that the cysteine modifying groups might have in maintaining, or allowing for, an **a**-factor structural conformation that is specifically recognized by the **a**-factor receptor. Recent studies (17) show that **a**-factor does not maintain a preferred conformation in solution. However, this result by no means rules out the possibility that the **a**-factor receptor itself recognizes a specific pheromone conformation. In addition, it is possible that **a**-factor might maintain a preferred conformation in the plasma membrane. Studies by other investigators have shown that some biologically active peptides, such as β-endorphin and dynorphin A, which do not exhibit conformational preferences in aqueous solutions or in crystalline form, do exhibit functionally important conformations upon interaction with macromolecular surfaces (i.e., lipid membranes) (39). NMR studies of **a**-factor in lipid vesicles should tell us whether **a**-factor might maintain a preferred conformation in the plasma membrane. However, only X-ray crystallographic studies of the ligand-bound form of the **a**-factor receptor will allow us to determine whether the farnesyl group or carboxy methyl ester play important roles in receptor-recognized **a**-factor conformation.

Role of **a**-Factor in Mating

Previous studies by other investigators (30) had suggested that **a** cells must actively produce **a**-factor in order to mate with α cells, since, under their assay conditions,

exogenous pheromone could not restore mating to a mixture of wild type α cells and **a** cells which had deletions of their **a**-factor structural genes (*mfa1 mfa2* mutants). However, in Part III of this dissertation, results are presented which show that exogenous **a**-factor does indeed restore mating of *mfa1 mfa2* mutants to wild type α cells, although mating was not restored to a wild type level.

As discussed in Part III of this dissertation, we believe that the inability of exogenous **a**-factor to restore mating of *mfa1 mfa2* mutants to a wild type level might be attributed to interference of the "courtship response". Recent studies by Jackson and Hartwell (19, 20) demonstrated that *S. cerevisiae* **a** and α haploids select as mating partners those cells of opposite mating type which produce the most pheromone. They termed this process courtship, and showed that it is one of the earliest steps of the mating process. Jackson and Hartwell concluded that the process of mating partner selection involves a response to pheromone gradients, such that shmoo morphogenesis occurs toward the source of a gradient (19, 20). Results from our laboratory, discussed in Part III of this dissertation, support their hypothesis. However, results of more recent experiments now argue against morphogenesis in response to pheromone gradients as being solely responsible for the courtship process. If **a** and α cells recognize pheromone gradients, we would expect that, at a certain range of pheromone concentration within a gradient, cells would direct their projections toward the source of the gradient (i.e., a filter disk or a patch of cells of opposite mating type). In recent experiments, we have observed that neither **a** nor α cells direct their shmoo projections toward pheromone sources (either synthetic pheromone from disks or natural pheromone from patches of cells of opposite mating type). In fact, even when cells were in direct contact with cells in patches of the opposite mating type, they appeared to direct their shmoo projections randomly (fig. 3, left panel), presumably because the pheromone levels produced by the patches were too high. When pheromone was supplied from paper disks, cells within several millimeters from the edges of the

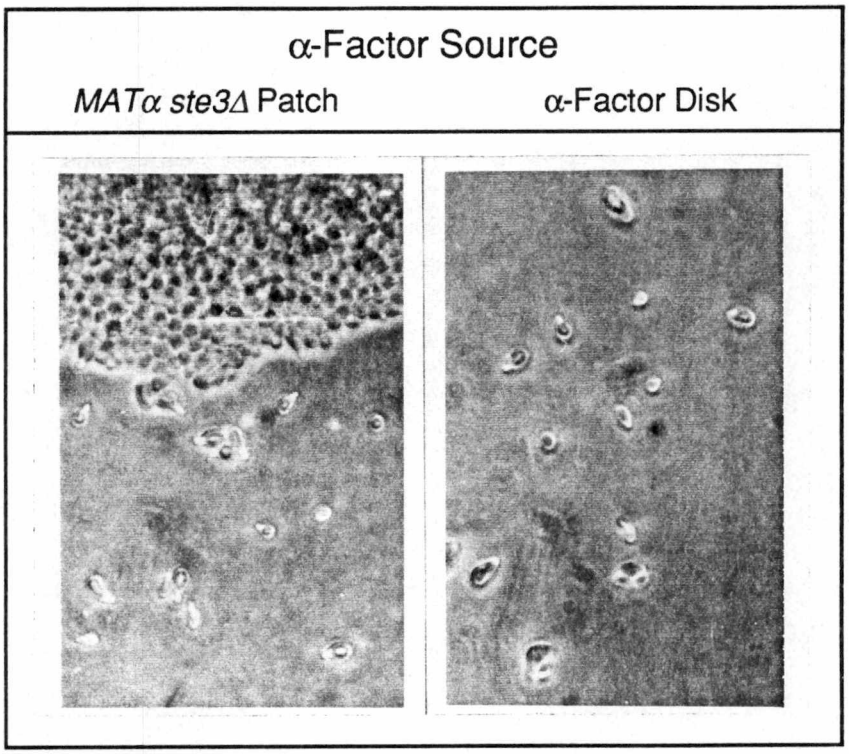

Fig. 3. *Saccharomyces cerevisiae* **a** and α cells do not appear to direct their shmoo projections in response to pheromone gradients. Left panel: *MATα ste3Δ* (**a**-factor receptorless mutants) were patched (top of photograph) onto a lawn of *MAT**a*** *sst1-2* cells . Photograph was taken approximately 5 hr after patching the *MATα* cells. Right panel: 0.1 μg of α-factor was placed on a sterile filter disk, then the disk was placed on a lawn of *MAT**a*** *sst1-2* cells. Cells shown were about 3 mm from the edge of the disk; cells at the top of the photograph were closest to the disk. Photograph was taken approximately 5 hr after placement of α-factor on the disk. Note that in both photographs, shmoo tips are oriented randomly and not specifically toward the α-factor source.

disks displayed a totally random directionality of shmoo projections (fig. 3, right panel). Even at the periphery of this "affected range", where ≤50% of cells were shmoos, projections were directed randomly (not shown). These results do not support the hypothesis that cells recognize pheromone gradients, and point to the likelihood that the process of courtship involves more than simply the recognition of pheromone gradients *per se*, but rather more complex interactions which might require cell-cell contact. It is also possible that cells recognize locally high concentration of pheromone as opposed to pheromone gradients. Furthermore, it is conceivable that responses observed in typical pheromone bioactivity assays may be the result of a default response mechanism that is activated above certain levels of pheromone and that is not dependent on courtship.

In light of our lack of evidence to support the hypothesis that *S. cerevisiae* haploids respond to pheromone gradients, other models to explain the courtship response must now be considered, especially those which would involve the requirement for cell-cell contact, instead of, or in addition to, pheromone gradients. For example, a haploid in direct contact with several different cells of opposite mating type may select as a mating partner the cell type presenting the most pheromone at its cell surface. Jackson and Hartwell's courtship assays were done using 4 to 8 layers of packed cells (19, 20), thus, cells were indeed in direct contact. Furthermore, based on the endpoint of response in the *FUS1-lacZ* assay, it is highly probable that fewer than ten molecules of **a**-factor are required to trigger the pheromone response! Thus, as opposed to a model in which cells respond to pheromone gradients, it should be considered possible, if not probable, that cell morphogenesis occurs toward the site where the first few molecules of pheromone bind to receptors. More elaborate experiments must now be devised to further study the process of courtship. For example, methods could be used which allow for the observation of cells of opposite mating type in close proximity or in direct contact with each other; the most direct means for achieving this would be by use of a micromanipulator. A second approach would be to

try the patch assay (fig. 3, left panel) with strains whose level of pheromone production can be controlled (i.e. under control of an inducible promoter). Because of the potential relationship to developmental processes of higher eukaryotes, an understanding of the cellular mechanisms involved in the courtship response is highly desirable. Our laboratory is now vigorously undertaking experiments aimed at better characterizing the process of courtship. A more detailed understanding of the courtship process should facilitate the designing of selection schemes for isolation of courtship-defective mutants, which will make it possible to clone genes involved this important intercellular communication process.

Identification and Characterization of an α-Cell Specific **a**-Factor-Degrading Protease

S. cerevisiae **a** cells degrade α-factor as one mechanism of recovery, or desensitization, from the pheromone response (8). The protease responsible for α-factor degradation is encoded by the *BAR1* gene (also called *SST1*), which is only expressed in **a** cells (8, 6, 7, 26). Expression of the *BAR1* gene is increased when **a** cells are exposed to α-factor (26). While there had been some speculation that α cells might similarly degrade **a**-factor, there was no direct evidence supporting such speculations. Part IV of this dissertation describes the first direct biochemical and physiological evidence of an α cell-specific **a**-factor-degrading endopeptidase, which we call **a**-factorase. Like the **a** cell-specific BAR protease, **a**-factorase appears to play a role in desensitization of α cells from the pheromone response, as agents which inhibited **a**-factor proteolysis also enhanced and prolonged the α cell response to the pheromone. In addition, preliminary experiments suggest that expression of **a**-factorase, like the BAR protease (26), increases when α cells are treated with **a**-factor. However, unlike the BAR protease, which is a secreted enzyme (8), **a**-factorase is cell-associated. Our results suggested that **a**-factor degradation is not required

for α cell response to the pheromone. This conclusion can be confirmed by cloning and subsequently disrupting or deleting the gene which encodes **a**-factorase (see below).

Since the radioiodinated **a**-factor degradation product exhibited chromatographic characteristics indistinguishable from the peptide Y(diiodo)IIKGV, we speculated that **a**-factorase cleaves **a**-factor between residues Val^6 and Phe^7. However, this result by no means allows us to definitively conclude on the **a**-factor cleavage site. Definitive determination of the **a**-factor cleavage site will require the isolation of quantities of degradation product(s) sufficient for structural characterization techniques such as amino acid analysis or mass spectrometry–such quantities will be most effectively obtained using purified or partially purified **a**-factorase.

Quite interestingly, **a**-factorase appears to have characteristics similar to those the mammalian peptide hormone-degrading protease, neutral endopeptidase (NEP), a cell-associated, metal-dependent enzyme which cleaves its peptide substrates at the carboxyl side of phenylalanine and, in some cases, aliphatic amino acid residues. NEP is thought to play a role in chronic acute lymphocytic leukemia (22) and is currently the subject of intensive study. In addition, it is one of only a handful of known biologically relevant peptide hormone-degrading proteases. We have recently determined that **a**-factorase is inhibited by the thiorphan, a potent inhibitor of NEP (22). Furthermore, Karen Marcus has determined that *S. cerevisiae* contains nucleic acid sequence(s) which hybridize to the NEP gene under highly stringent conditions. She is currently attempting to clone this potential NEP homolog to determine whether it encodes **a**-factorase.

If the *S. cerevisiae* NEP homolog does not encode **a**-factorase, other approaches will be taken. The first approach will involve screening for mutants supersensitive to **a**-factor, since **a**-factorase mutants would be expected to have phenotypes similar to those of *bar1* mutants (6, 7). As indicated in Part IV of this dissertation, Steden et al. (37) used a similar approach to identify the *SSL1* gene–the only α cell specific gene identified in their screen.

However, our results suggest that *SSL1* does not encode **a**-factorase. Consequently, additional screens for **a**-factor supersensitive mutants might be worthwhile. If such screens yielded **a**-factorase mutants, which would be easily identified by their inability to degrade **a**-factor, the **a**-factorase gene could be easily cloned by complementation of the supersensitive phenotype. An alternative, though not trivial, approach to cloning the **a**-factorase gene would involve purifying **a**-factorase by classical protein purification techniques, obtaining a partial amino acid sequence of the purified enzyme, then synthesizing a mixed oligonucleotide probe corresponding to the partial amino acid sequence. This probe would then be used to screen an *S. cerevisiae* α cell cDNA library so as to identify the **a**-factorase gene.

The identification of **a**-factorase is an important contribution to our overall understanding of the processes involved in the *S. cerevisiae* pheromone response. Very few mammalian peptide hormone-degrading proteases have been identified, and even fewer have been substantially characterized. It will be quite interesting to investigate whether **a**-factorase has homologs in higher eukaryotes and, if so, to determine their functional roles. By cloning **a**-factorase, we will be able to carry out such investigations.

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