To the Graduate Council:

I am submitting herewith a dissertation written by Guy Alexander Caldwell entitled "Functional Consequences of Structural Alterations to the Isoprenylated Yeast Mating Pheromone **a**-Factor". 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.

Dr. Jeffrey M. Becker, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of the Graduate School

FUNCTIONAL CONSEQUENCES OF STRUCTURAL ALTERATIONS TO THE ISOPRENYLATED YEAST MATING PHEROMONE **a**-FACTOR

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Guy Alexander Caldwell

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DEDICATION

This doctoral dissertation is dedicated to my parents, Marc and Victoria Caldwell, with the deepest gratitude and pride I can offer as their son; to the loving memory of all my grandparents, especially my grandmother, Barbara Constantinovna Rustamova, and my grandfather, Dr. Guy A. Caldwell, whose lives served as a constant inspiration for me to strive and pursue my dreams in the face of any adversity; and, to my colleague, my best friend, my love, and my wife, Kim, with whom I have shared this wonderful experience and without whom I could never imagine my life again.

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ABSTRACT

In the course of its biosynthesis, secretion, and biological action, the isoprenylated mating pheromone of *Saccharomyces cerevisiae,* **a**-factor, interacts with representative proteins of several biologically significant protein families. The **a**-factor is posttranslationally modified by the enzyme farnesyl transferase, exported by a mechanism requiring the action of a yeast homolog of the mammalian multidrug resistance P-glycoprotein, and induces mating via interaction with a protein that shares homology to the G protein-coupled family of receptors. The main objective of this research was to establish and employ a system for the analysis of the structure-function relationships involving **a**-factor as it serves as either a substrate or ligand for its cellular target proteins. An overview of the literature, as it is related to this work, is presented in Part I of this dissertation.

Part II of this dissertation outlines experimental procedures developed in order to establish a system for structure-function relationship studies on **a**-factor. This entailed the use of oligonucleotide-directed site-specific mutagenesis to introduce precise genetic mutations within the *MFa1* gene, encoding **a**-factor, coupled with a physiological assay for pheromone bioactivity to discern the consequences of such alterations. These experiments led to the characterization of two specific mutations which blocked the production of **a**-factor in vivo.

Part III of this dissertation describes an analysis of the biological consequences of altering the isoprene targeting motif within **a**-factor to code for posttranslational modification with a geranyl, geranyl isoprene group instead of the native farnesyl lipid. This study demonstrated that differential isoprenylation of **a**-factor with either farnesyl or geranyl, geranyl results in a secreted and bioactive peptide hormone. Furthermore, biochemical analysis of the peptide pheromones produced by yeast overexpressing **a**-factor

containing a specific geranyl, geranyl targeting sequence indicated that cross-prenylation of such an **a**-factor substrate peptide with either isoprene can occur in vivo.

Parts IV and V of this dissertation summarize the results obtained from an investigation into the bioactivity of **a**-factor peptides that contain amino acid substitutions or truncations. Specifically, Part IV discusses the identification of an **a**-factor analog which exhibits a stereospecific superactivity. In two separate bioassays, the [D-Ala^5] **a**-factor was found to have biological activity higher than both **a**-factor and a [L-Ala^5] **a**-factor analog. Part V reports the results of activity analyses on synthetic **a**-factor analogs containing truncations at the N-terminus of the peptide. In addition, a physiological screen was employed to identify non-active **a**-factor peptides selected from yeast expressing a pool of **a**-factor genes that were randomly mutated within the mature portion of the peptide. DNA sequence analysis of the genes encoding the selected peptides revealed amino acid substitutions predominantly at specific positions within the peptide, implicating these residues in pheromone bioactivity. These combined studies represent an initial inquiry into the relative contribution of specific amino acids to **a**-factor activity.

Finally, Part VI of this dissertation serves to summarize and relate the findings of this work to current studies in progress and acts as a forum for the proposal of future directions of **a**-factor related research.

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PART I

GENERAL INTRODUCTION

CHAPTER I

THE BIOSYNTHESIS , EXPORT, AND BIOLOGICAL ACTIVITY OF **a**-FACTOR: AN OVERVIEW

The common baker's yeast, *Saccharomyces cerevisiae*, has served as a model organism for the study of cellular processes shared by all eukaryotes (3). The ease with which this yeast is manipulated, in conjunction with the vast history of genetic and physiological information that has been acquired on its biology, makes it an ideal organism for experimental investigation into the molecular mechanisms underlying cellular function. Among the most interesting processes carried out by cells is intercellular signal transmission. In this regard, *S. cerevisiae* has adopted a mode of sexual reproduction which involves the secretion and recognition of peptide mating pheromones by cells of two opposing mating types (Fig. 1). Analysis of the molecular events surrounding the mating of haploid yeast cells to form diploids has provided much insight into the manner by which intercellular signals are propagated and disseminated in eukaryotic cells (14,19,39).

Specifically, the conjugation of haploid yeast cells is dependent upon the reciprocal action of mating pheromones termed **a**-factor, which is produced by **a**-cells (*MAT**a***) and α-factor, which is secreted by α-cells (*MATα*) (14,19,39). These peptides are recognized by receptor proteins, expressed on the surface of cells of the opposite mating type (5,13), and induce gene expression in their target cells resulting in responses that are required for mating: growth arrest at G_1 of the cell cycle, production of cell surface agglutinins, and morphological alteration (termed "shmooing") (19). These events lead to subsequent aggregation of cells, then cytoplasmic and, finally, nuclear fusion to form diploid zygotes . In the course of the yeast life cycle (Fig. 2), diploids may persist indefinitely; however,

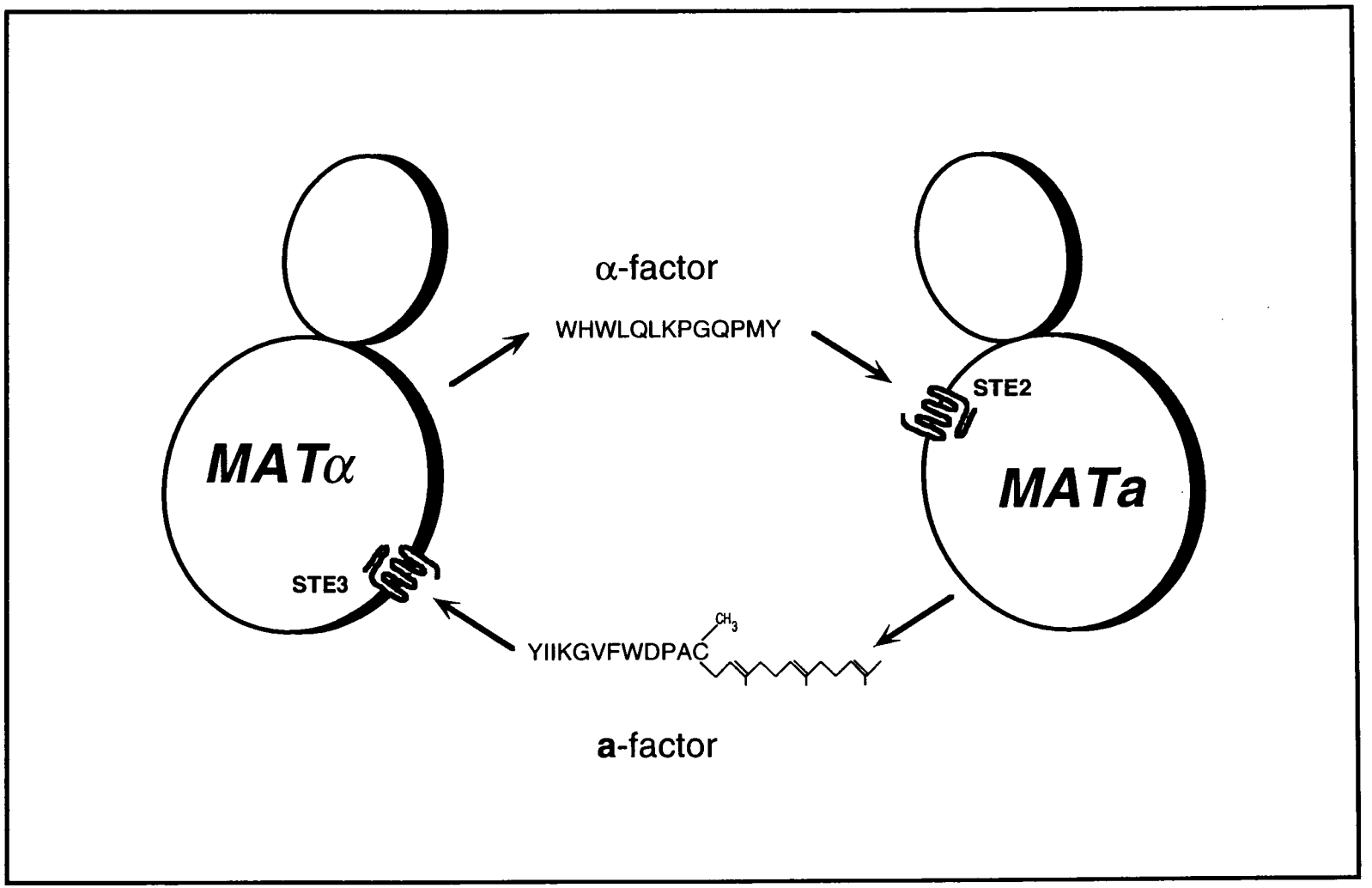


Figure 1. An overview of yeast mating

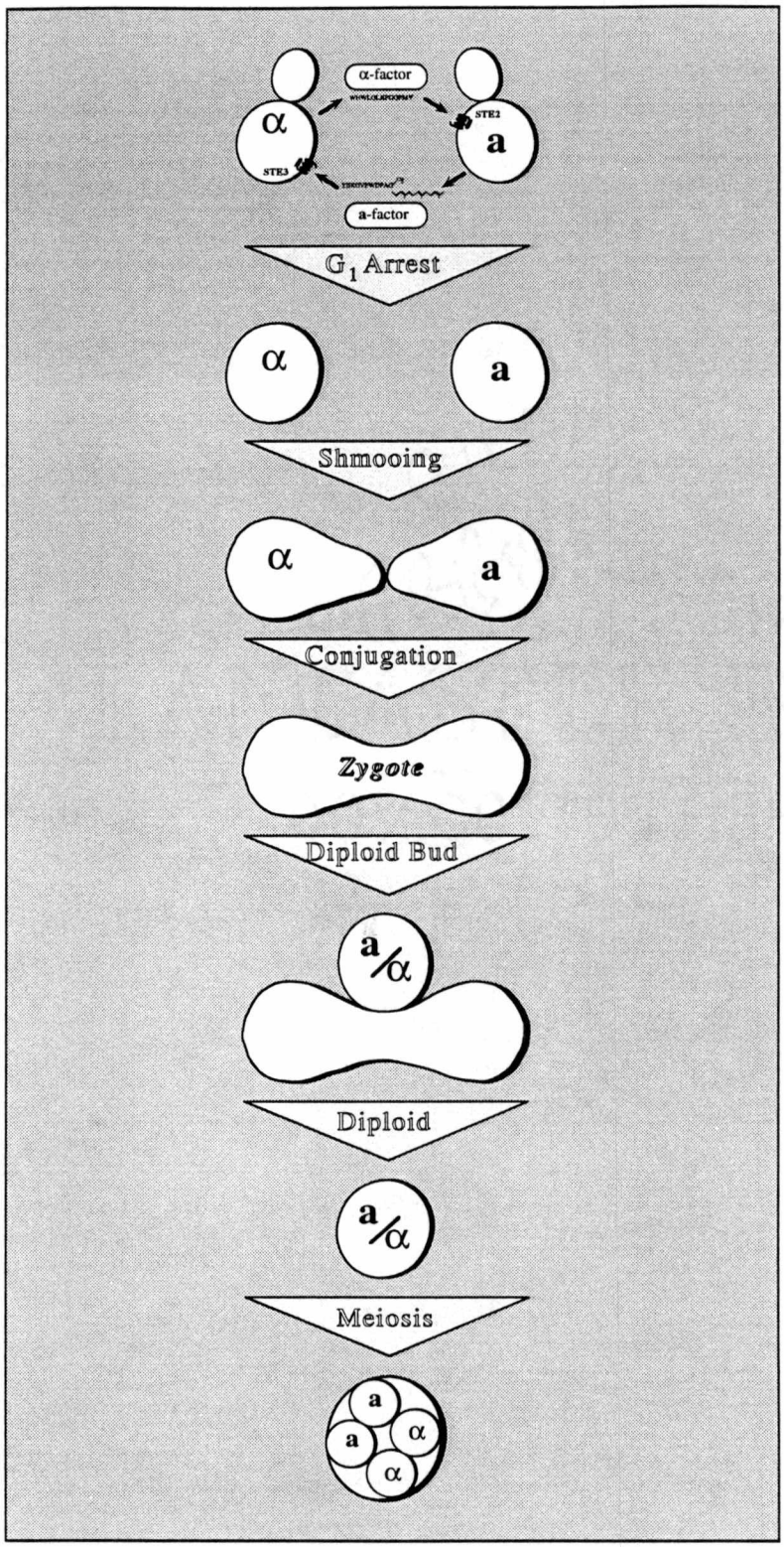


Figure 2. *S. cerevisiae* life cycle

under conditions of nutrient deprivation zygotes can commence meiosis and sporulation to return to a haploid state, as well (14,39).

Although the α-factor and **a**-factor appear to function in an equivalent fashion, these two peptides are surprisingly different in their structure (Fig. 3), biosynthesis, and mode of export from cells. A considerable number of studies has been performed on the biosynthesis and secretion of the α-factor which, in part, have greatly contributed to the dissection of the yeast secretory pathway (36). The α-factor is the product of two similar genes, *MFα1* and *MFα2*, which encode primary polypeptide precursors, each containing a hydrophobic leader sequence, and either four identical α-factor repeat sequences or two α-factor repeats, respectively (10,19). These identical α-factor repeats are separated by spacer amino acids which are extensively processed through the secretory pathway to yield mature pheromones in the form of unmodified tridecapeptides [WHWLQLKPGQPMY]. In contrast, the **a**-factor is the product of two nearly identical genes, *MFa1 and MFa2*, each encoding a thirty-six amino acid precursor which lacks a consensus signal sequence for directing its secretion through the classical secretory pathway (endoplasmic reticulum, Golgi, secretory vesicles) (4,26). This precursor is proteolytically cleaved and posttranslationally modified to yield the mature **a**-factor [YIIKGVFWDPAC(farnesyl)-OCH_3] (1,22,25,34). Furthermore, mutants which block secretion at various stages of the secretory pathway do not affect the production of **a**-factor (40,41). These observations suggest that the export of **a**-factor occurs by a novel mechanism, different than that utilized by α-factor.

The secretion of **a**-factor remained unexplained until the discovery of a gene which was found to complement an **a**-cell specific mutation, *ste6*, originally identified as sterile, due to its inability to secrete **a**-factor (42). This complementing DNA was sequenced and its predicted protein product appeared to share a high degree of amino acid similarity to the

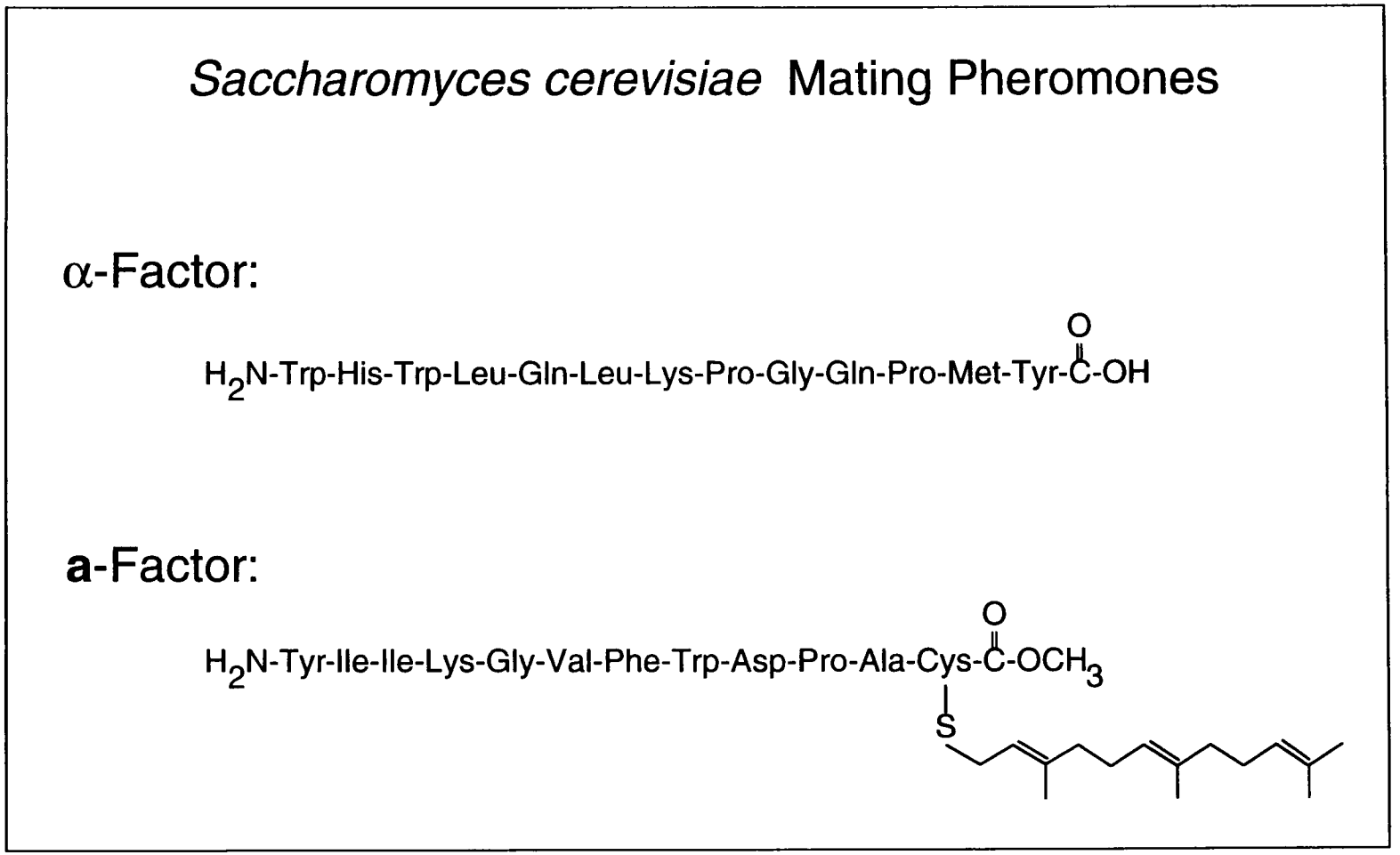


Figure 3. Structure of α-factor and **a**-factor

mammalian multidrug-resistance (MDR) P-glycoprotein (19,24). In mammalian cells, the MDR P-glycoprotein has been implicated in the resistance of cancer patients to treatment with multiple chemotherapy agents. These agents are exported from cells expressing the MDR P-glycoprotein by an undetermined mechanism (15,18). It became apparent that the **a**-factor was a natural substrate for this type of transport protein (31); and thus characterization of the substrate specificity exhibited by the yeast MDR homolog, the STE6 protein, should provide insight into MDR-related transport (18, 31).

The structural characterization of the mature **a**-factor (1) as a dodecapeptide containing modifications on its C-terminal cysteine led to several subsequent observations related to **a**-factor biosynthesis and export. The most significant finding was the discovery of mutants, originally identified on their inability to produce **a**-factor, that were also defective in production of the yeast homolog of mammalian oncogene product RAS (29). The identification of these mutants, termed *RAM* (for RAS-**a**-factor maturation), suggested that both RAS and **a**-factor possibly shared a common biosynthetic step. Structural comparison of RAS and **a**-factor showed that they contain identical C-terminal modifications in the form of a C_{15} isoprenoid group (farnesyl) and carboxyl methyl ester moiety. Farnesylation of RAS was later found to be required for its proper membrane localization and transforming activity (16,33,34,35). Similarly, the export and biological activity of **a**-factor was also found to be dependent on posttranslational modification (21,22,33,34,35). This focused much attention on the biosynthesis of **a**-factor as a model for understanding the molecular mechanisms involved in RAS membrane targeting, as well as for the interaction of all isoprenylated proteins with their cellular targets.

Among these cellular targets is the **a**-factor receptor protein, STE3 (13). The receptor proteins for both yeast mating pheromones are members of the seven-transmembrane-segment family of G protein coupled receptors. This family also includes

the rhodopsin, muscarinic, and adrenergic receptor proteins (8). The synthesis of α-factor analogs, development of ligand binding assays, and biological activity assays for α-factor have facilitated the analysis of structure-activity relationships between α-factor and its receptor, the STE2 protein (2,9,27,28,30). Similar analyses on the **a**-factor have not been extensive. However, bioactivity studies have shown that posttranslational modification of the **a**-factor is a prerequisite for its ability to induce growth arrest and mating (21). These results imply that farnesylation and carboxyl methylation serve a critical role in either the direct interaction of the **a**-factor with STE3 or indirect presentation of the pheromone to its receptor. The manner by which **a**-factor acts to trigger cellular responses via binding to the STE3 receptor serves as a model for studying lipid-protein interactions mediated by isoprene-modified proteins.

Clearly, in the course of its biosynthesis, export, and bioactivity, the **a**-factor serves as either a substrate or ligand for members of several biologically significant protein families: a farnesyltransferase enzyme, a homolog of a multidrug resistance transporter protein, and a G protein-linked receptor. The goal of this dissertation is to further our understanding of the structural requirements governing **a**-factor recognition by these targets and contribute to our basic understanding of their substrate specificity and function.

The initial stages of this study were focused on establishing a system for the mutagenesis of the gene encoding the **a**-factor (*MFa1*), expression of mutated gene constructs in yeast, and phenotypic characterization of the altered peptides, as described in Part II of this dissertation. This work not only served to establish a system for future structure-function analysis of the pheromone, but also resulted in the first published evidence that site-specific amino acid changes in the isoprenyl targeting motif (-CAAX box) of the **a**-factor precursor could abolish **a**-factor bioactivity in vivo (22).

During the course of these studies, an explosion of information occurred on the catalytic mechanisms, substrate specificity, purification, and gene cloning of the enzymes responsible for attachment of isoprene groups to proteins, prenyltransferases (6,11,32,34,37). Specifically, these enzymes were found to catalyze the transfer of two types of isoprenyl moieties, the C_{15} farnesyl and C_{20} geranyl, geranyl groups, to varied types of proteins (20,32,38). Although much work has centered on the enzymatic addition of these lipids to proteins, the functional consequences of differential isoprenylation of peptides with either farnesyl or geranyl, geranyl groups remain to be fully understood (7,23). Part III of this dissertation utilized the techniques established in Part II to generate specific changes in the **a**-factor gene. This allowed for the alteration of the isoprenyl targeting sequence in **a**-factor from the native farnesylation target to that encoding motifs for geranyl, geranyl addition. The results of a biochemical and physiological analysis of the functional effects of such changes on **a**-factor biosynthesis, export, and bioactivity are addressed in this portion of the dissertation.

Parts IV and V of this dissertation are devoted to the effect of changes in the peptidyl portion of the **a**-factor on pheromone bioactivity. In the course of this work, an analog of **a**-factor, termed [D-Ala^5] **a**-factor, containing a D-alanine residue substituted for glycine in the fifth position of the peptide, was shown to exhibit higher bioactivity than the native **a**-factor. The results of additional analyses on this stereospecific superactive **a**-factor are presented in Part IV of this dissertation. Part V specifically addresses the biological consequences of truncation and substitution of amino acids in the **a**-factor through a combination of molecular biology and synthetic peptide biochemistry experiments, in conjunction with physiological analyses of **a**-factor bioactivity. These studies represent an initial inquiry into the relative contribution made by the amino acids constituting the **a**-factor peptide toward pheromone activity, specifically in comparison to previous studies

performed on the C-terminal modifications of **a**-factor and their contribution toward pheromone function (21).

Finally, Part VI of this dissertation summarizes the studies outlined in Parts II-V and serves as a forum for the discussion of possible directions for future research. In addition, it attempts to place the conclusions of this dissertation in a contextual overview of lipopeptide-target protein interactions.

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PART II

ESTABLISHING A SYSTEM FOR STRUCTURE-FUNCTION ANALYSIS OF THE **a**-FACTOR MATING PHEROMONE OF *SACCHAROMYCES CEREVISIAE* *

*Portions of 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 produced by **a**-cells, and α-factor, which is secreted by α-cells (14). The biosynthesis of α-factor has been extensively examined and occurs via the classical yeast secretory pathway yielding the mature tridecapeptide product, WHWLQLKPGQPMY (5). In contrast, the **a**-factor, an isoprenylated peptide [YIIKGVFWDPAC(farnesyl)-OCH_3], is processed by an alternative pathway which involves posttranslational processing events shared by the *RAS* oncogene product (1,25,26,27) (Fig. 1). Studies have shown that isoprene modification is essential for normal RAS function (7). Likewise, isoprenylation is important for **a**-factor bioactivity, as removal of the C-terminal farnesyl moiety results in a marked decrease in pheromone activity (15). The target for this modification is a C-terminal consensus amino acid motif, -CAAX (C is cysteine, A is aliphatic amino acids, and X is any amino acid), contained in the primary precursor of either RAS or **a**-factor. Processing of the -CAAX sequence involves several steps: 1) isoprenylation of the cysteine sulfur, 2) proteolysis of the -AAX sequence, and 3) carboxyl methyl esterification (7,8,26). Work performed in our laboratory, in conjunction with this report, has clarified the order of these processing events, as listed above (16). Several gene products in yeast have been implicated in the biosynthesis and export of the **a**-factor (Fig.1): the *RAM1* and *RAM2* gene products have been demonstrated to successfully reconstitute farnesyltransferase activty in vitro (9,21); the *STE14* gene product appears to be responsible for the carboxyl methyl modification of both **a**-factor and RAS (4,10); and the STE6 protein, a homolog of

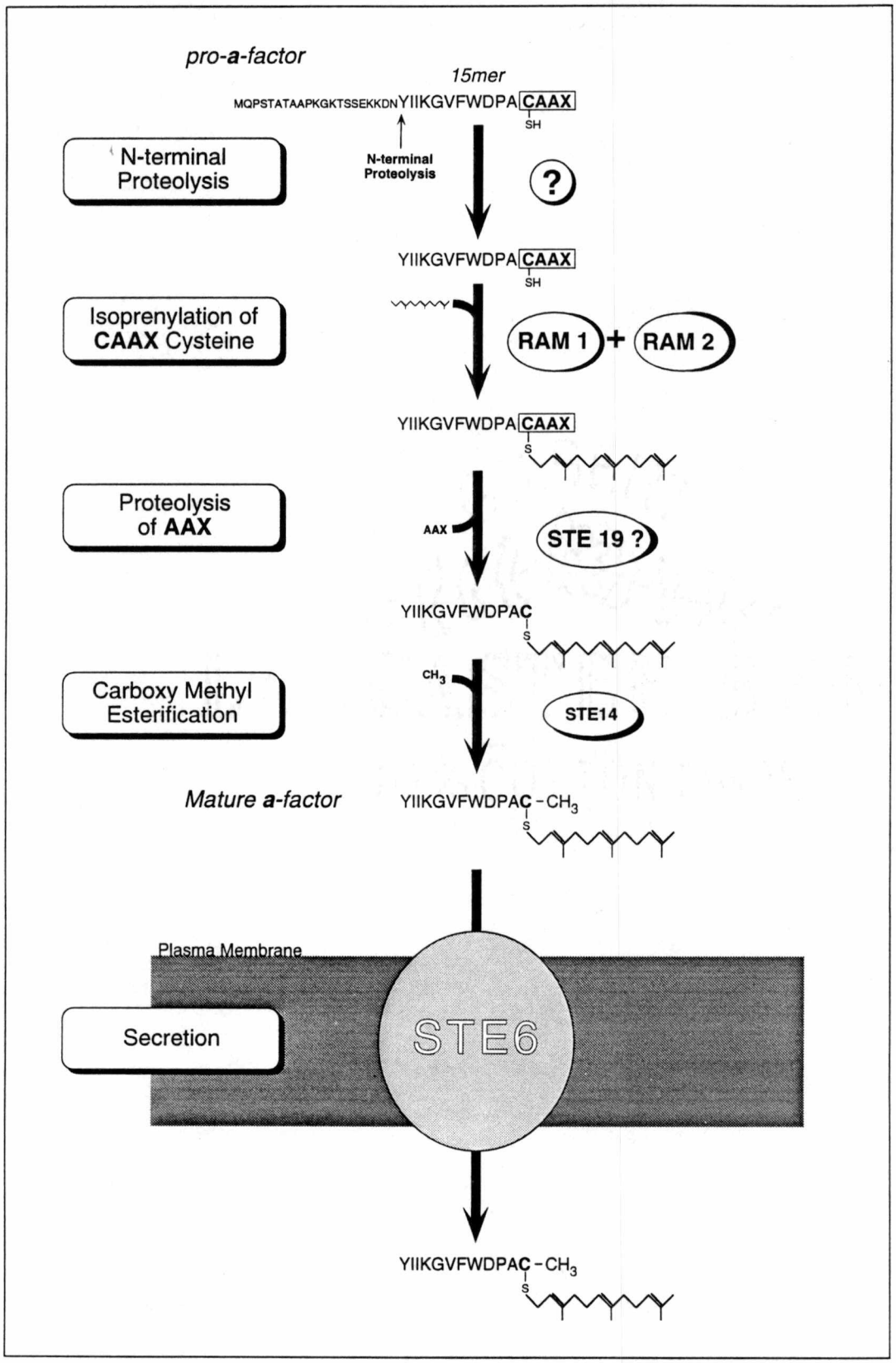


Figure 1. **a**-Factor biosynthesis and secretion

the mammalian multiple drug-resistance (MDR) P-glycoprotein, is required for export of the **a**-factor by an ATP-dependent mechanism (12,17). The maturation of **a**-factor, in combination with the ease with which yeast cells are manipulated, may prove useful for screening of agents which block any of the steps involved in the posttranslational modification of the pheromone and, by inference, may serve as anti-RAS therapeutic drugs (6). The following experiments represent an initial analysis of the functional effects that specific changes in the -CAAX motif have on **a**-factor biosynthesis and serve to establish a system for future structure-function analysis of the **a**-factor peptide.

CHAPTER II

MATERIALS AND METHODS

Yeast strains used in this study

The following strains were used in this study: RC757 (*MATα sst2-1 rme his6 met1 can1 cyh2*), a gift from Russell Chan (3); SM1058 (*MATa trp1 leu2 ura3 his4 can1*), SM1229 (isogenic to SM1058, but *Δmfa1::LEU2 Δmfa2::URA3*), gifts of Susan Michaelis (20); and X2180-1B (*MATα*) obtained from the Yeast Genetics Stock Center (University of California, Berkeley).

Transformation of yeast

Transformation of yeast was performed according a modification of the alkali ion procedure of Ito et al. (11). Briefly, cells grown overnight to exponential phase of growth (1-2 x 10^7 cells/ml) in 100 ml YEPD broth (1% yeast extract, 2% dextrose, 2% peptone) at 30°C were harvested at 2000 rpm for 5 minutes and washed with sterile distilled water once. The pellet was resuspended very carefully in 0.1 M lithium acetate at one third of the original volume. The culture was shaken slowly (about 40 rpm) at 30°C for 30 minutes and cells were pelleted at 2000 rpm for 5 minutes. The pellet was resuspended to 5 x 10^8 cells/ml in 0.1 M lithium acetate and 100 µl of these cells were placed into microfuge tubes. DNA (1-5 µg) was then added to the cells and after incubation at 30°C for 30 minutes (static) an equal volume of 70% polyethylene glycol 4,000 (Sigma) was added and incubated for 1 hour at 30°C (static). The cells were then placed for 15 minutes at 42°C and pelleted by centrifugation in a microcentrifuge for 20 seconds. The pellet was resuspended in 1 M sorbitol and 200-300 µl of cells were plated directly onto media selective for

growth of transformants. Plates were incubated at 30°C for 3-5 days until transformants appeared.

Growth arrest ("halo") assay

Active yeast mating pheromones induce growth arrest in cells of the opposite mating type, evidenced by the presence of a "halo" surrounding a patch of cells of one mating type grown on a lawn of cells of the opposite type after a period of about 24 hours at 30°C. In practice, a lawn of cells is prepared by resuspending cells from a freshly grown streak plate at a concentration of 1 x 10^6 cells/ml in sterile water. 1 ml of cells is added to 3 ml of melted and equilibrated (at 45°C) 1.1% Noble agar (Difco), mixed by inversion, and poured onto 20-25 ml agar plates that have been prewarmed to about 30°C. In the case of this study, patches of yeast cells were placed on lawns of supersensitive α-strain RC757 poured on agar plates with either 2% glucose or 2% galactose (with 0.05% glucose) as a carbon source. Cells actively producing **a**-factor, under the control of the galactose-inducible promoter *GAL1*, exhibit a clear zone of growth arrest when patched onto a lawn of RC757 grown on galactose following growth at 30°C for 24 hours. Corresponding patches on glucose containing medium are repressed for **a**-factor gene induction when under *GAL1* control and do not exhibit the formation of halos.

Isolation of ss DNA for in vitro mutagenesis

A DNA fragment containing the *MFa1* gene of *S.cerevisiae* (2) (obtained as a gift from Jeremy Thorner), and encoding the primary precursor of the **a**-factor mating pheromone, was subcloned as a 1.6 kb *Bam* HI gene fragment into phagemid pTZ19U (Bio-Rad) (18) using standard recombinant DNA techniques (23). This construct, named pGC001, was used for the generation of single-stranded template DNA, and subsequent in

vitro mutagenesis, following a modification of the method of Kunkel et al. (13) as outlined in the Muta-Gene kit from Bio-Rad.

Initially, *E. coli* strain CJ236 (*dut-, ung-*) (13) was transformed with phagemid pGC001 using the standard $CaCl_2$ method (23). Selection of transformants was performed using LB agar plates (1% tryptone, 1% NaCl, 0.5% yeast extract, 2% agar) containing ampicillin [50 μg/ml] and chloramphenicol [30 μg/ml]. This strain contains mutations in the genes encoding the enzymes dUTPase and uracil-N-glycosylase, which result in low-level incorporation of uracil into replicating DNA. All growth of CJ236 was performed in the presence of chloramphenicol, needed to maintain the F' plasmid, which carries a chloramphenicol resistance gene and is responsible for the production of the pili required for subsequent phage infection. An isolated transformant colony of CJ236, harboring vector pGC001, was grown overnight at 37°C in 20 ml LB broth containing ampicillin and chloramphenicol. The following day, 50 ml of 2X YT broth (1.6% tryptone, 1% yeast extract, 0.5% NaCl) containing ampicillin and chloramphenicol in a 500 ml flask, was inoculated with 1 ml of the overnight culture and grown to an OD_{600} of 0.3 (~1 x 10^7 cells/ml) with vigorous shaking (250 rpm) at 37°C. In order to induce single-stranded replication of the phagemid, kanamycin resistant helper phage M13KO7 [titer of 1-2 pfu/ml] (29) was added to the growing cells at a multiplicity of infection of 20 phage/cell. Phage adsorption and infection were permitted to occur by continued shaking at 37°C for 1 hour, at which time 70 μl of 50 mg/ml kanamycin was added to the flask to select for infected cells and incubation was continued for 6 hours. Cells were separated from the phage containing medium by centrifugation at 17,000 x g for 15 minutes and the supernatant clarified by a repeat spin. Single-stranded uracil-DNA-containing phage particles were precipitated by the addition of 1/4 volume 3.5 ammonium acetate/20% polyethylene glycol 6,000, mixed by inversion, and incubated on ice for 30 minutes. Phage

were pelleted by centrifugation at 17,000 x g for 15 minutes at 4°C. The supernatant was discarded and the pellet was air dried for at least an hour. This phage pellet was resuspended in 200 µl of a high salt buffer (300 mM NaCl, 100 mM Tris pH 8.0, and 1 mM EDTA). Finally, purification of ss phage DNA was performed by extraction (vortexing for 1-2 minutes) of the phage suspension 2X with an equal volume of phenol, 1X with phenol:chloroform:isoamyl alcohol (1:1:1/25), and at least 3 times with chloroform:isoamyl alcohol (1:1/25) until no visible protein interface was obtained. Precipitation of ss DNA by the addition of 1/10 volume 7.8 M ammonium acetate and 2.5 volumes ethanol followed by incubation at -70°C for 30 minutes and centrifugation for 15 minutes at top speed in a microfuge at 4°C was performed to pellet the purified DNA. This pellet was resuspended in 20 µl TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) and used in subsequent in vitro mutagenesis reactions following quantitation by gel electrophoresis band-brightness comparison to a known ss DNA standard.

In vitro mutagenesis of the *MFa1* gene fragment

All primers used for in vitro mutagenesis of the *MFa1* gene in this study were synthesized by the University of Tennessee Molecular Biology Resources Facility. Three specific primers were employed in the mutagenesis of the *MFa1* gene fragment:

1) 5'-CCAGAAATGAATTCCTGAGAGGGATCT-3' was utilized for the incorporation of a novel *Eco* RI site about 70 bases upstream from the translational start of *MFa1* ;

2) 5'-GACCCAGCATCTGTTATTGCT-3' was used to alter the cysteine codon, present at position 12 in the mature **a**-factor, to a codon specifying a serine residue; and 3) 5'-GACCCAGCATGTTAGATTGCTTAGTTTC-3' was designed to change the codon normally encoding the valine, within the -CAAX motif, to a "nonsense", or stop codon. Prior to use in mutagenesis, these oligonucleotide primers were phosphorylated using T4 polynucleotide kinase. Briefly, 200 pmol of primer was used in a 10 µl reaction mixture

containing 100 mM Tris pH 8.0, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP, and 4.5 units T4 polynucleotide kinase (New England Biolabs). The reaction was incubated at 37°C for 45 minutes followed by heat inactivation of the kinase at 65°C for 10 minutes. The phosphorylated oligonucleotide primers were stored at -20°C.

Annealing of mutagenic primers to the single-stranded uracil-containing DNA template was performed in a 10 µl reaction volume containing 9 pmol of phosphorylated primer, 200 ng ssDNA template, 1 µl annealing buffer (20 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 50 mM NaCl), and distilled water. The mixture was heated to 70°C in a heat block, then allowed to cool to room temperature at a rate of about 1°C/minute by removing the block from its heating unit and placing it on the lab bench top. Reactions were then placed on ice for 10 minutes. The following components were added to the annealing mixture in order to initiate second-strand synthesis in vitro: 1 µl synthesis buffer (0.4 mM each dNTP, 0.75 mM ATP, 17.5 mM Tris-HCl, pH 7.4, 3.75 mM $MgCl_2$, and 0.5 mM dithiothreitol), 1 µl T4 DNA ligase [3 units/µl], and 1 µl T4 DNA polymerase [1 units/µl]. This mixture was incubated on ice for 5 minutes, then at room temperature for 5 minutes, and finally at 37°C for 90 minutes. After 90 minutes, 90 µl of stop buffer (10 mM Tris pH 8.0 and 10 mM EDTA) was added to terminate the synthesis reaction. The resulting heteroduplex DNA was transformed into *dut*$^+$ *ung*$^+$ *E.coli* strain MV1190 (19) using between 3-10 µl of the synthesis reaction. Transformants were selected for their ability to confer ampicillin resistance to the MV1190 host and used as the source of plasmid DNA for dideoxy DNA sequence analysis (24) to verify the presence of mutant gene sequences. Sequencing of plasmid DNA was performed using Sequenase™ modified T7 DNA polymerase (United States Biochemical) on single-stranded template DNA, isolated by superinfection with helper phage M13KO7, as previously described. The approximate percentage of colonies identified to contain site-directed mutations was found to be near 70% using this selection scheme.

Expression of **a**-factor and mutated **a**-factor genes in yeast

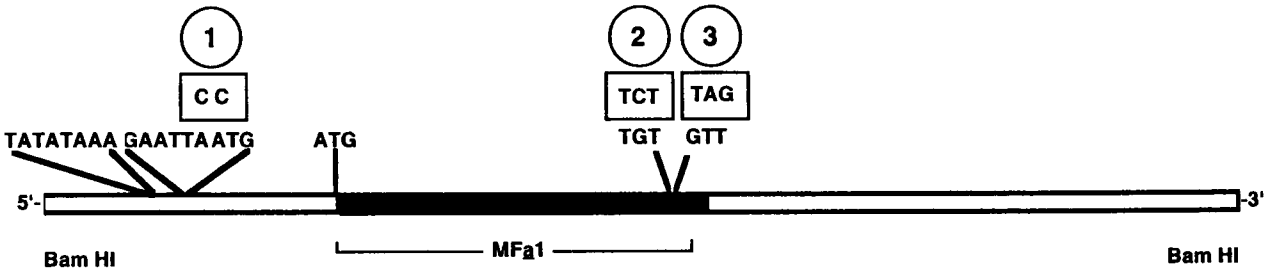
Yeast -*E.coli* shuttle vector pRS129 (a gift from Bill Michaud and Phil Hieter) was used for the overexpression of both wild-type and mutant **a**-factor gene constructs under the control of the galactose-inducible *GAL1* promoter. This vector is a derivative of plasmid pRSS56 with the *GAL1/10* promoter region subcloned into the *Kpn* I site of the polylinker (28). Site-directed mutagenesis was employed using pGC001, as outlined above, to introduce a novel *Eco* RI site upstream from the translational start site of the **a**-factor precursor, and downstream from the **a**-factor promoter, to enable subsequent subcloning of the **a**-factor gene into vector pRS129 in an orientation that facilitates galactose-promoted expression in yeast. Specifically, yeast strain SM1229 (*MATa Δmfa1 Δmfa2*), an **a**-strain which has had the chromosomal copies of the genes encoding **a**-factor deleted (20), was used for the transformation of the wild-type and mutant **a**-factor gene constructs. The only **a**-factor produced by this strain, therefore, is plasmid dependent. This strain is also a tryptophan auxotroph that allows for the selection of cells transformed with derivatives of pRS129 , which contains the *TRP1* marker gene.

CHAPTER III

RESULTS

Expression of the *MFa1* gene by galactose induction

In order to investigate the structural aspects of **a**-factor function, we established a system for mutagenesis and expression of **a**-factor and for assaying its biological activity. We used oligonucleotide-directed site-specific mutagenesis to alter a gene fragment containing the *MFa1* gene at specific nucleotide bases (Fig. 2). The first change we introduced into the **a**-factor gene fragment was designed to generate a novel restriction site for enzyme *Eco* RI in between the region of DNA sequence encompassing the **a**-factor promoter and the ATG codon signifying the translational start site of the peptide precursor. This specific sequence change served two purposes. First, it allowed for subsequent directional subcloning of a 1 kb *Eco* RI-*Bam* HI gene fragment encoding **a**-factor (but not its promoter) into a yeast expression vector (pRS129) in an orientation necessary and sufficient for galactose-inducible expression under control of the high-level expression yeast promoter, *GAL1*. Secondly, the oligonucleotide utilized in the mutagenesis of this region also was designed to simultaneously destroy an upstream ATG codon which might have served as an alternative, erroneous, translational start for transcripts initiated near the *GAL1* promoter, since, in general, yeast tend to translate the first ATG of an mRNA transcript. Figure 3 serves as an example of the DNA sequence verification we used in the identification of successful site-directed mutant clones. Following sequence verification, this altered cassette was isolated and subcloned into yeast expression vector pRS129 to generate the wild-type **a**-factor expression vector pGCGal003 (Fig. 4). This vector was transformed into yeast strain SM1229 (*MATa Δmfa1 Δmfa2*), an **a**-strain which has had the genes encoding **a**-factor deleted from its chromosomes by homologous recombination (20).



1 Introduction of a unique *Eco* RI site (GAATTC) and simultaneous disruption of an undesired ATG upstream from the *MFa1* translational start site

2 Codon substitution of serine (TCT) for cysteine (TGT) in the **a**-factor precursor C-terminal -CAAX motif

3 Codon substitution of a novel translational stop codon (TAG) instead of the codon for valine (GTT) in the C-terminus of the **a**-factor precursor

Figure 2. A representation of codon changes created within the *MFa1* gene fragment using site-directed mutagenesis.

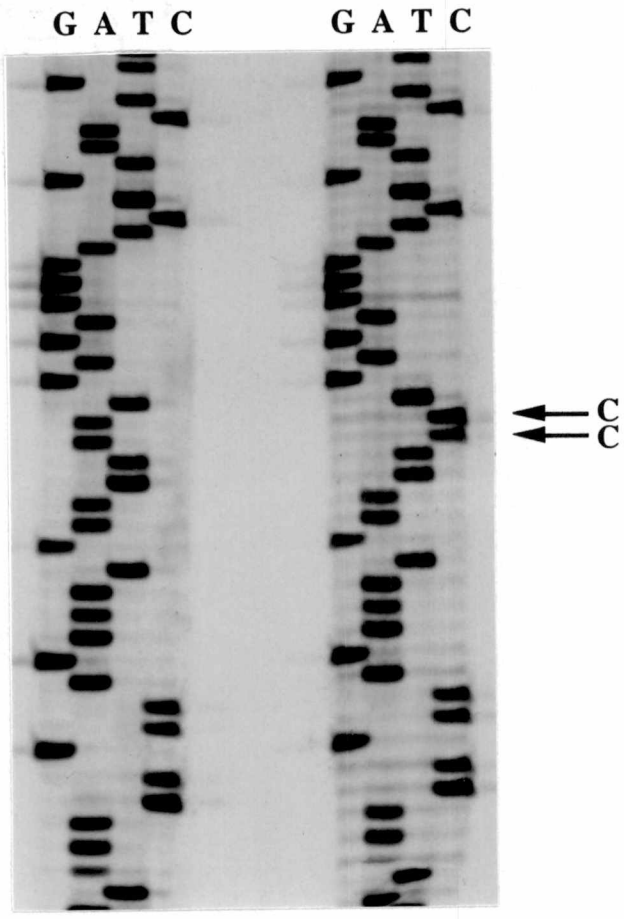


Figure 3. An example of a site-directed mutation within the *MFa1* gene fragment: generation of a novel *Eco*RI site. Dideoxy DNA sequencing was used to identify a clone containing a change of two nucleotides, as depicted above, resulting in the sequence : 5'-...GAATTCC...3'. The wild-type sequence appears on the left, whereas the altered gene is on the right. Mutagenesis and sequencing were performed as described in Materials and Methods.

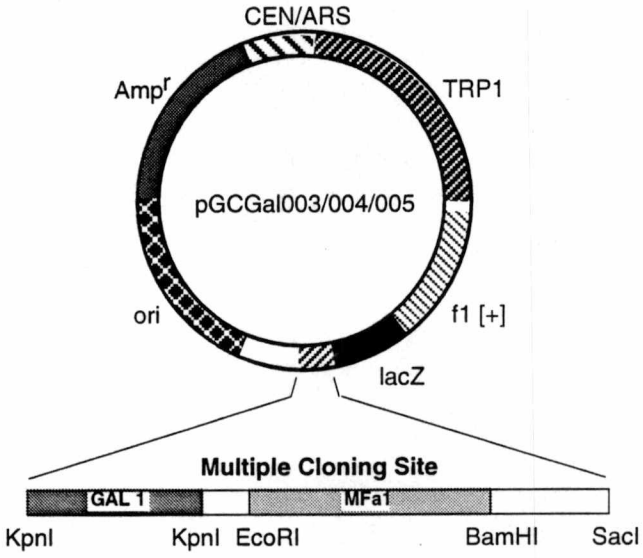


Figure 4. Overexpression vectors pGCGal003, 004, and 005. These were constructed by the ligation of a 1kb *Eco*RI-*Bam*HI ***MFa1*** gene fragment into vector pRS129. pRS129 is a derivative of plasmid pRSS56 with the *GAL1/10* promoter region subcloned into the *Kpn* I site of the polylinker. The ***MFa1*** gene was manipulated by site-directed mutagenesis to create a novel *Eco*RI site upstream from the ***MFa1*** translational start and downstream from its promoter sequence. This change enabled the ligation of the ***MFa1*** gene fragment into pRS129 in an orientation that facilitates overexpression under the control of the galactose inducible promoter, *GAL1*, while ensuring that translation of the **a**-factor precursor peptide will begin at the correct site. Additional attributes of this plasmid include: amp^r, ampicillin resistance for selection in *E.coli ;* ori, origin of replication for *E.coli* ; CEN/ARS, regions required for chromosome stability and replication in yeast; *TRP1*, selectable marker for yeast; f1[+], origin of replication for bacteriophage f1 for production of ss DNA for use in oligonucleotide-directed mutagenesis and sequencing; lac Z, gene encoding β-galactosidase [not useful for selection in this construct due to prior insertion of *GAL1* DNA into polylinker].

Note: Phagemids pGCGal003, 004, and 005 are identical with the exception of changes in the ***MFa1*** DNA encoding the codons in the -CAAX box:

-pGCGal003 (with the wild-type ***MFa1*** gene) encodes -CVIA at the C-terminus

-pGCGal004 encodes a C-terminus of -**S**VIA

-pGCGal005 encodes -C -**stop codon**

SM1229 (pGCGal003) transformant cells were patched onto a lawn of α-cells grown on medium containing either galactose or glucose as the primary carbon source. These cells exhibited a clear zone of growth inhibition surrounding the patch indicating bioactive **a**-factor pheromone was being produced in response to the galactose induction (Fig. 5). No growth arrest was found when these same cells were patched onto medium containing glucose instead of galactose (data not shown). The methodology developed for judging the bioactivity of this **a**-factor expression cassette enabled us to proceed in testing the effects of specific changes in the isoprenyl targeting sequence of the **a**-factor on pheromone production.

Site-directed mutagenesis of the **a**-factor -CAAX motif

Previous reports have shown that a synthetic peptide corresponding to an **a**-factor precursor peptide, YIIKGVFWDPACVIA, is farnesylated in vitro by cell extracts from *S. cerevisiae* (16,25). In contrast, a similar peptide with serine substituted for cysteine is not isoprenylated in vitro, suggesting that the cysteine sulfur is absolutely required for posttranslational modification with a farnesyl moiety. We decided to determine if a similar change in the gene encoding **a**-factor would have an effect on its production in vivo. In order to address this question, site-directed mutagenesis was performed to change a single base in the codon for cysteine (of the -CAAX box) in the **a**-factor precursor to yield the codon for serine, a base substitution of TGT for TCT, respectively (Fig. 2). Following verification of this sequence change by dideoxy DNA sequence analysis, the resulting gene fragment was subcloned into galactose-inducible yeast expression vector pRS129 to create plasmid pGCGal004 (Fig. 4). This vector was transformed into **a**-factor deficient strain SM1229 and transformants were tested for their ability to cause growth arrest in an α-cell lawn grown on galactose. No evidence of growth arrest was found surrounding these patches on either glucose or galactose containing media. These results demonstrate that substitution of serine for cysteine in the **a**-factor abolishes its production in vivo (Fig. 5).

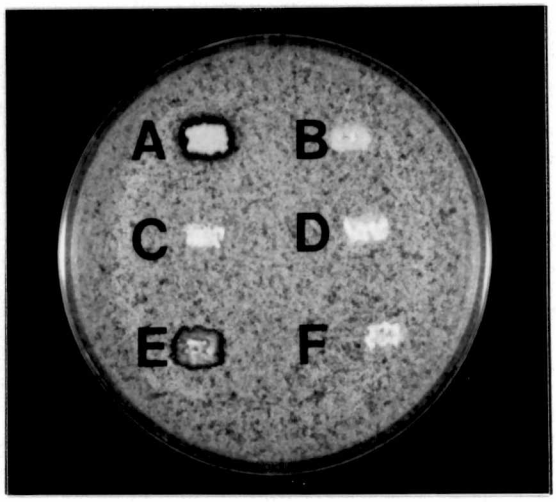


Figure 5. Results of site-directed mutagenesis of the *MFa1* gene. A lawn of strain RC757 (*MATα sst2-1*) cells was spread onto YPGal media (2% peptone, 1% yeast extract, 2% galactose, 0.05% dextrose). The following *S. cerevisiae* strains were then patched onto the lawns and incubated at 30°C for 24 hours: A, SM1058 (*MATa trp1 leu2 ura3 his4 can1*); B, X2180-1B (*MATα*); C, SM1229 (isogenic to SM1058, but *Δmfa1::LEU2 Δmfa2::URA3*); D, SM1229 (pGCGal004); E, SM1229 (pGCGal003); F, SM1229 (pGCGal005). Strains producing **a**-factor exhibit clear zones, or halos, at the periphery of their patch, an indication of the arrest of cell growth.

In addition to altering the actual amino acid residue to which farnesyl is attached, we investigated the in vivo consequences of removing the other three amino acids (-AAX) of the isoprene-targeting sequence in the **a**-factor while retaining a C-terminal cysteine residue in the **a**-factor. This entailed the design of a mutagenic oligonucleotide primer which was aimed at incorporating a stop codon at the second position of the -C<u>A</u>AX motif, thereby encoding a truncated peptide pheromone precursor lacking an intact putative isoprenylation recognition sequence. Specifically, the nonsense codon TAG was substituted for the native GTT codon encoding the aliphatic amino acid valine (Fig. 2). The successful incorporation of this change was verified by dideoxy DNA sequencing of the resulting mutagenized vector DNA. The altered *MFa1* gene was again subcloned as a 1 kb *Eco* RI-*Bam* HI fragment into yeast expression vector pRS129 and this plasmid, termed pGCGal005 (Fig. 4), was transformed into strain SM1229 for bioactivity analysis. Patches of transformant strain SM1229 (pGCGal005) did not exhibit clear zones of growth inhibition when placed on lawns of supersensitive α-strain RC757 grown on galactose media (Fig. 5). Therefore, the removal of the three C-terminal amino acids of the **a**-factor precursor (-VIA) serves to block subsequent production **a**-factor from **a**-cells.

CHAPTER IV

DISCUSSION

The isoprenylation of proteins appears to play a significant role in the localization and biological function of many eukaryotic proteins (26). Studies suggest that mutations which prevent the isoprenylation of activated RAS significantly decrease or abolish its ability to transform cells (7,8). Therefore, an understanding of the underlying structural and functional significance that isoprene modification may have in **a**-factor biogenesis contributes to our overall basic knowledge of the manner by which isoprenylated proteins are recognized as substrates and ligands in cellular processes.

In vitro studies have provided extensive evidence implicating the C-terminal consensus amino acid motif, -CAAX, as the target for posttranslational modification with isoprene moieties by prenyltransferase enzymes (7,22). In work perfomed in our laboratory, initally published simultaneously with this report, Marcus et al., have defined the order of events involved in -CAAX processing and have established an in vitro assay for studying the posttranslational modification of synthetic **a**-factor precursors (16). The results presented here complement the data obtained in vitro with in vivo studies implicating specific amino acid residues as necessary for **a**-factor production.

We have shown by genetic substitution of serine for cysteine at the C-terminus of the **a**-factor that replacement of a sulfhydryl attachment site in the form of cysteine, with a hydroxyl moiety as is present in serine, results in loss of **a**-factor activity. When taken in conjunction with the known requirement of farnesylation for successful secretion of **a**-factor (24,26), this result implies that the posttranslational modification of **a**-factor with farnesyl was blocked in this mutant, and no export of the peptide was possible. Even if

some **a**-factor may have been exported, farnesylation has been shown to be required for bioactivity of the pheromone as well (15), and therefore secreted peptides would have been biologically active only if they had been correctly processed. Similar data suggest that removal of the C-terminal three amino acids of the precursor, constituting the -AAX of the -CAAX isoprenylation targeting motif, prevents both farnesylation and secretion of **a**-factor. As with the cysteine to serine change, both in vitro maturation assays using corresponding synthetic precursors (16,26) and in vivo expression of a gene encoding this truncated **a**-factor peptide support these conclusions. Therefore, it is apparent that an intact -CAAX sequence is required for proper biosynthesis of the **a**-factor in vivo.

These experiments represent an initial inquiry into structural alterations to the **a**-factor and their effect on its function. The methodology established through these studies acts as a basis for which future investigations into **a**-factor peptide-target interactions may be carried out.

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PART III

CONSEQUENCES OF ALTERED ISOPRENYLATION TARGETS ON **a**-FACTOR EXPORT AND BIOACTIVITY

CHAPTER I

INTRODUCTION

Mating in the yeast *Saccharomyces cerevisiae* is mediated by the reciprocal interaction of secreted peptide pheromones with cell-surface receptors and has served as a model for the study of various cellular processes including signal transduction, gene expression, and secretion (24). Despite their apparent functional equivalence, the yeast mating pheromones, α- and **a**-factor, are structurally dissimilar. The α-factor, an unmodified tridecapeptide, is secreted by α-cells and undergoes maturation and secretion via the yeast secretory pathway (11,24). In contrast, the **a**-factor is a dodecapeptide [Tyr-Ile-Ile-Lys-Val-Phe-Trp-Asp-Pro-Ala-Cys(S-farnesyl)-OCH_3], which is post-translationally modified by farnesylation and methyl esterification of the carboxyl terminus (Fig. 1). These modifications are shared by other proteins including the RAS oncogene product, nuclear lamins, and rab vesicular trafficking proteins (8,27,38,39,40,43). Furthermore, the **a**-factor is exported from **a**-cells by a novel secretory mechanism requiring the action of the yeast STE6 protein, a homolog of the mammalian multiple-drug resistance P-glycoprotein, and a member of the ATP-binding cassette family of transporters (22,28).

The prenylation of oncogenic RAS has been identified as essential for its membrane localization and subsequent role in cellular transformation (20). In addition, recent evidence points to farnesylation as playing a critical role in the ability of yeast RAS2 to directly interact with, and activate, solubilized adenylyl cyclase, in vitro (25). Similarly, the farnesylation of **a**-factor has been shown to be required for both its secretion and biological activity (27,38,40). In this regard, the substrate specificity exhibited by **a**-factor

a-factor:



geranylgeranyl a-factor:

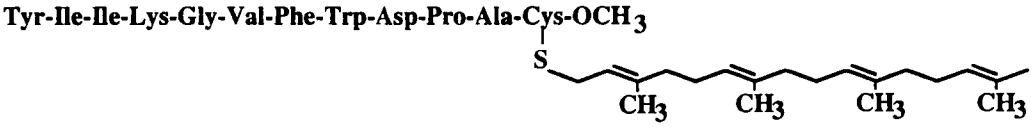


Figure 1. Structures of **a**-factor and geranylgeranyl **a**-factor.

in its recognition by both the STE6 transporter protein and its receptor on α-cells, the STE3 protein (14), provides a model for studying the molecular requirements involved in lipopeptide structure-function relationships.

Two distinct isoprenoids have been implicated in the posttranslational modification of proteins, the C_{15} farnesyl group and the C_{20} geranyl, geranyl isoprene (Fig.1). Through extensive in vitro prenylation studies, specific C-terminal cysteine-containing motifs have been identified as the targets for attachment of these lipids by one of at least three classes of prenyltransferases (8,39). Farnesyl prenyltransferase (FPT) and geranyl, geranyl prenyltransferase I (GGPT-I) catalyze the addition of either a C_{15} or C_{20} lipid, respectively, to terminal sequences of -CAAX (where C= cysteine, A= any aliphatic amino acid; X=predominantly Met, Ser, Ala, Cys , or Gln for FPT, and X= Leu or Phe for GGPT-I) (8,31,35,38). A third enzyme, geranyl, geranyl prenyltransferase II (GGPT-II), appears to be responsible for prenylation of -CXC or -CC terminal proteins with at least one, and perhaps two, geranyl, geranyl groups (18,32). Proteins containing the latter class of motifs do not appear to undergo carboxy methylation in vitro (8,19). In vitro evidence indicating the ability of prenyltransferases to cross-prenylate CAAX-related protein targets implies that peptide acceptor concentration may play a role in altering enzyme specificity (48). However, the functional consequences of altered isoprenylation targets in affecting the substrate specificity of these enzymes in vivo is not well understood.

Genetic and biochemical studies have identified several yeast genes which encode components of prenyltransferases: *CDC43* (10), *RAM1* (also called *DPR1*) (34), *RAM2* (16), and *BET2* (36, 39, 41). The observation that yeast *ram1* null mutants are viable, although RAS proteins are essential, suggests that some RAS is prenylated at a level sufficient for survival despite the absence of functional FPT (16,34). However, *cdc43/ram1* double mutants are inviable and overexpression of *CDC43* in *ram1* mutants acts to suppress associated growth defects (46).

Despite the similarities in posttranslational processing between **a**-factor and RAS-related proteins, the functional significance that differential isoprenylation might have for a small, secreted, bioactive peptide may differ from that of a membrane associated protein. In this report, we have employed molecular biology and synthetic peptide biochemistry to study the in vivo consequences of altered isoprenylation targets on the biosynthesis, secretion, and biological activity of **a**-factor.

CHAPTER II

MATERIALS AND METHODS

Yeast strains and methods

The following yeast strains were used: SM1058 (*MATa trp1 leu2 ura3 his4 can1*) and SM1229 (isogenic to SM1058 but *mfa1Δ::LEU2 mfa2Δ::URA3*) (29); JPY200 (*MATa his3-Δ200 leu2-3,-112 lys2-801 trp1-1 ura3-52*) and JPY201 (*MATa* isogenic to JPY200 but *ste6Δ-1::HIS3*) (28); RC757 (*MATα sst2-1 rme his6 met1 can1 cyh2*) (6); DBY746 (*MATα his3-Δ1 leu2-3 ,-112 ura3-52 trp1-289*) (4). Yeast strains were transformed by electroporation according to Becker and Guarante (2) or by the method of Gietz et al. (12). Growth arrest "halo" assays for *GAL1*-induced gene expression from cell patches were performed in the presence of either 2% galactose containing 0.05% glucose or with 2% glucose only, as described (27). Halo assays for testing of synthetic **a**-factor peptides or dilutions of purified fractions were done as previously outlined (26). Mating restoration assays were performed as described by Marcus et al. (26).

Peptide synthesis and characterization

The synthesis of synthetic geranyl, geranyl **a**-factor (Fig. 1) was carried out using a combination of solid-phase and solution-phase synthesis similar to that previously performed for the synthetic **a**-factor (47). Briefly, a protected decapeptide, (Fmoc)Tyr-Ile-Ile-Lys(Fmoc)-Gly-Val-Phe-Trp(For)-Asp(OFm)-Pro [where Fmoc = Fluorenylmethoxycarbonyl, For = formyl, and OFm = Fluorenyl methyl ester] was coupled to Ala-Cys (geranyl, geranyl) -OCH_3. The protecting groups were removed and the peptide purified by HPLC. The final product was > 99% homogeneous in two solvent

systems on gradient HPLC and gave the correct molecular ion in FAB mass spectroscopy. Stocks of **a**-factor and geranyl, geranyl **a**-factor were dissolved at 1 mg/ml directly in methanol.

Plasmid constructions and mutagenesis

Plasmids used for galactose-induction experiments were constructed from a 1.6 kb *Bam* HI fragment containing the *MFa1* gene (5) into which a novel *Eco* RI site was introduced by site-directed mutagenesis as previously described (27). Mutagenic oligonucleotides designed to alter the **a**-factor gene sequence encoding the wild-type CAAX box (Cys-Val-Ile-Ala) to both Cys-Val-Ile-Leu (CVIL) and Ser-Val-Cys-Cys (SVCC) were utilized in site-directed mutagenesis by the method of Kunkel (23). The resulting mutated *MFa1* genes were separately subcloned as an 1.0 kb *Eco* RI-*Bam* HI fragment lacking the endogenous **a**-factor promoter into vector pRS129 (42) for *GAL1*-dependent overexpression. Simultaneously, for use in **a**-factor isolation studies, the two mutagenized 1.6 kb *MFa1* *Bam* HI fragments, containing the **a**-factor promoter, were also subcloned into the vector pRS424 (7). Large-scale plasmid preparations were performed according to Becker et al. (3). DNA sequencing, according to Sanger (37), was employed to verify all site-specific mutations, both prior to and after, yeast transformation.

Isolation of **a**-factor from yeast cultures

Cultures of log-phase yeast cells expressing either wild-type or mutant **a**-factor genes were grown in 1 L YEPD in the presence of Amberlite XAD-2 polystyrene resin as previously described (45). Since **a**-factor as been shown to bind to glass, all glassware used in **a**-factor isolation was treated as described by Sterne (44). Elution of **a**-factor from washed beads/glass was performed with 100 ml 1-propanol and the eluate was

concentrated to approximately 5 ml using a rotary evaporator. The concentrated eluate was filtered through an Acrodisc CR PTFE syringe filter. Reverse-phase HPLC (Beckman Instruments) was performed by injection of 1 ml of concentrated, filtered eluate over a Zorbax Protein Plus C_3 column using either a previously described acetonitrile/water gradient (27) or equivalent methanol/water gradient. Fractions (1.5 ml) were collected, dried by vacuum centrifugation, resuspended in 1-propanol, and tested for bioactivity on lawns of α-strain RC757 as described (26). Fractions exhibiting bioactivity (or corresponding fractions from control strain SM1229, an **a**-factor gene deletion strain) were pooled and applied to a Sephadex LH-20 column prepared according to Sterne (44). Fractions showing bioactivity were pooled, dried, resuspended in 300 µl 1-propanol, and repurified by HPLC, as above. Individual fractions were collected, dried, resuspended in 1-propanol, and serial dilutions were prepared to quantify the bioactivity of each purified fraction.

CHAPTER III

RESULTS

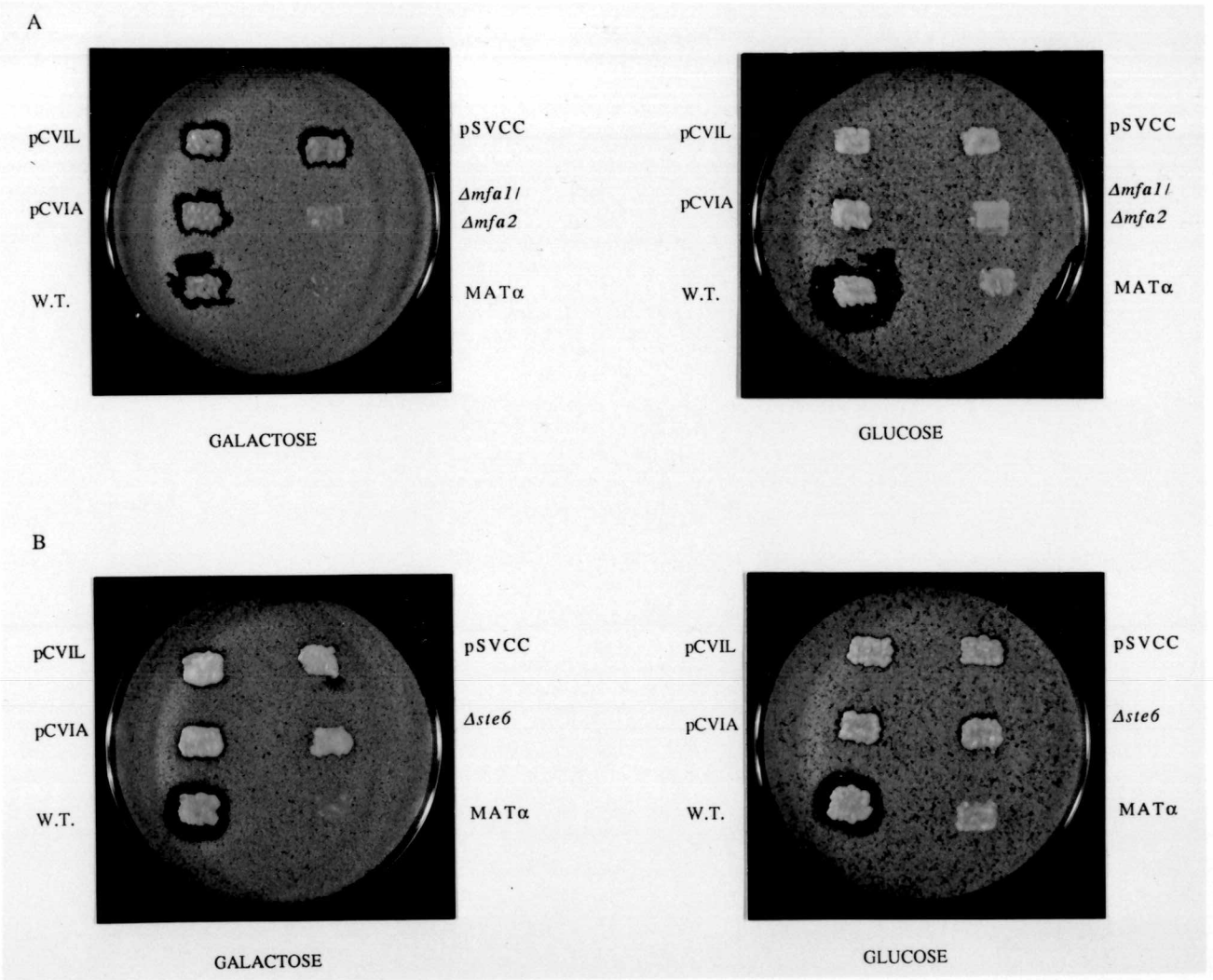
Biological activity of **a**-factors with altered isoprenylation targets

The expression of mutagenized **a**-factor genes under the control of a galactose-inducible promoter was performed to determine the consequences of alternative isoprenylation target sequences on **a**-factor secretion, or bioactivity. Plasmids containing *MFa1* gene constructs encoding **a**-factor precursors that end in either the wild-type -CVIA, or mutant -CVIL or -SVCC residues were transformed into yeast strain SM1229 (*MATa Δmfa1, Δmfa2*). Transformants were patched onto lawns of supersensitive α-cells containing either galactose, to induce expression, or glucose, for repression of mutant **a**-factor production (Fig. 2A). Evidence of growth arrest, in the form of a halo surrounding the patches, indicated that cells expressing both -CVIL and -SVCC **a**-factor exhibited both transport and biological activity, specifically under galactose induction. As expected, both chromosomally-expressed, wild-type **a**-factor and galactose-induced **a**-factor activity was detected from strains SM1058 and SM1229 [pCVIA], respectively. The double deletion host strain (SM1229 *Δmfa1, Δmfa2*) alone exhibited no biological activity. These results suggest that replacement of the native **a**-factor isoprenylation target sequence (CVIA) with either CVIL or SVCC does not impair secretion or biological activity of the pheromone.

Secretion of -CVIL and -SVCC **a**-factor via STE6

The secretion of **a**-factor occurs by an ATP-dependent mechanism utilizing the STE6 transport protein (22). Farnesylation of **a**-factor is a prerequisite for its secretion and

Figure 2. Biological activity and export of **a**-factors with alternative isoprenylation targeting sequences. Patches of cells were placed onto lawns of α-strain RC757 containing either galactose (plates on left) or glucose (plates on right) and incubated for 24 hours at 30°C. Panel A : Bioactivity produced by SM1229 (*Δmfa1, Δmfa2*) transformed with plasmids encoding **a**-factors containing the CAAX motifs: pCVIL, pSVCC, pCVIA. Controls included *Δmfa1/Δmfa2* = SM1229 (no plasmid) , W.T. = SM1058, the parent strain containing chromosomal genes encoding wild-type -CVIA **a**-factor, and MATα = DBY746 (*MAT*α) which grows poorly on galactose. Panel B: Export of **a**-factors from *Δste6* mutant strain JPY201 transformed with the same plasmids as above. Controls included *Δste6* = JPY201(no plasmid), W.T. = parent strain JPY200 (with wild-type chromosomal copies of the -CVIA **a**-factor and wild-type *STE6* genes), and MATα = α-strain DBY746 (*MAT*α).

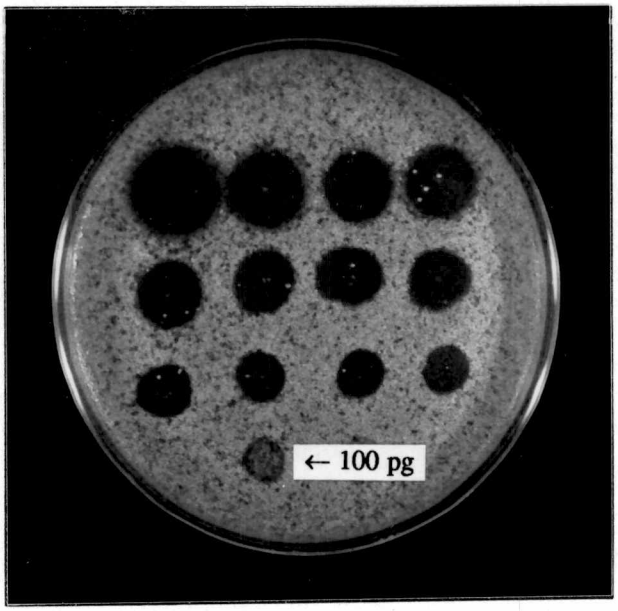

A
pCVIL
pCVIA
W.T.
pSVCC
Δmfa1/
Δmfa2
MATα
GALACTOSE
pCVIL
pCVIA
W.T.
pSVCC
Δmfa1/
Δmfa2
MATα
GLUCOSE
B
pCVIL
pCVIA
W.T.
pSVCC
Δste6
MATα
GALACTOSE
pCVIL
pCVIA
W.T.
pSVCC
Δste6
MATα
GLUCOSE

is believed to be involved in either specific or non-specific membrane localization of **a**-factor in conjunction with export and recognition by STE6 (22,28,38,40). In order to investigate the effect on STE6-dependent secretion of interchanging the farnesyl targeting motif (CVIA) in **a**-factor to those presumed to call for modification with geranyl, geranyl groups (CVIL and SVCC), we transformed a yeast strain containing a deletion of the *STE6* gene with our expression constructs. The galactose-induced expression of **a**-factor activity was blocked in both of the mutant patches, as well as in the *GAL1*-induced wild-type patch and the deletion strain control, whereas the parent strain containing a functional *STE6* gene produced a typical growth arrest response (Fig. 2B). These results indicate that the peptides resulting from the biosynthesis of both the -CVIL or -SVCC **a**-factors are secreted by a mechanism dependent upon the presence of the yeast *STE6* gene product.

Biological activity of synthetic geranyl, geranyl **a**-factor

A geranyl, geranylated **a**-factor peptide containing a substitution of a C_{20} isoprenoid for the C_{15} farnesyl normally linked to the sulfur of the terminal cysteine was synthesized and tested for its ability to cause growth arrest and to exogenously restore mating between the **a**-factor gene deletion strain and a supersensitive α-strain. Dilution analysis of geranyl, geranyl **a**-factor indicated that this peptide was able to induce growth arrest in α-cells, as well as restore mating (Fig. 3). This result implies that intracellular events that would lead to the biosynthesis and secretion of a geranyl, geranylated **a**-factor of equivalent structure would produce a functional peptide. It should be noted that geranyl, geranylated **a**-factor is about one-fourth as active as synthetic farnesylated **a**-factor in both assays with this tester strain.

A



B

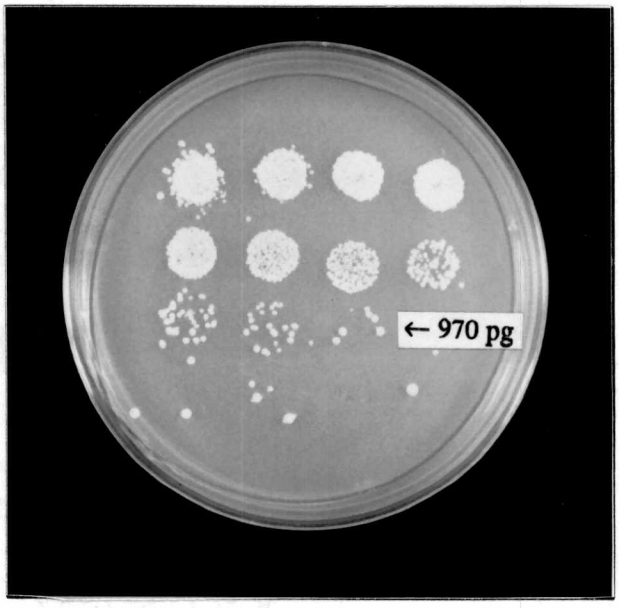


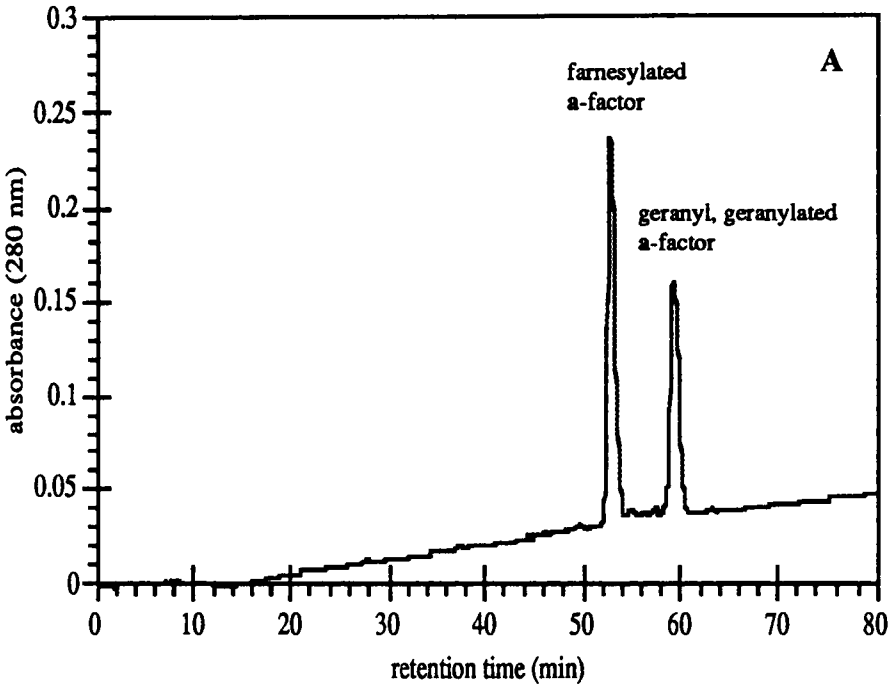
Figure 3. Synthetic geranyl, geranyl **a**-factor induces growth arrest and restores mating.
Panel A : Endpoint of bioactivity as determined by growth arrest of α-strain RC757.
Panel B: Endpoint of exogenously added synthetic geranyl, geranyl **a**-factor required to restore mating between **a**-factor gene deletion strain SM1229 and α-strain RC757 (as evidenced by formation of a constellation of diploids on diploid selective media).

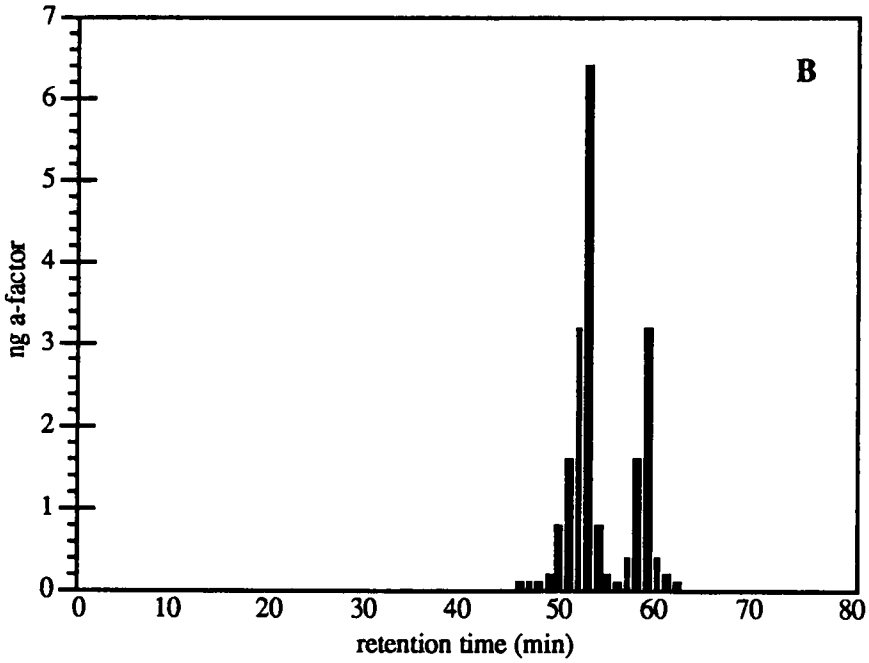
HPLC analysis of synthetic and in vivo produced **a**-factors

Using synthetic **a**-factor and geranyl, geranyl **a**-factor, we established conditions suitable for the chromatographic separation of these pheromones by reverse-phase HPLC. In an acetonitrile/water gradient, farnesylated **a**-factor eluted at a retention time of 52 min., whereas the peak absorbance of geranyl, geranylated **a**-factor eluted at approximately 58 min., when injected either separately or as a mixed sample (Fig. 4A). Similarly, these peptides were separated in a methanol/water gradient on a reverse-phase column and exhibited retention times of 73 min and 78 min, respectively (data not shown).

Although we have shown that switching the isoprenylation target sequence at the C-terminus of **a**-factor results in the biosynthesis of secreted and bioactive peptides, it remained unclear whether this activity was due to the presence of geranyl, geranylated **a**-factor, farnesylated **a**-factor, or perhaps a mixture of both. To answer this question, we proceeded to isolate and purify **a**-factor activity from culture supernatants of strain SM1229 (*Δmfa1, Δmfa2*) expressing the -CVIL mutation, as well as wild-type parent strain SM1058 (expressing the chromosomal copies of the gene) and the double **a**-factor gene deletion strain, as controls. Concentrated samples of supernatant were injected onto a C_3 reverse-phase column and eluted using either an acetonitrile/water gradient or methanol/water gradient. Individual fractions were tested for their endpoints of bioactivity in the halo assay. Comparison of the peaks of biological activity from the eluted sample fractions to the retention time of the synthetic standards indicated that two peaks of activity were present in the -CVIL mutant **a**-factor preparation and that these correlated directly with the elution times of farnesylated and geranyl, geranylated **a**-factor (Fig. 4B). In contrast, no activity was recovered from the **a**-factor gene deletion strain, and only one peak of activity, at the retention time of farnesylated **a**-factor, was recovered from the parent strain (data not shown). These samples were applied to two different solvent gradients, in order

Figure 4. HPLC elution profiles of synthetic **a**-factor and geranyl, geranyl **a**-factor (Panel A) and biological activity from culture supernatant recovered from -CVIL **a**-factor mutant (Panel B). Panel A : Peak absorbance of a mixed sample of approximately 5-10 μg of both synthetic geranyl, geranyl **a**-factor and synthetic farnesylated **a**-factor when separated by an acetonitrile/water reverse-phase HPLC gradient. Panel B: Bioactivity of concentrated supernatant from SM1229 [pCVIL] recovered in each fraction of an equivalent HPLC gradient, as determined by serial dilution of fractions and endpoint analysis in the growth arrest of α-strain RC757. The approximate quantity of pheromone in each fraction was calculated on the basis of the known endpoint value (25 pg) of synthetic **a**-factor with this tester strain.


A
farnesylated
a-factor
geranyl, geranylated
a-factor
absorbance (280 nm)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
60
70
80
retention time (min)


B
ng a-factor
7
6
5
4
3
2
1
0
0
10
20
30
40
50
60
70
80
retention time (min)

to rule out the possibility of chance co-elution; identical elution profile comparisons with the synthetic standards were obtained from either the acetonitrile or methanol gradients. It should be noted that calculation of the approximate quantity of **a**-factor in each fraction was performed on a comparative basis to the known quantity of synthetic farnesylated **a**-factor required to yield an endpoint. Since geranyl, geranyl **a**-factor is about 4-fold less active than **a**-factor, equivalent quantities of secreted geranylated peptide would induce less growth arrest than farnesylated **a**-factor. Thus, overexpression of the -CVIL **a**-factor in vivo results in cross-prenylation and secretion of biologically active pheromones. In the absence of an appropriate synthetic standard, no similar comparative studies were feasible for the -SVCC **a**-factor mutant. However, given the structure of the putative biosynthetic peptide, this mutant must produce a pheromone that differs markedly in structure in comparison to the natural **a**-factor.

CHAPTER IV

DISCUSSION

In the course of its biosynthesis and through its mode of action, the yeast **a**-factor interacts with various proteins including farnesyltransferase, STE6, and STE3, which are representative members of three biologically significant protein families. The mammalian homolog of the yeast FPT is a putative target for interventive cancer therapy using drugs designed to inhibit membrane localization of RAS (8,13,39). The STE6 protein, required for **a**-factor export, is a member of the ATP-binding cassette family of transport proteins whose members include the multi-drug resistance P-glycoprotein pump and the cystic fibrosis transmembrane conductance regulator protein (17,22,28). Finally, the **a**-factor receptor on α-cells, STE3, is a protein that is structurally analogous to the β-adrenergic and rhodopsin receptors, as well as HMG-CoA reductase, an important cholesterol pathway regulatory protein (39). An analysis of the structural and functional requirements governing the association of this small lipopeptide with these proteins, for which it acts as either a substrate or ligand, provides us with a basis for further study of lipid-protein interaction.

Several conclusions may be drawn regarding the overexpression of **a**-factors with alternative isoprenylation target sequences. Clearly, the substitution of a geranyl, geranyl moiety for the farnesyl of **a**-factor results in a peptide with high biological activity, and thereby implies recognition by the STE3 receptor protein. In contrast to the trend we previously observed in which changes from prenyl (C_5) to geranyl (C_{10}) to farnesyl (C_{15}) groups on **a**-factor increased bioactivity, a change to geranyl, geranyl (C_{20}) slightly decreases bioactivity implying that an optimum size of isoprene may have been reached

with the wild-type **a**-factor (26). If this is true, overall hydrophobicity may not be the characteristic most important for ligand binding to its receptor protein. Perhaps, either the farnesyl lipid is recognized in a specific manner, or it may provide a specific presentation of the peptidyl portion of the pheromone to the receptor.

Biosynthetically, the specificity of the geranyl, geranyl group is functionally equivalent to the farnesyl group in directing secretion via the STE6 protein. This may indicate that either a C_{15} or C_{20} isoprene is sufficient for interaction with this target protein. Alternatively, the STE6 substrate recognition requirements may lie in the peptidyl portion of the **a**-factor precursor. In fact, although yeast mutants that block **a**-factor farnesylation also block secretion, no direct evidence for isoprenyl interaction with STE6 has been shown and, perhaps, proteolysis of the **a**-factor precursor is required for export (39). However, the idea that generalized isoprenyl modification of a small peptide may be enough to direct secretion would correlate well with what is known about drug transport by the MDR P-glycoprotein and its wide range of substrates, all of which are hydrophobic as well (17).

There has been considerable interest in developing inhibitors of farnesyl transferase as putative anti-cancer agents (15). The rational design of prenyltransferase inhibitors as drugs will require a complete understanding of the enzymatic catalysis and substrate specificity of this class of proteins. In this regard, the observation that cross-prenylation of -CAAX substrates occurs both in vitro and in vivo may have consequences on inhibitor design or dosage requirements. Recently, a microbial screen for farnesyl transferase inhibitors has been developed (15). However, the preferential inhibition of farnesyl transferase may not completely abolish the ability of geranyl, geranyl transferase to cross-prenylate protein substrates and such proteins may subsequently retain bioactivity. Yokoyama et al., have clearly demonstrated that cross-prenylation is markedly influenced

by peptide acceptor concentration even with separated FPT and GGPT-I enzyme preparations (48). Overexpression of -CAAL targets of GGPT-I, specifically CDC42 and RHO1, suppresses *cdc43* mutants (which lack GGPT-I activity), implying that these recover due to cross-prenylation of the targets by either FPT or GGPT-II (33). Our biochemical identification of both farnesylated and geranyl, geranylated **a**-factors from cells overexpressing the -CVIL **a**-factor peptide indicates that cross-prenylation can occur in vivo and may have physiologically relevant consequences for other proteins.

Altered isoprenylation targets in mammalian RAS affect the function of the protein or its ability to interact with other molecules and membranes (1,9,21). The substitution of the -CVLS motif in RAS for -CVLL or -CAIL leads to correct membrane association of either oncogenic or normal RAS; however, geranyl, geranyl modification of normal RAS exhibits growth inhibitory effects, whereas oncogenic RAS retains the ability to cause cellular transformation when geranyl, geranylated (9). In addition, substitution of -CAAX with either -CC or -CXC does not promote membrane association of RAS, implying that additional information is required for correct prenylation (9). Another -CAAX containing protein, $p21^{rho}$, has been shown to be both farnesylated and geranyl, geranylated and the substrate specificity of prenyl addition for this protein is altered by mutation of upstream sequences (1). In contrast, yeast YPT1 protein is still active in ER to Golgi transport when its C-terminal -CC motif is replaced with a farnesyl targeting motif (30). It is important to note that overexpression of the gene encoding the -SVCC **a**-factor results in production of a bioactive pheromone. Although we have not characterized the exact product formed as a result of this mutation, it is clear that the mature pheromone must differ drastically from **a**-factor in that Ser replaces Cys^{12} and the prenyl moiety must have a different distance relationship to the peptide portion of the molecule. This finding extends our knowledge of the requirements for recognition by the STE6 transporter and STE3 receptor proteins.

The results of our study have various implications on the overall understanding of the in vivo role of isoprene modifications. Specifically, isoprenyl modification of the small bioactive peptide **a**-factor with either a farnesyl or geranyl, geranyl moiety is functionally equivalent with respect to secretion and bioactivity. The STE6 transporter non-specifically recognizes and exports at least two kinds of isoprene-modified peptides. Since **a**-factor is the only known natural substrate for the MDR-family of transporters (22,28), this system serves as a model for the broad substrate recognition exhibited by the MDR P-glycoprotein. In addition, the bioactivity of geranyl, geranylated **a**-factor demonstrates that a similar role may be played by either type of isoprene in the peptide-receptor interaction events which induce signal transduction and mating in yeast. Finally, the attachment of specific isoprenes to peptides by prenyltransferases in vivo apparently involves factors other than target sequence specificity alone, as evidenced by the cross-prenylation of -CVIL **a**-factor. These results must be considered in the design of effective prenyltransferase inhibitors.

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PART IV

AN ANALOG OF THE YEAST MATING PHEROMONE **a**-FACTOR EXHIBITS STEREOSPECIFIC SUPERACTIVITY

CHAPTER I

INTRODUCTION

Mating between haploid cells of the yeast *Saccharomyces cerevisiae* is controlled by diffusible peptides termed α-factor and **a**-factor (9). Although these peptides appear to be functionally equivalent in their roles as ligands which induce the receptor-mediated signal transduction cascade involved in mating, they are structurally distinct. While α-factor is an unmodified 13 amino acid peptide (WHWLQLKPGQPMY), the **a**-factor is a 12 amino acid lipopeptide modified with a 15-carbon isoprenoid moiety, farnesyl, and a carboxyl methyl ester at its C-terminus (YIIKGVFWDPAC[farnesyl]-OCH_3) (1,6).

Numerous structure-activity studies on the α-factor have provided important insights into the relationship between peptide sequence and bioactivity (5,16,23). Hypotheses concerning the bioactive conformation of this pheromone have been previously published (16). In contrast, few analyses of **a**-factor structure-function have been performed and these have focused primarily on the effects of C-terminal modifications in bioactivity (11). The posttranslational modification of **a**-factor has been shown to be essential for its secretion and biological activity (11,18,19). Isoprenylation, in general, has been implicated in the membrane association and function of various other proteins including nuclear lamins, rab vesicular trafficking proteins, and the oncogene product RAS (21).

The contributions to biological activity made by the peptidyl portion of the **a**-factor have yet to be clarified. In the course of such studies, we have synthesized and tested an **a**-factor analog which has been shown to exhibit superactivity in biological assays including growth arrest and mating restoration. Furthermore, this 12 amino acid peptide,

YIIK(D-A)VFWDPAC[farnesyl]-OCH_3, was also found to be susceptible to degradation by the α-cell associated protease, **a**-factorase (14). Interestingly, a stereospecific isomer of this peptide, [L-Ala^5] **a**-factor, exhibited lower activity than both native pheromone and the superactive peptide. In light of these results, we presume that the superactive nature of [D-Ala^5] **a**-factor is a consequence of either a heightened ability to bind or attain access to its receptor, the STE3 protein (8). This represents the first report to date of a superactive yeast pheromone of either mating type.

CHAPTER II

MATERIALS AND METHODS

Peptide synthesis

Both the [D-Ala^5] and [L-Ala^5] **a**-factors were synthesized by coupling the appropriately protected amine terminal decapeptide with Ala-Cys(farnesyl)-OCH_3 following the procedure originally used in the synthesis of the synthetic **a**-factor by Xue et al. (22). Crude product was deprotected with 20% piperidine in DMF and isolated by reversed-phase HPLC using a water/acetonitrile/trifluoroacetic acid gradient from 30% acetonitrile to 100% acetonitrile. The product was homogeneous in the purification gradient and also in a water/methanol/TFA gradient. It gave the expected amino acid ratios and the correct molecular ion in fast atom bombardment spectroscopy analysis. It was further characterized by ^{1}H NMR spectroscopy and exhibited peaks characteristic of *trans,trans*-farnesyl and the methyl ester.

Yeast strains and genotypes

The following yeast strains were used in this study: strain RC757 (*MATα sst2-1 rme1 his6 met1 can1 cyh2*) was a gift from Russell Chan (2); strain SM1229 was a gift from Susan Michaelis (*MATa Δmfa1::LEU2 Δmfa2::URA3 trp1 his4 can1*) (14); strains X2180-1A (*MATa SUC2 mal mel gal2 CUP1*) and X2180-1B (*MATα SUC2 mal mel gal2 CUP1*) were obtained from the Yeast Genetics Stock Center (University of California, Berkeley).

Biological assays

All **a**-factors were assayed for bioactivity using growth arrest ("halo") and mating restoration assays. Growth arrest is indicated by the presence of a halo in lawns of *S. cerevisiae* grown on agar plates onto which was spotted a quantity of mating pheromone of the opposite mating type. The endpoint of bioactivity is defined as the minimum quantity of peptide resulting in the formation of an observable halo. All halo assays were performed as previously described by Marcus et al. (11) using supersensitive α-strain RC757. Mating restoration is assessed as the ability of exogenously added synthetic peptide to induce the formation of constellations of diploid yeast colonies from a mixture of an α-strain and an **a**-strain in which the structural genes for **a**-factor, *MFa1* and *MFa2*, have been deleted. The endpoint is termed the minimum quantity of peptide required to form a diploid cluster on appropriately selective media. Mating restoration was performed between α-strain RC757 and **a**-strain SM1229 (*Δmfa1 Δmfa2*) supplemented with the addition of serial dilutions of either [D-Ala^5], [L-Ala^5] **a**-factor, or wild-type **a**-factor, and treated as previously described (11). All peptide stock concentrations were verified to be approximately equal by absorbance at A_{279}.

Degradation assays

The degradation of [D-Ala^5] **a**-factor by both **a**- and α-cells (X2180-1A and X2180-1B, respectively) was assayed, following iodination, using the method described by Marcus et al. (14). After a 4-hour reaction time, assays were terminated by centrifugation to remove the cells. A total of 50 µl of the supernatant was applied by repeated spotting and drying of 10 µl aliquots onto Whatman 3MM Chr paper and resolved with 65% acetonitrile in water. After drying, the paper was cut according to Rf value and each strip counted in a LKB Wallac Clini-Gamma 1272 gamma counter.

CHAPTER III

RESULTS

Biological activity of [D-Ala^5] **a**-factor

The effects that alterations in peptide structure may have on yeast mating pheromone bioactivity can be assessed by a variety of methods including growth arrest, cell morphology, gene expression, and mating response. All of these methods act as measures of potency in judging ligand-receptor interaction. As determined by two biological assays, the substitution of D-alanine for glycine in the **a**-factor peptide resulted in a pheromone which exhibited biological activity exceeding that of the native **a**-factor. In the halo assay, which assesses the ability of **a**-factor to induce growth arrest of α-cells, [D-Ala^5] **a**-factor displayed an endpoint of activity higher than that of the wild-type **a**-factor on a supersensitive α-cell lawn (Fig. 1A; Table 1). Although growth arrest serves as a good representation of peptide potency, the definitive functional test for analog bioactivity is the ability to induce mating. In this respect also, the [D-Ala^5] **a**-factor demonstrated a capacity to restore mating at levels above that of wild-type pheromone (Fig. 1A; Table 1). Furthermore, corresponding analyses of both the growth arrest and mating restoration capability of [D-Ala^5] **a**-factor with wild-type α-cells yield similar results (data not shown).

Biological activity of [L-Ala^5] **a**-factor

While it was apparent that the [D-Ala^5] **a**-factor was a superactive analog, it remained to be seen whether this activity was a consequence of the mere substitution of alanine for glycine or if the observed results were due to the stereospecific character of the peptide. In order to address this question, the same bioassays were performed on synthetic

Figure 1. Biological activity of [D-Ala^5] **a**-factor exceeds that of [L-Ala^5] **a**-factor, and **a**-factor. Panel A: Growth arrest of α-strain RC757 induced by serial dilutions of [D-Ala^5] **a**-factor, [L-Ala^5] **a**-factor, and **a**-factor performed as described in the Materials and Methods. Equal 5 μl quantities of each peptide were spotted at the same positions on each lawn. Endpoints of activity are reported in Table 1. Panel B: Restoration of mating between α-strain RC757 and **a**-strain SM1229 (*Δmfa1, Δmfa2*) in the presence of exogenously added dilutions of [D-Ala^5] **a**-factor, [L-Ala^5] **a**-factor, and **a**-factor. The assay was performed as described in the Materials and Methods. Equal 5 μl quantities of each peptide were spotted at the same positions on each lawn. Endpoints of activity are as reported in Table 1.

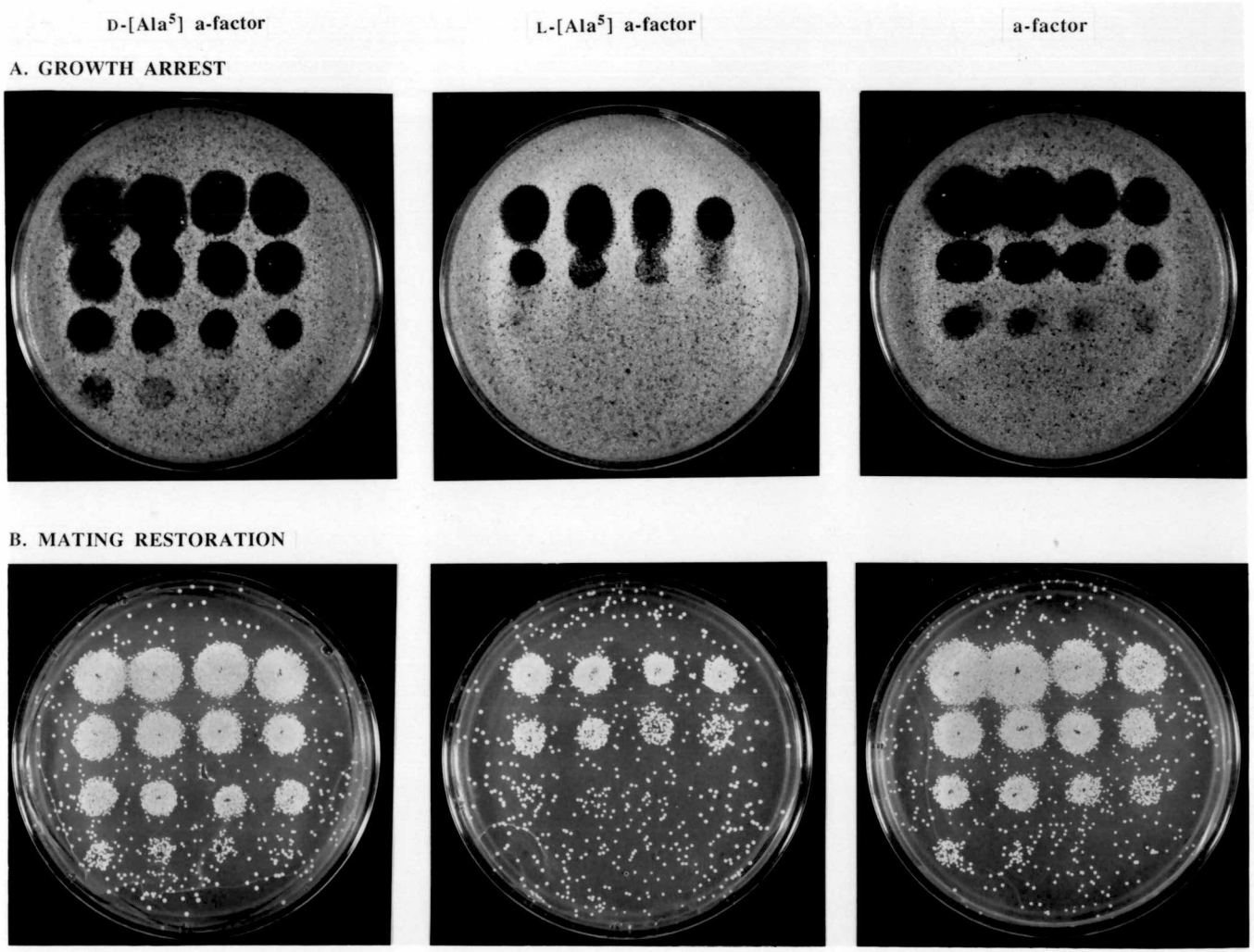

D-[Ala5] a-factor
L-[Ala5] a-factor
a-factor
A. GROWTH ARREST
B. MATING RESTORATION

TABLE 1. Endpoints of biological activity for **a**-factor analogs

	Growth arrest[a]	Mating restoration[a]
[D-Ala^5] **a**-factor	0.01 ng	0.06 ng
[L-Ala^5] **a**-factor	0.10 ng	3.90 ng
a-factor	0.03 ng	0.24 ng

[a] performed in triplicate, as described in Materials and Methods.

[L-Ala^5] **a**-factor. The results from halo and mating restoration assays were interesting in that they indicated simple substitution of alanine for glycine causes a clear decrease in **a**-factor bioactivity in both assays (Table 1; Fig. 1A and 1B). Therefore this suggested that the superactive nature of [D-Ala^5] **a**-factor was indeed related to the stereochemical rotation of the alanine substituted at the fifth position.

[D-Ala^5] **a**-factor is degraded by **a**-factorase

We have previously reported the discovery of an α-cell associated endoprotease, **a**-factorase, capable of degrading **a**-factor in a manner which suggests it serves a role in the recovery of α-cells from the sensitization induced in pheromone response (14). One possible explanation for the observed superactivity of the [D-Ala^5] **a**-factor might presume this analog has an inherent ability to evade cleavage by **a**-factorase, thereby avoiding this means of cellular desensitization. We proceeded to investigate this possibility by radiolabeling [D-Ala^5] **a**-factor with [^{125}I] and utilizing this peptide in an assay for mating-type-specific degradation. The radiolabeled [D-Ala^5] **a**-factor retained biological activity in the growth arrest assay (data not shown). The results of this experiment indicated that the analog is successfully cleaved by α-cells, specifically (Fig. 2), and that its superactivity is probably not a result of increased stability to peptidase. On the basis of this result, it is reasonable to conclude that the [D-Ala^5] **a**-factor is superactive because it either has a higher affinity for the STE3 receptor or because the bound ligand is in a conformation which is especially conducive in promoting receptor-mediated signal transduction via the G-protein pathway. In the absence of an available **a**-factor binding assay, it is currently impossible to distinguish between these possibilities.

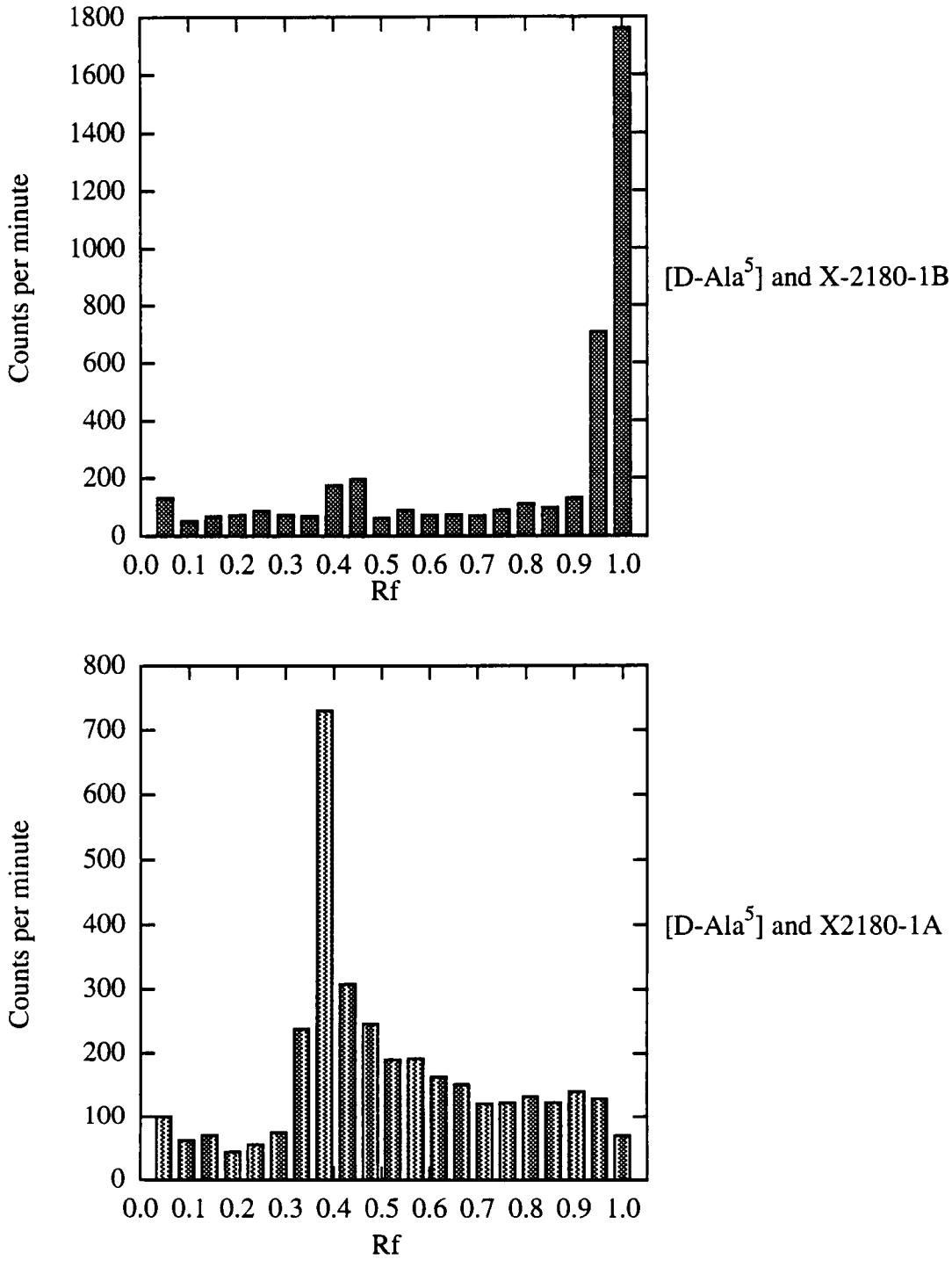


Figure 2. Degradation of iodinated [D-Ala^5] **a**-factor after incubation with α-cells (X2180-1B, top) and **a**-cells (X2180-1A, bottom). The vertical axes refer to the counts per minute detected using a gamma counter after separation of the [D-Ala^5] **a**-factor degradation reaction by paper chromatography in 70% acetonitrile in water. The horizontal axes represent the relative distance from the solvent front of each section of paper counted.

CHAPTER IV

DISCUSSION

The **a**-factor receptor, or STE3 protein, is a member of the seven transmembrane-domain G protein-linked family of receptor proteins which includes the adrenergic, muscarinic, and rhodopsin receptor molecules (3). Due to its lipopeptide structure, **a**-factor represents a novel form of natural ligand for this type of receptor, with respect to the relative influences of the C-terminal modifications and peptide portions of its structure on its function. In this study, we have replaced a single amino acid, glycine at position 5, with two stereospecific (D- and L-) forms of the amino acid alanine to evaluate the effect this may have on **a**-factor biological activity.

The results of this study clearly indicate that replacement of Gly^5 with D-alanine yields a highly bioactive pheromone, whereas an equivalent substitution of L-alanine at this position results in a significant decrease in activity. Additional evidence indicates that the increased bioactivity of [D-Ala^5] **a**-factor is probably not due to an increased stability of this peptide since it remains susceptible to cleavage by the **a**-factorase α-cell specific protease. Additional experiments are in progress to determine if the rate of **a**-factor degradation by **a**-factorase may be affected with this analog. In all, these results suggest that the superactivity demonstrated by this analog represents a stereospecific conformational preference in either ligand binding or receptor-mediated response to pheromone.

An increasing amount of information is accumulating regarding a possible physiological role for naturally occurring D-amino acid residues in proteins (15). Specifically, one class of opioid neuropeptides, whose members include dermorphin and dermenkephalin, have been found to contain either D-Ala or D-Met at position 2,

respectively (10,15,17). In stark contrast to their virtually inactive [L-Ala^2] or [L-Met^2] counterparts, these highly active peptides exhibit a marked increase in receptor affinity and selectivity (17). Furthermore, the presence of the D-residues has been shown to influence the conformation and stabilization of these opioids in solution (10). It has been proposed that these peptides interact with opioid receptors in a manner which involves putative membrane-peptide related interactions according to the model of Schwyzer (22), in addition to receptor-ligand binding. Similarly, we have previously invoked this model in attempts to conceptualize a role for C-terminal isoprenylation in **a**-factor-membrane-receptor interaction (4,10).

In an earlier study of **a**-factor conformation, it was concluded that this pheromone is disordered in solution (7). However, by nuclear magnetic resonance, a weak interaction between protons in the side chains of Val^6 and Trp^8 indicated a possible bend in the center of the molecule. The replacement of Gly by D-Ala might be expected to stabilize turn structures (7). Therefore, it may be possible that the superactivity exhibited by this peptide may reflect a preference for the biologically active conformation. Clearly, additional structure-activity studies focused on the relative contribution that different amino acid residues have toward the biological function of **a**-factor will lead to a further understanding of lipopeptide-membrane-receptor interactions.

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PART V

BIOLOGICAL CONSEQUENCES OF TRUNCATION AND SUBSTITUTION OF AMINO ACIDS IN THE *SACCHAROMYCES CEREVISIAE* MATING PHEROMONE **a**-FACTOR

CHAPTER I

INTRODUCTION

The yeast *Saccharomyces cerevisiae* has served as a model organism for the analysis of the molecular events that occur in receptor-mediated signal transduction (21). Mating between haploid yeast cells to form diploids is controlled by the reciprocal action of peptide mating pheromones, termed α-factor and **a**-factor, which are secreted by either α-cells or **a**-cells, respectively. These peptides bind to their respective receptor proteins on cells of the opposite mating type and subsequently induce growth arrest, morphological change, and mating (21,42). Both pheromones are ligands for receptors that are members of the G protein-coupled receptor family, which includes the rhodopsin and adrenergic receptors. Analyses of this protein family has revealed that its members share the common structural organization of seven transmembrane domains, although their amino acid sequences are not similar (9). This appears to be the case for the yeast pheromone receptors as well, if comparing the amino acid identity between the STE2 protein, the α-factor receptor, and STE3 protein, the **a**-factor receptor (3,13,32).

Although the two yeast pheromones appear functionally equivalent, they are dissimilar in their structure, secretion, and biosynthesis. The α-factor is an unmodified 13 amino acid peptide [WHWLQLKPGQPMY] which is secreted through the classical yeast secretory pathway involving the endoplasmic reticulum, Golgi, and secretory vesicles (12). In contrast, the **a**-factor is a 12 amino acid lipopeptide which is posttranslationally modified by the addition of a C_{15} farnesyl isoprenoid and a carboxyl terminal methyl group [YIIKGVFWDPAC(farnesyl)-OCH_3] (Fig. 1) (1). Furthermore, **a**-factor is exported via a mechanism that requires the action of the yeast *STE6* gene product, a homolog of the mammalian multidrug resistance P-glycoprotein (19,26).

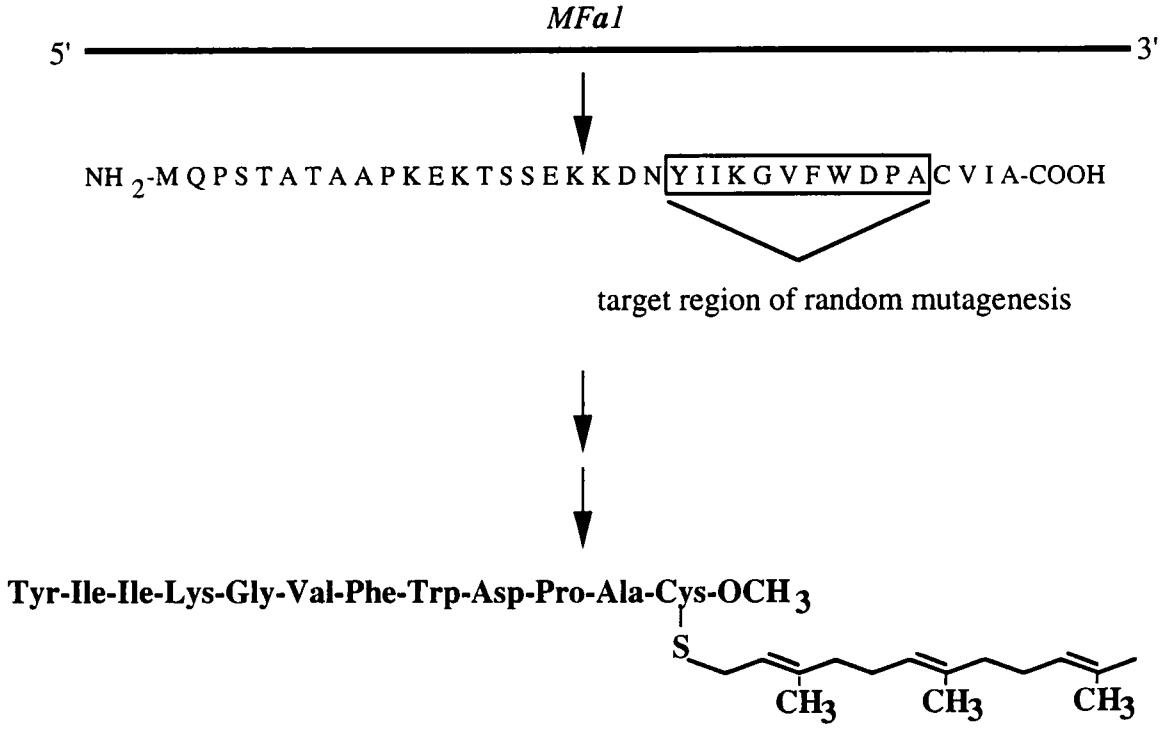


Figure 1. Representation of the primary product of the **a**-factor gene, the region of this gene targeted for random mutagenesis, and the mature **a**-factor pheromone following intracellular processing and post-translational modification.

Extensive studies have been performed on the structural aspects of α-factor bioactivity and receptor binding (11,31,45), whereas relatively few studies on **a**-factor bioactivity have been published (23). It has been previously shown that isoprenylation of **a**-factor is a prerequisite for its export and bioactivity (23,37,38,39). Removal of both the C-terminal farnesyl and methyl ester groups on **a**-factor results in a 100,000 fold drop in bioactivity (23). The presence of C-terminal modifications on the **a**-factor imply that this peptide may interact with its receptor in a manner different from that of a simple unmodified peptide. The presence of the isoprenyl moiety on **a**-factor may play a role in either the direct interaction of the pheromone with the STE3 receptor or indirect presentation of the amino acid portion of **a**-factor to the receptor. Isoprenylation has been implicated in the membrane localization and function of various proteins including **a**-factor, nuclear lamins, rab vesicular trafficking proteins, and the oncogene product RAS (7,37,38,39,41). In fact, RAS-mediated cellular transformation is dependent upon membrane localization via farnesylation (8,17).

Several recent studies indicate that farnesylation of proteins directly enhances their ability to interact with specific targets. For example, the attachment of a farnesyl group to RAS increases its ability to bind and activate adenylyl cyclase, even in the absence of membranes (22). Similarly, the interaction between the α subunit and βγ subunits of transducin is highly favored specifically when the γ subunit is farnesylated and carboxyl methylated (34). However, the function of protein prenylation remains to be fully elucidated and very little is known about factors affecting the interaction of lipopeptides with their recognition proteins (25). In this study, we utilize a combined approach of molecular biology and synthetic peptide biochemistry to analyze the biological effects of amino acid truncations and substitutions within the peptidyl portion of the **a**-factor as a model system for the analysis of isoprene-mediated peptide-protein interaction.

CHAPTER II

MATERIALS AND METHODS

Yeast strains and methods

The following yeast strains were used: SM1229 (*MATa Δmfa1::LEU2 Δmfa2::URA3 trp1 his4 can1 leu2 ura3*) (29) and RC757 (*MATα sst2-1 rme his6 met1 can1 cyh2*) (5). Transformation of yeast strains was performed by the LiAc method of Gietz et al. (13). Growth arrest or "halo" assays for measuring the bioactivity of synthetic **a**-factor and **a**-factor analogs was done by testing serial dilutions of peptides on lawns of supersensitive α-strain RC757, as previously described (23). Growth arrest assays for the selection of bioactive **a**-factor producing colonies under galactose-induced gene expression control were carried out either in the presence of 2% galactose containing 0.05% glucose (induced condition) or with 2% glucose only (repressed condition), as described (24). Mating restoration assays were performed by the exogenous addition of serial dilutions of **a**-factor or **a**-factor analogs to a mixture of α-strain RC757 and **a**-strain SM1229 (*Δmfa1 Δmfa2*) on media selective for growth of diploid colonies of these strains, as previously outlined by Marcus et al. (23).

Synthesis and characterization of truncated **a**-factor peptides

The chemical synthesis of **a**-factor peptides was performed essentially as previously described (43,44,45). Briefly, the synthesis of truncated **a**-factor analogs was perfomed by coupling the amino terminus of a modified dipeptide Ala-Cys (farnesyl)-OCH_3 to a series of separately synthesized and protected oligopeptides (length = n) of varying lengths in a n + 2 solution phase fragment coupling strategy. For example, the synthesis of des-Y **a**-factor was accomplished by coupling the protected peptide I-I-K-G-V-F-W-D-P to the

amine of dipeptide A-C(farnesyl)-OCH_3. All coupled peptides were subsequently deprotected and their structure confirmed by amino acid analysis and fast atom bombardment mass spectroscopy. Stocks of **a**-factor peptides were dissolved in 100% methanol and maintained at -20°C.

Random oligonucleotide-targeted mutagenesis of the *MFa1* gene

A 1.6 kb *Bam* HI DNA fragment containing the *MFa1* gene of *S. cerevisiae* (2), which encodes the primary precursor of the **a**-factor, was used as the template for oligonucleotide-targeted mutagenesis of a specific region of this gene by a variation (33) of the method of Kunkel (20). This fragment was subcloned into phagemid vector pTZ19U (28) at the *Bam* HI site of the polylinker and the resulting vector used as a source of single-stranded DNA for subsequent *in vitro* mutagenesis primed by a specific pool of mutagenic synthetic oligonucleotides (24,33).

This pool of oligonucleotides was prepared by "doping" or contaminating each of the nucleoside phosphoramidites used in the synthesis of the single-stranded DNA with a specific percentage of an equimolar mixture of all four phosphoramidites (G,A,T,C) prior to synthesis of the target DNA region of interest. A 33 base oligonucleotide specifically corresponding to the region of the *MFa1* gene that encodes the mature, secreted form of the **a**-factor, with the exception of the C-terminal cysteine (Fig. 1), was synthesized using nucleoside phosphoramidite mixtures that were contaminated at a level of 6.0% equimolar mixture. This predetermined level of doping was designed to approximate the introduction of 1.5 random base substitutions per oligonucleotide calculated on the basis of the procedure of McNeil and Smith (27). Oligonucleotides used in this procedure were obtained from Genosys Biotechnologies (The Woodlands, Texas).

Phenotypic screening for **a**-factors lacking biological activity

Mutagenized *MFa1* -containing plasmid DNA was isolated from an amplified pool of several thousand *E.coli* colonies and a 1.0 Kb *Eco* RI- *Bam* HI gene fragment containing the **a**-factor coding sequence, but lacking its promoter, was isolated and subcloned into yeast expression vector. This plasmid, pRS316GU (a gift from Phil Hieter), contains the galactose-inducible *GAL1* promoter in the *Kpn* I site of the polylinker of vector pRS316 which carries *TRP1* as the selectable marker (40). The *Eco* RI site in the *MFa1* gene was previously introduced by site-directed mutagenesis, as described (24). Again, over 1000 colonies containing the ligated *MFa1* gene fragment were pooled, amplified and their plasmid DNA isolated. This pool of randomly-mutated **a**-factor encoding expression vectors was transformed into yeast strain SM1229 (*Δmfa1 Δmfa2*). Close to 1000 tryptophan-selected transformants were individually transferred into microtiter plates containing media selective for growth of the yeast and plasmid maintenance. The yeast in these plates were grown to high density and 25 µl portions were removed and spotted onto lawns of α-strain RC757 in the presence of either galactose or glucose. Plates were incubated overnight at 30°C and scored the following day for evidence of growth arrest on both glucose and galactose.

Sequencing of plasmid DNA directly from phenotypically-selected yeast colonies

Individual colonies of yeast cells that did not exhibit **a**-factor bioactivity were identified and used as the source of DNA from which part of the *MFa1* gene was amplified and sequenced. We developed a procedure for the rapid isolation and sequencing of plasmid DNA directly from yeast colonies (4). Transformants were picked directly from plates, resuspended in 10 µl H_2O and mixed well. This suspension was added to a tube containing 100 µl of a lysis buffer [2.5 M LiCl, 50 mM Tris-HCl (pH 8), 4% Triton X-100, 62.5 mM EDTA], 100 µl phenol:chloroform (1:1), and 0.2 g glass beads (0.45 mm).

The mixture was vortexed well for 2 min, separated by centrifugation for 1 min at 12,000 x g, and the supernatant was transferred to a fresh tube containing 2.5 volumes ethanol. The nucleic acid was precipitated at -20°C for 10 min, then pelleted by centrifugation for 15 min. at 12,000 x g at 4°C. The pellet was washed with cold 70% ethanol, dried under vacuum for 2-5 min, then resuspended in 20 µl H_2O. This solution is heated at 94°C for 10 min., then immediately placed on ice. Amplification of the *MFa1* gene was performed by mixing 5 µl 10X reaction buffer [500 mM KCl, 100 mM Tris-HCl (pH 9), 1% Triton X-100] with 1 µl of the nucleic acid preparation. This mixture was overlayed with mineral oil, placed in a thermocycler, and held at 94°C for 15 min. A mixture of the following components was prepared and then added through the oil overlay: 0.5 µM of two primers (designed to flank the **a**-factor sequence), 50 µM dNTPs, 1.5 mM $MgCl_2$, 2.5 units *Taq* polymerase (Perkin-Elmer Cetus), and H_2O to bring the final volume to 50 µl. These samples were subjected to 30 cycles of: 94°C for 1 min., 45°C for 1 min., and 72°C for 2 min. Cycle sequencing reactions were performed using the *fmol* sequencing kit (Promega) with 1 µl of the amplified yeast template and a ^{32}P end-labeled primer (36) just upstream to the **a**-factor sequence.

CHAPTER III

RESULTS

Biological activity of truncated **a**-factor peptides

We have previously studied the significance of C-terminal modifications to the biological activity of **a**-factor and determined that the farnesyl and methyl groups contribute approximately equally to, and are required for, biological activity (23). However, the relative contribution to **a**-factor activity made by the individual amino acids present in the pheromone has not been addressed. A series of synthetic **a**-factor peptides containing serial truncations from the N-terminus of the pheromone was synthesized in order to ascertain the effect changes in peptide structure and length would have on **a**-factor bioactivity. It should be noted that all of these truncated peptides retained an intact C-terminus correctly modified with both the C_{15} farnesyl and carboxyl methyl moieties.

We employed two different assays to measure the biological activity of **a**-factor and its analogs. The halo, or growth arrest assay is an agar diffusion assay which serves to determine the minimum quantity of peptide required to induce growth arrest of α-cells. The mating restoration assay is a measure of the minimum quantity of exogenous peptide required to stimulate mating between α-cells and **a**-cells which do not have the ability to produce their own **a**-factor. Mating is scored by the evidence of colonies on plates that select for growth of only diploids in the presence of exogenously added peptide. The results of testing N-terminal truncations of **a**-factor in both of these assays suggest that sequential loss of specific amino acid residues negatively affects pheromone activity (Table 1). Removal of the N-terminal tyrosine residue reduces bioactivity to approximately 2% of wild-type **a**-factor in the halo assay (Table 1; Fig.2). Truncation to remove the first isoleucine results in an additional order of magnitude decrease in activity. The further

TABLE 1. Bioactivity of truncated **a**-factor analogs

Peptide	Growth Arrest[a,d] (ng)		Mating Restoration[b,d] (ng)	
YIIKGVFWDPAC(farn)-OCH_3	0.025	(1X)[c]	0.25	(1X)[c]
YIIKGVFWDPAC	2500	(100,000X)	> 2500	(10,000X)
IIKGVFWDPAC(farn)-OCH_3	1.0	(40X)	1.0	(4X)
IKGVFWDPAC(farn)-OCH_3	10.0	(400X)	1.0	(4X)
KGVFWDPAC(farn)-OCH_3	10.0	(400X)	2.0	(8X)
GVFWDPAC(farn)-OCH_3	10.0	(400X)	4.0	(16X)
VFWDPAC(farn)-OCH_3	100	(4,000X)	31.0	(125X)
FWDPAC(farn)-OCH_3	250	(10,000X)	31.0	(125X)
WDPAC(farn)-OCH_3	1,000	(40,000X)	250	(1,000X)
DPAC(farn)-OCH_3	> 1,000		> 1,000	

[a] Growth arrest assay perfomed using supersensitive α-strain RC757. The amount listed in the table represents the smallest amount of peptide giving a response in the growth arrest assay.

[b] Mating restoration assay performed by selecting for diploid constellations between α-strain RC757 and **a**-factor gene deletion **a**-strain SM1229 (*Δmfa1 Δmfa2*) in the presence of exogenously added peptide. The amount listed in the table represents the smallest amount of peptide giving a response in the mating restoration assay.

[c] Number in parentheses represent fold change in activity compared to wild-type **a**-factor.

[d] The values represented in the table are the average of at least three determinations with each test yielding a result variant by not more than one two-fold dilution.

A



B



Figure 2. Bioactivity of truncated **a**-factor analog [IIKGVFWDPAC(farnesyl)-OCH_3]. Panel A: Growth arrest assay. Growth arrest of α-strain RC757, which is supersensitive to **a**-factor, is monitored as 5 µl of serial dilutions of peptide are spotted onto a lawn of this strain (see Materials and Methods). Panel B: Mating restoration assay. The mating restoration assay serves as a means for determining pheromone potency by judging the minimum quantity of exogenously added peptide required to induce the formation of diploid cells between haploid α-strain RC757 and **a**-strain SM1229 (*Δmfa1 Δmfa2*).

removal of the I^3 and K^4 residues has no effect on activity. However, removal of G^5 results in an order of magnitude reduction of activity and subsequent removal of V^6 and F^7 further lowers activity resulting in a peptide with 1/40,000 the activity of **a**-factor in the halo assay. The successive removal of amino acids from the N-terminus of **a**-factor results in a gradient of activity loss until virtually all bioactivity appears lost with the tetrapeptide DPAC(farn)-OCH_3 peptide. This peptide does not cause growth arrest even when tested at 1 μg. Overall changes in bioactivity are not as dramatic in the mating restoration assay (Table 1). Nevertheless, the relative effect of sequential truncation of N-terminal residues follows approximately the same trends as in the halo assay. Thus, changes to the peptide portion of the **a**-factor cause only moderate effects on the activity of the pheromone until more than half of the residues are removed.

Identification of substitution mutations within the peptide portion of **a**-factor that do not exhibit biological activity

To further investigate the functional significance of individual amino acids in the **a**-factor peptide, we used a genetic screen to identify residues that contribute to the bioactivity of the pheromone. This required the generation of random mutations in the specific region of the *MFa1* gene which corresponds to the mature, secreted form of the **a**-factor peptide (Fig. 1), followed by expression of mutated gene products in yeast, and phenotypic selection of colonies which lack **a**-factor bioactivity. Plasmid DNA from yeast colonies which exhibited a loss of **a**-factor activity was then directly sequenced and the mutations responsible for the observed phenotype were identified.

Random oligonucleotide-targeted mutagenesis (33) was employed to generate a mutant pool of **a**-factors which contained base substitutions in the **a**-factor gene, specifically covering all the residues of the mature **a**-factor, with the exception of the C-terminal cysteine. This mutagenesis scheme was designed to maintain an intact target for

isoprenylation, or -CAAX box, which is required for export of the **a**-factor (24, 37, 39), while, theoretically, altering all other residues in the mature pheromone. Changes within the primary sequence of **a**-factor have been shown to have little impact on **a**-factor secretion, as indicated in preliminary studies (6,30). Prior to pooling the putative mutants, eight individual *E.coli* transformants were sequenced to verify evidence of mutagenesis. The results of this analysis indicated that mutations were present at a rate of 1.4 mutants per gene and exhibited a relatively even spread over the bases encoding **a**-factor (data not shown). Following the subcloning of the altered gene pool into a yeast expression vector, expression of the mutated *MFa1* genes in a yeast strain deleted in the **a**-factor genes (strain SM1229 *Δmfa1 Δmfa2*) was controlled by a galactose-inducible promoter. We have previously employed this methodology in the characterization of site-directed mutations in the *MFa1* gene that effect the biosynthesis of **a**-factor (24). Transformant colonies were isolated on selective medium and screened for evidence of loss of **a**-factor bioactivity in the presence of galactose, on the basis of an inability to induce growth arrest of α-cells. About 1000 colonies were screened and approximately 300 were identified as putative mutants. Plasmid DNA from a random selection of about 50 of these putative mutant colonies was then directly sequenced to identify the altered DNA sequences in the *MFa1* gene responsible for the phenotypic loss of **a**-factor bioactivity.

The results of this selection indicated that substitution mutations predominated at specific codons (Fig. 3; Table 2). Thus, certain amino acids appear to have a critical role in maintaining **a**-factor bioactivity. Most of the phenotypically selected colonies contained *MFa1* genes in which the predicted gene product would contain changes at either glycine (at position 5), valine (at position 6), tryptophan (at position 8) or aspartic acid (at position 9) (Fig. 3). Many of the sequenced genes contained multiple changes at these positions with a variation of substitutions in the case of some residues, but not others. For example, whereas the changes at glycine and tryptophan exhibited a mixture of both conservative and

TABLE 2. Substitution mutations[a,b] identified in screen for colonies lacking **a**-factor bioactivity

a-factor:	YIIKGVFWDPAC
	YIIK**DI**FW**N**PAC
	YIIK**A**VF**L**DPAC
	YIIKGVFW**N**PAC
	YIIKG**I**F**K**DPAC
	YIIK**SI**FWDP**T**C
	YIIKGVF**L**DPAC
	YIIK**S**VFWDP**T**C
	YIIK**N**VFW**N**PAC
	YIIKGVF**C**DPAC
	YIIKGV**V**W**N**PAC
	YIIKGVF**Q**DPAC
	YIIKG**I**F**L**DPAC
	YIIKG**I**FWD**S**AC
	YIIK**SI**FW**N**PAC

[a]amino acids in bold and underlined represent positions where changes in codons predict the shown substituiton in the resulting peptide.

[b]in some cases, identical mutations were identified more than once, yet only represented once here.

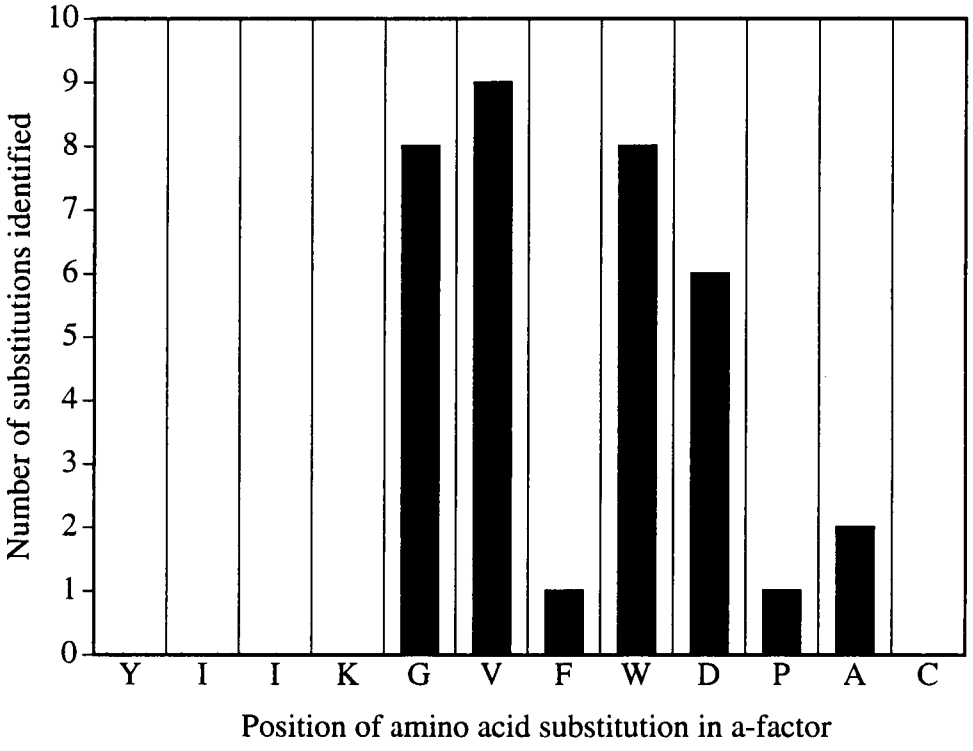


Figure 3. Graphic representation of the relative distribution and frequency of substitution mutations resulting in altered codons for specific amino acids in the predicted **a**-factor peptides produced by phenotypically selected colonies exhibiting a loss of **a**-factor bioactivity.

non-conservative amino acid substitutions, all changes at either valine or aspartic acid were to either isoleucine or asparagine, respectively. The identification of mutant genes encoding several changes may imply that multiple substitutions are required before a detectable loss of activity is observed in the case of some positions using this selection scheme. Furthermore, although single substitution mutations at the position encoding aspartic acid were identified, all colonies containing substitution mutations of isoleucine for valine were associated with an additional mutation at the position of either glycine or tryptophan. The *MFa2* gene of *S. cerevisiae* also encodes a biologically active **a**-factor peptide which differs from the *MFa1* gene product only at position 6, where it codes for leucine instead of valine (2). This suggests that the conservative change to isoleucine would not be expected to affect bioactivity.

All mutations identified were present within the region defined by the oligonucleotide used for random mutagenesis, however, as would be expected from such a mutagenesis procedure, a number of "nonsense" mutations resulting in the presence of in-frame stop codons were identified as an obvious source of inactive **a**-factors. These were found exclusively at the codon encoding tryptophan at position 8 of the mature pheromone, in which case a single base substitution in the wild-type codon (TGG) could result in an in-frame nonsense mutation (to either TAG or TGA). The resulting **a**-factors would be truncated prior to the translation of the -CAAX box and, thereby, not be posttranslationally modified and subsequently exported by the cell. It should be emphasized that this selection scheme was designed simply to identify amino acid residues that appear to significantly affect **a**-factor bioactivity and by no means represents a complete saturation analysis of amino acid tolerance or preference within the peptide. However, it is equally important to consider that a lack of substitutional mutations found at any specific residue(s) may imply that these positions are not critical for bioactivity, and may indicate a tolerance for changes or combinations at a specific site(s), as judged by our screen.

CHAPTER IV

DISCUSSION

Various models have been proposed for the mechanism of targeting isoprene-modified proteins (7,38). These include the possibility that prenylated proteins might be directed to membranes by interaction with specific membrane-bound receptors, by lipid-lipid interactions in membranes, or by secondary modifications subsequent to prenylation (38). The ease with which **a**-factor biological activity is assayed, along with our established methodologies for chemical synthesis of its analogs (43,44,45) and mutagenesis of the gene encoding the pheromone (24), provide an attractive system by which structure-function relationships in lipopeptide-protein interaction may be studied.

The results of our truncation analysis of **a**-factor indicate that only extreme changes in the peptidyl structure of the pheromone have as great an effect on biological activity as does removal of the C-terminal modifications. Over two-thirds of the peptide must be removed from the N-terminus to lose nearly total activity. In contrast, an equivalent loss of bioactivity is found with the complete peptide missing only the farnesyl and methyl ester moieties. This strongly suggests that these posttranslational modifications play a direct role in either the interaction with, or presentation of, the **a**-factor to its receptor. It is also clear that different amino acids contribute in varying degrees to the overall activity of the **a**-factor. For example, removal of residues 1-3 results in only an 87% drop in activity as judged in the mating restoration assay (Table 1) in comparison to a 99% drop on removal of either the methyl ester or farnesyl moiety and a complete absence of activity with the totally unmodified peptide (23). It is somewhat suprising that nearly 50% of the peptidyl portion of the pheromone can be removed with retention of measurable biological activity. Despite this observation, the synthetic peptides do not provide insights into the efficiency

of biosynthesis, the intracellular stability, and the export of such molecules from yeast. Nevertheless, it seems clear that the STE3 receptor can be triggered by lipopeptides containing very small portions of the peptide sequence of mature **a**-factor.

It has been previously demonstrated by two-dimensional NMR methods that the structure of synthetic **a**-factor is apparently not effected by the presence of a farnesyl on the terminal cysteine in solution (15). However, it might be possible that the **a**-factor assumes a specific structural orientation within the lipid bilayer as it interacts with the STE3 protein. Recent studies involving the interaction of **a**-factor with membranes vesicles in vitro suggest that the the farnesyl group bestows a particular physical interaction of **a**-factor with membranes (10). Specifically, studies on the intrinsic fluorescence of the tryptophan residue at position 8 of the **a**-factor indicated that isoprenylation causes this residue to be more deeply imbedded into bilayers. Furthermore, truncation of the N-terminal amino acids of **a**-factor reduced the penetration of the peptide into membranes as measured by tryptophan fluorescence. This reduced penetration correlates with the lower bioactivity of N-terminal truncations of **a**-factor.

The results of our studies indicate that the interaction of the **a**-factor with its receptor is affected by amino acid substitutions and truncations. The cumulative results from synthetic peptide analysis and molecular mutagenesis experiments suggest that removal or substitution of specific residues within the pheromone exhibit deleterious effects on bioactivity. These analyses are not intended to represent a complete examination of amino acid tolerance or deletion analysis to reveal all possible positional effects involved in **a**-factor function. Rather, these analyses serve as an initial inquiry into the nature of the contribution made by the peptidyl portion of the pheromone toward its bioactivity. We have not shown directly in our studies that the mutant **a**-factors identified are secreted; however, preliminary studies (6,30) have reported that substitution mutations in each position of the mature **a**-factor, with the exception of position 12 (Cys), are successfully secreted.

Therefore, we believe that it is reasonable to assume that mutations within amino acids one to eleven of the translated, mature **a**-factor result in a loss of bioactivity due to altered pheromone structure, leading to non-optimal peptide-receptor recognition. We are currently attempting to synthesize several of the non-active **a**-factor peptides (Table 2) in order to determine whether these conclusions are valid.

Glycine residues in proteins are often found in regions of protein structure which correspond to turns or bends. Our screen for substitution mutations which affect the bioactivity of **a**-factor also identified this residue as a putatively important site for the ability of the peptide to induce growth arrest. In a separate study, we have identified an analog of the **a**-factor containing a substitution of glycine with D-alanine which exhibits stereospecific superactivity (Part IV of this thesis). In contrast, L-alanine at this same position is less active than the wild-type pheromone. These combined data suggest that the glycine in position 5 profoundly affects **a**-factor function and may serve a role in determination of the biologically active conformation of the peptide. Single substitution mutations which resulted in colonies that lacked **a**-factor bioactivity were also identified at the codons for tryptophan and aspartic acid. Therefore, these residues may also play a significant role in the structure of the pheromone or recognition by its receptor. It should be noted that our screen, though not exhaustive, did not identify mutants in residues 1-4 of the **a**-factor (Fig. 3). This may reflect a tolerance for changes in these residues, as further indicated by the truncated synthetic analogs, which were active despite removal of these residues entirely.

Previous studies of Kuchler et al. have pointed to a region of amino acid similarity (approximately forty residues) shared between the **a**-factor receptor, STE3, and the **a**-factor transporter, STE6, which may represent a putative pheromone recognition domain (19). The results of our structure-function studies on the relationship between synthetic **a**-factor analogs and the STE3 receptor could serve as the basis for a structure-function

analysis of such peptides with the STE6 transporter. For example, truncated **a**-factor mutants, designed on the basis of our studies, could be generated genetically by expressing mutated *MFa1* gene constructs in yeast cells. It could then be determined whether or not these peptides were secreted via the STE6 protein.

Goldstein and Brown (14) have suggested that prenylated proteins may directly interact with specific membrane proteins and might serve a regulatory role in the pathway for cholesterol biosynthesis. The key regulatory protein in this pathway is the membrane-bound enzyme HMG-CoA reductase. Cellular depletion of isoprenoid containing proteins results in an elevated level of HMG-CoA expression, thereby suggesting a prenyl-dependent feedback inhibition mechanism may be involved in HMG-CoA regulation (14). It is interesting to note that the receptor for the **a**-factor and HMG-CoA reductase share a similar structural organization, since both are seven transmembrane domain containing proteins. In this sense, the interaction of **a**-factor with its receptor may serve as a model for our future understanding of how prenylated proteins may interact with other cellular targets, perhaps in a regulatory role, as in the case of HMG-CoA reductase, or as previously described for RAS-adenylyl cyclase interaction (22).

The manner in which isoprene modification of proteins and peptides affects their substrate specificity and targeting will play an important role in our understanding of lipid-protein interactions (25). The possibility of specific receptors or recognition domains within receptors for lipids has been proposed (25,38). The **a**-factor receptor is a good candidate for the presence of structurally significant regions by which such interactions are mediated. Mutational analysis of STE3, in conjunction with the use of synthetic **a**-factor and its analogs in the development of binding assays for **a**-factor and in continued bioactivity analyses, will facilitate an understanding of this recognition process. The results presented here begin to address these concerns at a structural level and provide a foundation for future, more-detailed molecular analyses of **a**-factor structure and lipopeptide-target interactions.

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PART VI

SUMMARY

CHAPTER I

THE **a**-FACTOR AS A MODEL FOR THE INTERACTION OF A PRENYLATED PROTEIN WITH ITS CELLULAR TARGETS: FUTURE DIRECTIONS

The cumulative research into the biosynthesis, export, and biological activity of the *Saccharomyces cerevisiae* **a**-factor mating pheromone serves as an example of the manner by which investigation into basic biological processes may yield significant information, which in turn can contribute to future practical applications. In this case, an understanding of the mechanisms by which a mating hormone of a simple single-celled microbe is produced, secreted, and functions has provided insights into possibilites for anti-cancer drug design and hormone-receptor signal transmission. The research presented in this dissertation represents an inquiry into structural aspects of **a**-factor function and provides a foundation for future experimentation in the rapidly expanding field of prenylated protein-target protein interaction. This final chapter summarizes the major conclusions of this dissertation in the context of their implications for the future direction of **a**-factor-related research.

The substrate specificity of the **a**-factor transporter STE6

One of the most intriguing discoveries related to **a**-factor was the identification of a requirement for the yeast *STE6* gene product for its secretion from **a**-cells (11,17,27). The eventual characterization of this protein as a member of the ATP-binding cassette (ABC) family of transport proteins led to much speculation on its role as a molecular pump with a specificity for a peptide substrate (7,11,17). The cellular rejection of chemotherapy drugs in cancer patients has been found to correlate with the incidence of a specific member

of this protein family, the human multidrug resistance (MDR) P-glycoprotein (9). On the basis of amino acid identity, hydropathy analysis, and organization of structural domains, the yeast STE6 protein appears to be homologous to the MDR P-glycoprotein (11,17). Furthermore, expression of the mammalian MDR1 gene in yeast is capable of functionally complementing a *Δste6* mutant strain (which has had the chromosomal copy of its *STE6* gene deleted) and thereby restoring the ability of this strain to secrete **a**-factor and mate (21). These data strongly suggest that **a**-factor is a substrate for the MDR-family of transport proteins. Since the native biological function of MDR proteins and characterization of their natural substrates has eluded investigators for many years, the identification of **a**-factor as a specific, natural substrate for this class of molecules invites further experimentation into its structural specificity as a target for export via this mechanism.

Upon initial examination, the obvious structural characteristic shared between **a**-factor and the vast majority of chemotherapeutic agents exported by MDR-containing cells is their overall hydrophobic nature (4). In the case of **a**-factor, this is generally accounted for by its posttranslational modification with both a farnesyl isoprenoid moiety and carboxyl terminal methyl group. Studies have shown that blocking the farnesylation of **a**-factor prevents its membrane localization and subsequent export (15,22,24). In light of this, it is possible that farnesylation of the **a**-factor contributes to either the direct or indirect recognition of the peptide by STE6. In Part III of this dissertation, we examined the consequences on **a**-factor export of altering the isoprene-targeting motif, or -CAAX box, at the C-terminus of **a**-factor from that encoding farnesyl addition to a sequence for geranyl, geranyl modification. The results of this experiment indicated that a geranyl, geranyl group can functionally substitute for farnesyl in mediating **a**-factor export and that this export remains dependent on STE6. This implies that STE6 does not exhibit a strict structural

specificity for the farnesyl moiety on the **a**-factor and that additional factors must be involved in the recognition of this pheromone as an export substrate. Furthermore, although the difference between a farnesyl and geranyl, geranyl group is not dramatic, the functional consequences of alternative isoprenylation with either group remains to be elucidated in the case of many cellular proteins (16). The lack of specificity exhibited by STE6 in the recognition of **a**-factors with either modification may represent an inherent broad substrate specificity, typical of MDR-like proteins, or may be a consequence of the functional equivalence of differential isoprenoid modification.

Alternatively, it is possible that the recognition of **a**-factor by the STE6 protein is dependent upon a specific amino acid sequence within the peptide. In this regard, an extensive substitutional analysis of amino acids within the mature **a**-factor peptide sequence has recently revealed that specific codon replacements in this region of the peptide do not appear to have a dramatic effect on export (18). This does not, however, preclude the possibility that N-terminal amino acid sequences within the precursor of **a**-factor mediate peptide recognition by STE6, and that subsequent N-terminal proteolysis to yield the mature pheromone acts as a molecular trigger to release the pheromone (23). Similar substitution analyses within the N-terminus of the precursor may provide further information to address this possibility. It would be equally interesting to identify amino acid changes in **a**-factor that would yield peptides which could act as in vivo antagonists of STE6-mediated export. We have already described a system for the in vitro mutagenesis of **a**-factor, expression, and analysis of its bioactivity in Part II of this dissertation and have applied this system in the random mutagenesis of the mature **a**-factor peptide sequence in Part V. An equivalent use of this technique could be used to create a pool of randomly mutagenized **a**-factor genes containing substitutions in the N-terminal region of the pheromone precursor. Subsequent overexpression, via high-copy plasmids, of this peptide pool in a wild-type **a**-strain (naturally producing chromosomally encoded **a**-factor) could

lead to the identification of intracellular competitors for **a**-factor secretion. An examination of such transformant colonies may disclose that they phenotypically exhibit decreased - or a total lack - of **a**-factor bioactivity in a physiological screen for growth-arrest inducibility. The identification of putative in vivo competitors of **a**-factor export would contribute to our knowledge of MDR protein substrate specificity and could serve as the basis for the design of effective inhibitors to reverse MDR-mediated drug translocation.

The role of farnesylation in directing **a**-factor secretion

As previously mentioned, isoprenylation of **a**-factor is a prerequisite for its export and biological activity (15,22,23,24). However, it is not clear if the presence of an isoprene group on **a**-factor acts simply to localize the peptide to the plasma membrane in a non-specific manner or if it is necessary for the direct interaction of **a**-factor with the STE6 transporter. Several models have been proposed for the interaction of isoprenylated molecules with their cellular targets (23). One hypothesis centers on the presence of intracellular "receptor" proteins which specifically recognize the lipid moiety on isoprenylated proteins by means analogous to a ligand-binding "pocket". The STE6 protein might be considered a candidate for this type of receptor protein. However, it remains to be shown that **a**-factor directly interacts with the STE6 protein in an isoprene-dependent fashion.

Recently, a genetic system by which protein-protein interactions can be detected has been described and successfully implemented in yeast (3). Briefly, this entails the construction of two separate gene fusions: one between a specific protein of interest and the DNA-binding domain of yeast transcription factor GAL4, and the other between the other protein of interest and the transcriptional activation domain of GAL4. For these separate domains of GAL4 to form a functional complex, protein-protein interaction between the

two target proteins of interest must occur. GAL4 activity is easily detected by a chromosomally integrated reporter gene near the site of GAL4 transcriptional activation. This elegant genetic system could be used to elucidate the dependence of **a**-factor on isoprenylation for interaction with STE6 by constructing gene fusions of these respective proteins with the appropriate domains of GAL4. A specific requirement for isoprenylation could be ascertained by generating a fusion between a GAL4 domain and the gene encoding the **a**-factor mutant containing a substitution of serine for cysteine in the -CAAX box (which does not get isoprenylated), previously constructed in Part II of this dissertation. Alternatively, co-immunoprecipitation or crosslinking studies on in vivo labeled STE6 and **a**-factor could lead to experiments which would clarify any putative peptide-protein interactions biochemically, providing the availability of appropriate antibodies.

The export of **a**-factor via STE6 clearly represents a novel secretory mechanism for cellular factors (12). In the classical secretory pathway, a hydrophobic signal or leader amino acid sequence at the N-terminus of a secreted protein serves to direct the nascent protein into the lumen of the endoplasmic reticulum for subsequent passage to the Golgi, secretory vesicles, and eventually out of the cell (25). In contrast, it is not yet apparent if either the isoprenoid group of **a**-factor or an unidentified amino acid sequence is responsible for directing STE6-mediated secretion of the pheromone since the **a**-factor precursor lacks any consensus signal peptide sequence (2). It is intriguing to consider whether one of these secretory paths takes precedent over the other within the cell. To address this question, we have constructed a gene fusion between the DNA encoding the α-factor signal sequence and the portion of the **a**-factor gene encoding the mature **a**-factor plus the additional three C-terminal amino acids contributing to the -CAAX box. This chimeric mating pheromone, therefore, contains both a signal for translocation into the endoplasmic reticulum into the classical secretory pathway, and also contains an intact

isoprenylation targeting motif for putative plasma membrane association and subsequent interaction with STE6. An analysis of the secretion, intracellular localization, and biological activity of this α/**a** hybrid mating pheromone construct should provide us with interesting information on the varied influence of these "molecular addresses" on intracellular protein transport.

The **a**-factor and the STE3 protein: a model for lipopeptide ligand-receptor interaction

It has recently been shown that the isoprenylation of RAS apparently enhances its ability to directly interact with and stimulate adenylyl cyclase production of cyclic AMP within cells (13). Furthermore, this protein-protein interaction and isoprene-dependent stimulation has been demonstrated to take place in the absence of membranes. The implications of this observation are profound when considering the role an isoprene group may have in the presentation of a peptide or protein to its cellular targets. We have previously shown that the presence of the farnesyl group on **a**-factor exhibits a critical influence on the biological activity of the pheromone (14). This activity is mediated by binding of the pheromone to its receptor, the STE3 protein, on cells of the opposite mating type (5). In this sense, the interaction of **a**-factor with its receptor represents the only known system in which alterations within the structure of a isoprenylated ligand may be analyzed for their effects on recognition by a target receptor coupled with a simple assay for bioactivity. In Parts IV and V of this dissertation, we have attempted to demonstrate the functional consequences of structural alterations within the amino acid sequence of **a**-factor. Specifically, we have identified a stereospecific superactive **a**-factor analog, [D-Ala^5] **a**-factor; we have studied the biological activity of a series of **a**-factor peptides that have been serially truncated from the N-terminus of the mature peptide; and we have performed a mutational analysis in which amino acid substitutions within the pheromone were coupled with a physiological screen to identify residues putatively important to the

bioactivity of the pheromone. These experiments represent the first structure-function analysis of the peptidyl portion of **a**-factor and complement our previous studies on the biological significance of C-terminal modifications on **a**-factor (14). They also contribute to the growing base of information on the manner in which prenylated proteins interact with their effectors.

The use of synthetic peptide analogs has greatly enhanced our ability to understand the structural specificity of both α-factor and **a**-factor as they are recognized by their respective receptors (14,19,20). However, clearly, the lack of an available binding assay for **a**-factor with STE3 has impeded progress on this front. Work is in progress to develop such an assay, although the hydrophobic nature of **a**-factor presents a variety of complications to limiting the degree of background, non-specific binding. Assuming such an assay will be developed, however, the continued use of structural analogs of **a**-factor will greatly facilitate our understanding of the affect of both the peptidyl and C-terminal modifications on ligand binding.

Mutational analysis of the STE3 receptor also serves as an alternative route in defining the structural specificity of individual residues and putative domains within this protein. We have initiated a collaboration with the laboratory of Dr. George Sprague at the University of Oregon, where specific mutations within the **a**-factor receptor, which have apparent effects on signal transduction, have been generated. The use of these receptor mutants, in conjunction with our synthetic analogs, will provide us with additional information on how the receptor may serve to transmit ligand-induced signals via coupling to G proteins. In addition, with the advent of an **a**-factor binding assay, we may be able to distinguish between structural alterations that affect ligand-binding from those that directly influence receptor-mediated signalling.

The identification of antagonists of **a**-factor bioactivity would also represent a significant contribution and serve as an important tool in the elucidation of ligand-receptor recognition mechanisms. The strongest antagonist of **a**-factor bioactivity identified to date was a palmitoylated synthetic **a**-factor pentadecapeptide, YIIKGVFWDPAC (palmitoyl)VIA (1). However, this acylated peptide only exhibits an effect on **a**-factor bioactivity at high peptide amounts. Interestingly, this peptide is not carboxyl methylated and, perhaps, the presence of a non-isoprene lipid moiety suggests a lipid preference in its presentation to, or recognition by, the receptor. In contrast to the rationale design of synthetic peptide antagonists, another approach to identify possible antagonists once again involves the random mutagenesis of the peptide portion of **a**-factor, coupled with a physiological screen for antagonists of **a**-factor bioactivity. The mutagenesis system established in Part II of this dissertation, with the modifications employed in Part V, would suffice to generate a pool of randomly mutagenized **a**-factor gene fragments that, when appropriately overexpressed (on high-copy plasmids), could serve as a source for the identification of novel antagonists. A two-step screening process would successfully identify putative antagonists: mutagenized **a**-factor expressing colonies would be initially assayed for the production of inactive pheromones (by halo assay), followed by a secondary screen in which these colonies would be patched next to spots of synthetic **a**-factor (at a concentration just barely necessary to induce growth arrest). Patches expressing putative **a**-factor antagonists would interfere with the ability of the synthetic **a**-factor to cause growth arrest on a lawn of α-cells and would allow for growth of α-cells near the border of the halo, normally formed by a spot of synthetic **a**-factor. The successful isolation of a potent **a**-factor antagonist would facilitate binding studies and possibly provide additional structural information that could be used in addressing the functional role of isoprenylated protein-target interactions.

Implications of this work

A historical perspective of **a**-factor related research leads one to conclude that through continued analysis of the basic mechanisms governing the biological processes associated with **a**-factor function additional insights into varied areas of cell biology will be discovered. Furthermore, the application of this information towards drug design and disease prevention is already beyond the conceptual stages and is now a pharmacological reality. A genetic screen in yeast has already been established and is currently being applied in the identification of putative farnesyltransferase inhibitors (6). Very recently, two highly encouraging studies report the discovery of -CAAX motif based farnesyltransferase inhibitors which appear to effectively reverse the transformation of cancerous cells induced by activated RAS in cell culture (8,10). The identification of these compounds will highlight the debate regarding in vivo crossprenylation of proteins, as well. In this regard, a study just published supports our observations on in vivo crossprenylation of **a**-factor (Part III) and substantiates the existence of this phenomenon (26). However, in this recently published study, it appears as though there may be a fortuitous difference in the ability of oncogenic RAS to be crossprenylated in vivo vs. normal RAS, which may allow for successful inhibition of only the cancerous form of the protein at specific doses of an anti-farnesyltransferase agent.

In addition to the design of farnesyltransferase inhibitors, the investigation of **a**-factor biogenesis and activity has prompted studies into a novel mechanism of peptide export from cells (12). This area of **a**-factor related research may provide its own insights into cancer therapy through gaining a better understanding of how **a**-factor serves as the only known natural substrate for the MDR-family of transport proteins. Finally, investigation into the functional consequences of protein prenylation from a structural perspective has, and will continue to benefit from studies on **a**-factor bioactivity. The practical insights derived from the basic analysis of this mating pheromone from a simple yeast justifies our commitment to basic research and the underlying opportunities it affords us in the future.

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VITA

Guy Alexander Caldwell was born in Upper Montclair, New Jersey on December 15, 1962. He grew up in the New York City metropolitan area and graduated from James Caldwell High School in his hometown of West Caldwell, New Jersey in 1981. Following high school, Guy attended Washington and Lee University in Lexington, Virginia from where he graduated with honors in 1986 while obtaining a Bachelor of Sciences degree with a major in Biology. While at Washington and Lee, he was twice the recipient of a Robert E. Lee Research Scholarship and served as the founder and editor of *The Washington and Lee Journal of Science* .

Guy initially entered The University of Tennessee, Knoxville as a student in the Graduate Master's Degree Program in Biotechnology in the Fall of 1986. During the course of his time in this program, he served as a Graduate Teaching Assistant and worked as an intern in molecular biology at the Burroughs Wellcome Company in Research Triangle Park, North Carolina. As an aside to his graduate studies, Guy also co-authored an introductory laboratory textbook in molecular biology, *Biotechnology: A Laboratory Course* , which has sold several thousand copies while being marketed world-wide by Academic Press, Inc. Guy taught and supervised the graduate Biotechnology teaching lab at The University of Tennessee from 1987 to 1990. In 1988, he switched to the Ph.D. Program in Cellular, Molecular, and Developmental Biology and began his dissertation research in the laboratory of Dr. Jeffrey M. Becker. He was a five-time recipient of the Science Alliance Research Achievement Award, having received this honor in 1988, 1990, 1991, 1992, and 1993. In December 1993, he received a Doctor of Philosophy degree in Cellular, Molecular, and Developmental Biology. Dr. Caldwell has accepted a temporary post-doctoral associateship in the laboratory of Dr. Becker and will continue his research there until he leaves The University of Tennessee for a permanent post-doctoral position.