To the Graduate Council:

I am submitting herewith a thesis written by John Robert Wilgus entitled " Structure-function Studies of the *Saccharomyces cerevisiae* **a**-factor Lipopeptide Mating Pheromone." I have examined the final copy of this thesis for form and content and recommended that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Sciences.

Jeffrey M. Becker Ph. D, Major Professor

We have read this thesis
and recommend its acceptance:

W. S. Biggs

Robert N Moore

Cynthia B. Peterson

Accepted for the Council:

Associate Vice Chancellor
and Dean of the Graduate
School

STRUCTURE-FUNCTION STUDIES OF THE *Saccharomyces cerevisiae* **a**-FACTOR LIPOPEPTIDE MATING PHEROMONE

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

John Robert Wilgus

May 1997

Dedication

This thesis is dedicated to my wife Catherine Wilgus. Her love, encouragement, support, and patience have made my completion of this degree possible. My life was forever changed by her entering it. I would also like to thank my parents, James and Peggy Wilgus. Their support, both financially and emotionally, throughout the successes and failures in my college career gave me the courage to further pursue my education. Last, but not least, I would like to thank all my family and friends who have always supported and helped me. To all of you I give my sincerest thanks and my deepest love. Thank you.

Acknowledgments

I would like to thank my major professor, Dr. Jeff Becker, for his encouragement and wisdom. Without his professional, personal guidance, and his belief in my abilities, my endeavors would not have made it to this endpoint. To Dr. Fred Naider, I greatly appreciate the peptides he always provided and for all of his assistance. I would also like to extend my most heartfelt appreciation to all the members of the Becker lab. Everyone in the lab at one point or another has helped me in some way, either large or small, in my research. I would particularly like to thank David Barnes and Angus Dawe for being not only a idea wall to bounce things off of, but being two of the closest friends I've ever had. I would also like to thank Lub for his support and interest in my endeavors as well as a meal ticket every fifteenth. Last, but not least, I would like to thank Greg Anderson with helping me out with all the statistical analysis.

Abstract

Mating in *Saccharomyces cerevisiae* is initiated by extracellular peptides, known as mating pheromones. The mating factors are recognized by membrane-bound receptors which are similar to receptors in mammalian cells. The pheromone system of the *S. cerevisiae* can be used as a model to understand the mode of action of peptide hormones in more complex mammalian systems. The purpose of this thesis is to provide information regarding the yeast lipopeptide pheromone, **a**-factor. The first part of this project, described in Chapter 2, was the development of an assay which could be used to define the binding of **a**-factor to its receptor. The assay, which made use of membrane preparations in place of whole cells, was able to show that a larger percentage of the radioactive **a**-factor pheromone did bind to membranes which contained the **a**-factor receptor (Ste3p) when compared to membranes which lacked the receptor. However, further experiments are needed to more clearly define the pheromone/receptor interaction. The second part of this project, described in Chapter 3, was the examination of the role of the farnesyl group in **a**-factor bioactivity, particularly mating restoration. This examination made use of **a**-factors with altered farnesyl groups that contained the novel lipid substitutions of the 3-methyl moiety with bulkier additions. The study suggested that there is a specific preference for a particular relationship between the membrane and the ligand with regard to bioactivity, and that

this preference is mediated via the farnesyl group. Thus, the farnesyl group may also play a role in presentation of the peptide portion to the receptor in addition to its role in membrane localization.

TABLE OF CONTENTS

CHAPTER		PAGE
1	INTERCELLULAR COMMUNICATION BETWEEN THE TWO MATING TYPES of *Saccharomyces cerevisiae*	1
2	DEVELOPMENT OF AN ASSAY TO MEASURE THE BINDING OF **a**-FACTOR TO ITS RECEPTOR.....	8
	Introduction.....	8
	Materials and Methods.....	13
	Strains.....	13
	Iodination of **a**-factor.....	13
	Membrane Preparation.....	15
	Binding Assay.....	16
	Statistical Analysis.....	17
	Results.....	18
	First group of binding assays.....	18
	Second group of binding assays.....	24
	Discussion.....	33
3	COMPARISON AND ANALYSIS OF VARIOUS FARNESYL ANALOGS OF **a**-FACTOR VS. **a**-FACTOR IN AN AQUEOUS MATING ASSAY.....	37

Introduction..... 37
Materials and Methods..... 43
Yeast Strains and Genotypes 43
Mating Induction Assay..... 43
Synthesis of Modified **a**-factor
Analogs..... 43
Statistical Analysis..... 44
Results..... 44
Mating induction assay
(0-3000nM)..... 44
Mating induction assay
(0-200nM)..... 44
Mating induction assay
(**a**-factor vs. farnesyl
analogs (10nM))..... 44
Mating induction assay
(**a**-factor vs. farnesyl
analogs (0-50 nM))..... 51
Discussion..... 56
REFERENCES..... 64
VITA..... 75

LIST OF TABLES

TABLE		PAGE
1.	First group of binding assays	20
2.	Statistical analysis of first group of binding assay data (experimental similarity)	21
3.	Statistical analysis of first group of binding assay data (experimental difference)	22
4.	Statistical analysis of first group of binding assay data (complete analysis)	23
5.	Second group of binding assays	28
6.	Binding assays on heat-inactivated membranes	29
7.	Statistical analysis of second group of binding assay data	30
8.	Statistical analysis of **a**-factor vs. modified **a**-factor analogs	54
9.	Mating induction assay with a-factor and farnesyl analogs covering the concentration range from 0 to 50 nM	55

LIST OF FIGURES

FIGURE PAGE

1. Two opposite *S.cerevisiae* mating types secrete pheromones which interact with their respective receptors as a requirement for mating..... 2
2. Graph of first group of binding assays..... 27
3. Graph of second group of binding assays..... 33
4. **a**-factor maturation..... 42
5. Structure of **a**-factor and lipopeptide analogs used in this study..... 48
6. Mating induction assay (0-3000 nM)..... 50
7. Mating induction assay (0-200 nM)..... 52
8. Mating induction assay (**a**-factor vs. farnesyl analogs (10nM))..... 55
9. Mating induction assay (**a**-factor vs. farnesyl analogs (0-50nM)..... 59

CHAPTER 1

INTERCELLULAR COMMUNICATION BETWEEN THE TWO MATING TYPES OF *Saccharomyces cerevisiae*

Saccharomyces cerevisiae, commonly referred to as baker's yeast, has served as a model organism for the study of cellular processes of eukaryotic cells (Botstein and Fink 1988). The combination of the ease with which yeast is manipulated, in conjunction with the vast amount of genetic and physiological information available, makes it an ideal organism for experimental investigation of the molecular mechanisms which underlie cellular function. This study concentrates on various aspects of one of the most interesting processes carried out by cells: the intercellular signal transmission.

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 (Figure 1). Analysis of the molecular events which surround 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 (Herskowitz 1988; Kurjan 1992; Sprague and Thorner 1992).

The conjunction 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-

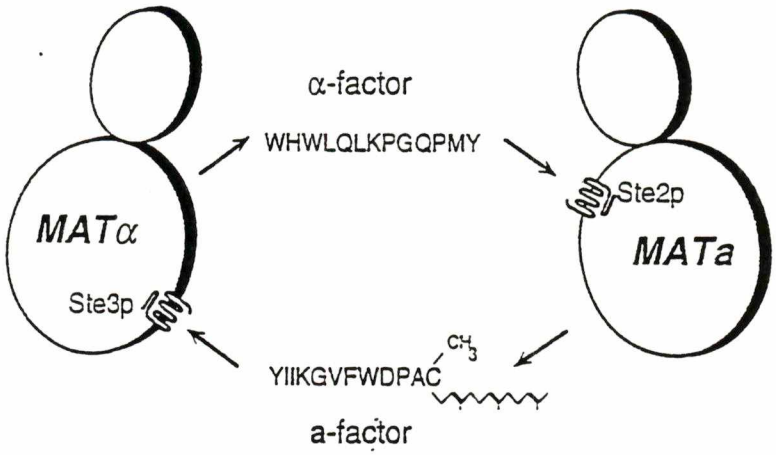


Figure 1. Two opposite *S. cerevisiae* mating types (*MATα* and *MATa*) secrete pheromones (α-factor and **a**-factor) which interact with their respective receptors (Ste2p = α-factor receptor, Ste3p = **a**-factor receptor) as a requirement for mating.

cells (*MAT*α) (Herskowitz 1988; Kurjan 1992; Sprague and Thorner 1992). These peptides are recognized by receptor proteins, which are expressed on the surface of cells of the opposing mating type (Burkholder and Hartwell 1985; Hagen, McCaffrey, and Sprague 1986). Following recognition of the peptide by its receptor, there is an induction of gene expression in 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 morphologic alteration (termed "Shmooing") (Kurjan 1992). These cellular responses lead to the subsequent aggregation of cells and their subsequent fusion of cytoplasm and nucleus. Ultimately the formation of diploid zygotes occurs.

Although α-factor and **a**-factor appear to be functionally equivalent, these two peptides are very different in their structure, biosynthesis, and mode of export from cells. A number of studies have been performed on the biosynthesis and secretion of α-factor which has greatly contributed to the dissection of the yeast secretory pathway (Scheckman 1985). The α-factor is encoded by two similar and functionally redundant genes, *MF*α*1* and *MF*α*2*. Each of these genes encode primary polypeptide precursors containing the mature, secreted α-factor. The *MF*α*1* gene encodes a hydrophobic leader sequence, and four identical α-factor repeat sequences (Kurjan 1992). The *MF*α*2* gene encodes a hydrophobic leader sequence and two α-factor repeats (Kurjan 1992). 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, **a**-factor is the product of two functionally redundant, nearly identical genes, *MFa1* and *MFa2*, encoding a 36 or 38 amino acid chain, respectively. These gene products lack a consensus signal sequence for directing its secretion through the classical secretory pathway (Endoplasmic Reticulum, Golgi, Secretory Vesicles) (Brake *et al.* 1985; Michaelis and Herskowitz 1988). These precursors are proteolytically cleaved and post-translationally modified to yield the mature **a**-factor [YIIKGVFWDPAC(farnesyl)-OCH3] from MF**a**1 and [YIIKGIFWDPAC(farnesyl)-OCH3] from MF**a**2 (Anderegg *et al.* 1988; Marcus *et al.* 1990; Michaelis *et al.* 1992; Schafer and Rine 1992). Furthermore, mutants which block secretion at various stages of the secretory pathway do not affect the production of **a**-factor (Sterne 1989; Sterne and Thorner 1986). These observations suggested that the export of **a**-factor occurs by a novel mechanism involving a MDR-like efflux pump, which is different than that which is used by α-factor.

Isoprene modifications are found not only in the mating pheromones of fungi, but also in eukaryotic cells where a significant number of proteins, including the monomeric and heterotrimeric G proteins, are isoprenylated at the carboxy-terminal cysteine in a manner similar to **a**-factor (Caldwell 1995).

One protein of particular interest is the product of the ras oncogene. Farnesylation of *RAS* was found to be required for its proper membrane localization and transforming activity (Kato *et al.* 1992; Schafer and Rine 1992; Schafer *et al.* 1989; Schafer *et al.* 1990). Similarly, the export and biological activity of **a**-factor was found to be dependent on post-translational modification (Marcus *et al.* 1991; Marcus *et al.* 1990; Schafer *et al.* 1989; Schafer and Rine 1992; Schafer *et al.* 1990). The similarities between *RAS* and **a**-factor post-translational focused attention on the **a**-factor as a model for understanding the molecular mechanism involved in *RAS* membrane targeting, as well as for the interaction of all isoprenylated proteins with their cellular targets.

One known interaction of **a**-factor is with its receptor protein Ste3p (Hagen *et al.* 1986). Evidence that Ste3p was the **a**-factor receptor came from three findings. First, wild-type Ste3p is required for response to the pheromone: mutants which contain one of five different mutations to *STE3* are unresponsive to **a**-factor. Second the hybrid products encoded by fusion of the *STE3-lacZ* gene fractionated to the particulate fractions of yeast cell extracts, which suggested the Ste3p is a membrane protein. Third, the DNA sequence of *STE3*, encoded a protein of 470 amino acid residues that contains seven distinct hydrophobic segments of sufficient length to span a lipid bilayer (Hagen *et al.* 1986; Nakayama *et al.* 1985). 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 (Dohlman *et al.* 1991). The synthesis of α-factor analogs, development of ligand binding assays, and a biological activity assays for α-factor have greatly facilitated the analysis of structure-activity relationships between α-factor and its receptor, Ste2p (Blumer *et al.* 1988; Eriotou-Bargiota *et al.* 1992; Naider and Becker 1986; Naider *et al.* 1989; Raths *et al.* 1988). Similar analyses on **a**-factor have not been as extensive. Bioactivity studies, however, have shown that post-translational modification of **a**-factor is a prerequisite for its ability to induce growth arrest and mating (Marcus *et al.* 1991). The above study used four different assays: the halo assay, an agar diffusion assay which measures pheromone response based on growth arrest of α cells; the shmoo assay, an assay which measures pheromone response by morphological alteration (shmooing) and growth arrest of α cells; the *FUS1-lacZ* induction assay, which measures pheromone response by induction of a *FUS1-lacZ* gene; and the mating restoration assay, which measures the ability of exogenously added pheromone to induce mating and diploid formation between cells of opposite mating types which are unable to mate in the absence of pheromone (Marcus *et al.* 1991). These results imply that farnesylation and carboxyl methylation serve a critical role in either the direct interaction of the **a**-factor with Ste3p or interaction with other cellular components involved in

presentation of the pheromone to its receptor. The manner by which **a**-factor acts to trigger cellular responses via binding to the Ste3p receptor may serve as a model for studying the interactions between isoprene-modified proteins and their cellular targets, because similar biophysical interactions between the isoprene group and its membrane target may occur in these interactions.

The purpose of this thesis was two-fold. The first goal of this project was the development of an assay which could measure the binding of **a**-factor to its receptor. This assay could provide information on the interaction between **a**-factor and its receptor. The second goal of this project was the examination of the role of the farnesyl group in **a**-factor through its effect on mating. The further examination could provide insight as to whether the farnesyl moiety of **a**-factor has a greater role than just membrane association of the lipopeptide pheromone.

CHAPTER 2

DEVELOPMENT OF AN ASSAY TO MEASURE THE BINDING OF **a**-FACTOR TO ITS RECEPTOR

Introduction

Studies involving the structure-function relationships of **a**-factor have been carried out extensively in the laboratories of Jeff Becker, University of Tennessee, and Fred Naider, City University of New York. These studies included the synthesis of a number of **a**-factor analogs, elucidation of the bioactivities of these analogs, the generation of mutant **a**-factors and studies of their expression and bioactivities. These studies have contributed to our knowledge of substrate specificity in protein prenylation, the discovery of a novel means of peptide export by cells, and the elucidation of molecular determinants of bioactivity (Caldwell *et al.* 1993, 1994a, 1994b; Zhang *et al.* 1996).

Synthetic **a**-factor and analogs of **a**-factor have proven to be excellent tools for investigating the consequences of hydrophobic modification on lipopeptide bioactivity (Marcus *et al.* 1991; Caldwell *et al.* 1994a, b). Detailed structure-activity studies involving both the S-farnesylation and carboxy-terminal methyl esterification have provided insight to the role of these modifications to the biological activity of the pheromone. Marcus *et al.* (1991) have shown that synthetic **a**-factor analogs that lack

either the carboxyl methyl or farnesyl groups display a 100- and 1000-fold reduction in bioactivity, respectively. When both modifications are absent, the resulting analog is 40,000-fold less active than the naturally occurring **a**-factor pheromone. These results suggested that the post-translational modifications of **a**-factor have profound effects on the ability of this peptide to interact with its receptor, or the presentation of the peptide to its receptor.

Further experiments, however, have revealed that the farnesyl group per sé is not required for biological activity (Marcus *et al.* 1991). In this study Marcus *et al.* (1991) showed that incremental increases in prenyl group number on **a**-factor, from C_5 (prenyl) to C_{10} (geranyl) to C_{15} (farnesyl), corresponded to an increase in bioactivity with each rise in fatty acid chain length as judged by both growth arrest and morphology change assays (Marcus *et al.* 1991). The replacement of the farnesyl group with a prenyl group resulted in a pheromone which was 15- to 70-fold less active than **a**-factor, while a pheromone with the replacement of the farnesyl with a geranyl group was only 2- to 8-fold less active (Marcus *et al.* 1991). In addition to the results just discussed, an **a**-factor analog having the farnesyl group replaced by an S-hexadecanyl group was more active than the **a**-factor analog which contained a S-methyl replacement. Thus with two types of modifications, **a**-factor activity increased as the S-alkyl group became bulkier and more hydrophobic. This

study showed that the farnesyl moiety can be substituted by other hydrophobic moieties and still remain active. However, in contrast to the trend seen above, the addition of the geranylgeranyl (C_{20}) to **a**-factor slightly decreased the bioactivity (25% activity as compared to wild-type **a**-factor)(Caldwell *et al.* 1994). The decrease in activity implied that hydrophobicity itself was not the only property conferred to the pheromone by the farnesyl group of **a**-factor.

In order to understand the interaction between **a**-factor and its receptor, a binding assay must be developed. This assay would provide a tool which would allow determination of the binding affinity of **a**-factor to the **a**-factor receptor (encoded by the *STE3* gene) and elucidate the number of receptors per cell. The development of a binding assay could give insight into how the **a**-factor interacts with its receptor by providing a method for describing the relationship between the pheromone and its binding site in its receptor. For example, whether the farnesyl moiety is recognized specifically or perhaps the farnesyl moiety is providing a specific presentation of the peptidyl portion of the pheromone to the receptor. These interactions could be detailed by analysis of **a**-factor analogs or mutated **a**-factor receptors.

The hydrophobic nature of **a**-factor has resulted in difficulty in the development of the **a**-factor binding assay in this laboratory. One previous attempt made use of whole cell suspensions to which (^{125}I) **a**-factor was added (Lovin 1994).

These suspensions were then filtered through glass filters using a vacuum apparatus to remove all the medium from the suspension. The cells adherent to the filters were then counted using a gamma counter. This attempt was unable to show specific binding of radiolabeled **a**-factor to whole cells containing the pheromone receptor in comparison to cells lacking the receptor due to high amounts of radioactivity associating with the cells non-specifically and/or radioactivity associating with the filters. Attempts to lower non-specific adherence by the use of a variety of filters, and by the addition of bovine serum albumin acting as adsorbent of non-specifically bound **a**-factor were not successful in attaining a binding assay.

Another attempt in the development of an **a**-factor binding assay involved oil centrifugation as described by Fasoli and Kerridge (1991). This attempt again used whole cells. A cell suspension which contained the cells and radiolabeled **a**-factor was centrifuged through an inert oil mixture (dibutyl phthalate/dionyl phthalate at 55:45). Following centrifugation the supernatant, which contained any unbound **a**-factor, was removed and the layer containing cells and "bound" **a**-factor was washed with the oil mixture. After a second wash, the oil layer was removed leaving a cell pellet which was then counted by gamma counter. As with the previous attempts, this procedure failed to show specific binding of the radiolabeled peptide to cells containing its receptor; the amount of label associated with cells

containing functional **a**-factor receptor was the same as the amount of **a**-factor associated with receptorless cells within experimental error.

Thissen and Casey, using a radiolabeled prenylated peptide ([^{3}H] REKKFFCAIL (geranyl-geranyl) peptide, which corresponds to the gamma-2 subunit of the heterotrimeric G-protein) as a ligand, identified a protease- and heat-sensitive binding site on microsomal membranes from bovine brain tissue (Thissen and Casey 1993). This putative receptor, which is a binding protein that may serve as the initial component in the processing/trafficking pathway for newly prenylated proteins, appeared to exhibit saturable binding (with a dissociation constant of 34 nM and a B_{max} of 0.95 nmol of [^{3}H] REKKFFCAIL (geranyl-geranyl) peptide bound per mg of protein) that could be specifically competed by farnesylated and geranyl-geranylated peptides, but not by unprenylated peptides and prenyl diphosphates. These results demonstrated that the binding to the microsomal membranes was specific for prenylated peptides, specifically recognizing both geranylgeranylated and farnesylated peptides, and that both the peptide and the isoprenoid were required for binding. Using this assay as a model, I attempted to use cell membrane preparations instead of whole cells in the development of an **a**-factor binding assay.

Materials and Methods

Strains:

The strains used in this chapter were *S. cerevisiae* SY1953 (*MATα* Δste2 Δste3::*URA3* Δmfa1 Δmfa2::fus1-lacZ fus1-*HIS3* ade1 leu2-3 leu2-112 ura3-52) and *S. cerevisiae* SY2035 (SY1953 with *STE3 LEU2* inserted at the *STE3* locus). *STE3* is marked downstream of the open reading frame by *LEU2*. Selection for leu+ and screening for ura- phenotype was used to integrate these constructions at the *STE3* locus in strains carrying a Δste3::*URA3* allele. Thus strain SY1953 contained a deletion of Ste3p, the **a**-factor receptor, and strain SY2035 contained a fully functional Ste3p. The ability of SY2035 to respond to **a**-factor was tested and showed growth arrest to a normal endpoint of 10ng of **a**-factor. It was also determined that SY1953 was unable to respond to **a**-factor even at a concentration of 50 μg/ml. Both of these strains were gifts from Dr. George Sprague at the University of Oregon.

Iodination of **a**-factor:

The **a**-factor peptide was synthesized in the laboratory of Dr. Fred Naider at the College of Staten Island and was provided for experimental purposes. The glass tubes in which the reaction was performed were prepared by first rinsing them with chloroform to remove impurities from the glass. Iodo-Gen™

Iodination reagent (available from Pierce) was dissolved in chloroform to a concentration of 50µg/ml. 200 µl of the above solution was then dispensed in each tube. The tubes were gently dried under a stream of N_2. When the tubes were completely dry they were rinsed with 50mM Tris (pH 7.4) and then desiccated overnight at 4°C. To each of the above reaction tubes 50 µl of **a**-factor (2 mg/ml), 440 µl H_20, and 50µl of 10 X PBS were added. 10 µl of ^{125}I (100mCi/ml, ICN) was added to the reaction tube, which was then swirled for 5 minutes. After 5 minutes 50 µl of NaI solution (2.75mM) was added to the reaction which was then placed in a 30°C shaking bath for 30 minutes to allow the reaction to proceed. Once the reaction was complete the contents were transferred to a siliconized microfuge tube. The radiolabeled **a**-factor was then separated from unreacted **a**-factor and ^{125}I and purified using reversed-phase high-pressure liquid chromatography (HPLC). The column used in the separation was a C3 column. The flow rate in the separation was 1.5 ml/minute. The gradient of acetonitrile (mobile phase) was 48% ACN from 0 to 5 minutes, 48 to 65% from 5 to 17 minutes, and 65 to 80% from 17 to 22 minutes. The labeled fractions were removed from 16 to 19 minutes. Once purified, the radiolabeled **a**-factor samples were then stored at -20°C in a lead pig until used.

Membrane Preparation:

Each strain was inoculated into 1 liter of YEPD (yeast extract, 2%, peptone, 1%, dextrose 2%) and allowed to grow at 30°C with shaking until they reached a density of between $5x10^6$ to $1x10^7$ cells/ml. Once the cells reached this density they were collected by centrifugation for 10 minutes at 5,000 rpm. The cells were then resuspended 1:4 (w/v) in lysis buffer which contained PIPES (Piperazine-N,N'-bis[2-ethanesulfonic acid])(10mM), $MgCl_2$ (1mM), EDTA (Ethylenediaminetetraacetate)(0.1mM), Glycerol (10%), PMSF (phenyl-methyl sulfonyl fluoride)(0.1 mM), Benzamidine (0.1 mM), TPCK (tosyl-phenylalanine chloromethyl ketone)(20μM), and Pepstatin (5μM). An equal volume of glass beads were added and the cells were lysed by vortexing until 99% breakage (as verified by haemocytometer count). The vortexing was for one minute followed by one minute on ice repeated 15-20 times. The cell lysate, which consisted of cell debris, was removed by centrifugation for 15 minutes at 10,000 rpm. The remaining supernatant, which contained the membranes, was centrifuged at 120,000g (Beckman L8-M Ultracentrifuge) for 20 minutes at 4°C to collect the membranes. The collected membranes were then resuspended in lysis buffer. The protein concentration of the membrane preparation was determined by Bradford analysis. The membranes were then resuspended in lysis buffer in stocks of 1mg. protein/ml for storage at -80^0C.

Binding Assay:

The membrane preparations of both SY1953 and SY2035 were resuspended in lysis buffer mentioned above. This buffer was the same buffer as that used in the binding assay with the exception of the addition of 0.05% Triton X-100, which was added to reduce non-specific binding. Membranes containing a 2 μg concentration of protein (2 μl of a 1 mg/ml stock) were added to the binding buffer to a final volume of 48 μl. 2 μl of the radiolabeled **a**-factor was then added (25 nmoles per ml.) to the mixture. For the first group of assays this reaction was done in triplicate for each experiment. For the second group of assays this reaction was done in quintuplet for the experiment. The binding reaction was then allowed to incubate at room temperature for 30 minutes and then cooled on ice. The reaction mixture was then brought up to a volume of 1 ml. to facilitate better centrifugation. Following centrifugation at 150,000g for 20 minutes at 4°C (Beckman TL-100 Ultracentrifuge) the supernatant was removed and the pelleted membranes washed and resuspended in the above buffer. The resuspended membrane pellet was then centrifuged as above. The supernatant was removed and added to the initial supernatant fraction. The pellet was solubilized in 50μl of 2% SDS. Both the supernatant fraction and the solubilized membrane fraction were counted using the gamma counter (LKB Wallac 1272 Clinigamma, Wallac 1400 DSA ver2.4) to determine bound vs. unbound radioactive peptide.

The first group of assays consisted of six experiments, with each reaction in the experiment done in triplicate. These assays were performed over the span of three days (two experiments per day). The second group of assays consisted of one experiment with each reaction repeated in quintuplet. This experiment was performed over two days. In the second group of assays heat-inactivation of membranes were used to determine the amount of radiolabeled peptide that was non-specifically binding to the lipid component of the membranes. Heat inactivation is performed prior to the addition of the radiolabeled peptide by placing the assay tubes containing the membranes in a boiling water bath for 10 minutes, then allowing them to cool to room temperature.

Statistical Analysis:

Statistical analysis consisted of the Students T Test as described by Ott (1993). These tests were performed by computer through the Statistical Analysis System (SAS) on the UNIX mainframe at the University of Tennessee. The T value was calculated using the formula listed below, where y is the mean of the sample, s is the sample variance (which is the difference in measurement from the mean, and n is the sample size.

$$T = \frac{\overline{y}_1 - \overline{y}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

$$P = \frac{N_e}{N}$$

The above formula was used to calculate the P value, where Ne is the number of favorable outcomes, and N is the number of possible outcomes.

Results

The strains used in the binding assay experiments were SY1953 (from which the *STE3* gene has been deleted) and SY2035 (which contains the wild type *STE3* gene). Halo assays were performed on these strains to test for growth arrest when exposed to exogenously added **a**-factor. As expected, the SY1953 strain, which contains no **a**-factor receptor, showed no growth arrest. SY2035, which does contain the **a**-factor receptor, showed growth arrest to the normal endpoint of 10ng of **a**-factor.

Table 1 shows the results of the first group of binding assays performed on membranes. Although the actual number of bound counts and total counts varies between experiments in the group, the percentage of bound counts are consistent. Statistical analysis (Students T Test) (shown in Table 2, 3, and 4) was used to determine two things: First, whether the difference or similarity of experiments within each set of strains was statistically valid; second, whether or not the difference seen in comparing the entire set of values of both strains was statistically valid. In

comparing samples, the smaller T score represents samples that are similar, and a larger T score represents samples that are different (Ott 1993). In order to compare the values of each strain as a whole (all experiments combined), the similarity of the values from each strain had to be determined (as shown by a low T score). The following experiments were compared in both SY1953 and SY2035: Experiment 1 to experiment 2, experiment 1 to experiment 5, experiment 5 to experiment 6, experiment 2 to experiment 4, and experiment 3 to experiment 6 (see Table 2). From this comparison it is evident that these values are similar, as shown by their low T score. The same experiment between both strains was then compared (ie. experiment 1 (SY1953) to experiment 1 (SY2035), experiment 2 (SY1953) to experiment 2 (SY2035), etc.) to show that the percentage values were different (as shown by a high T score)(Table 3). From this comparison it is evident that the percentage of bound counts are different, as shown by high T scores. The P score in this situation can be disregarded due to the low sample size. The percentage of bound counts could now be compared between both strain sets (ie. values from each experiment (SY1953) compared to values from each experiment (SY2035) to determine if the percentages of bound counts were statistically different (See Table 4). This comparison showed that the difference in percentage bound between SY1953 and SY2035 is statistically different (as shown

Table 1: First group of binding assays.

Strain[a]	Expt[b]/Rxn[c]	Unbound counts[d]	Bound counts[e]	Total counts[f]	% bound[g]
1953	1/1	94630	550	95180	0.578
1953	1/2	108456	672	109128	0.618
1953	1/3	97372	526	97898	0.537
1953	2/1	69958	300	70258	0.427
1953	2/2	70295	372	70667	0.525
1953	2/3	87203	425	87629	0.485
1953	3/1	145929	810	146739	0.552
1953	3/2	151451	758	152209	0.498
1953	3/3	147412	778	148190	0.525
1953	4/1	129896	578	130474	0.443
1953	4/2	111812	528	112340	0.470
1953	4/3	119382	618	120000	0.515
1953	5/1	132303	812	133115	0.610
1953	5/2	149668	858	150526	0.570
1953	5/3	146980	798	147778	0.540
1953	6/1	50836	312	51148	0.610
1953	6/2	68571	382	68953	0.554
1953	6/3	59451	360	59801	0.602
2035	1/1	90106	715	90821	0.787
2035	1/2	84040	719	84759	0.848
2035	1/3	92257	699	92956	0.752
2035	2/1	80149	526	80675	0.652
2035	2/2	81192	574	81766	0.702
2035	2/3	90386	628	91014	0.690
2035	3/1	134882	1022	135904	0.752
2035	3/2	110337	910	111247	0.818
2035	3/3	131547	946	132493	0.714
2035	4/1	103570	728	104298	0.698
2035	4/2	102362	678	103040	0.658
2035	4/3	106599	732	107331	0.682
2035	5/1	131421	1132	132553	0.854
2035	5/2	124661	1018	125679	0.810
2035	5/3	121973	944	122917	0.768
2035	6/1	85326	624	85950	0.726
2035	6/2	77855	582	78437	0.742
2035	6/3	62700	498	63198	0.788

a- Strains SY1953 (ste3Δ) and SY2035 (*STE3*).
b- Experiments 1 and 2, 3 and 4, and 5 and 6 were duplicate experiments performed on three consecutive days.
c- Triplicate reactions in each experiment.
d- Radioactivity associated with supernatant fraction.
e- Radioactivity associated with membrane pellet.
f- Total counts in binding assay (unbound + bound counts).
g- percentage of radioactivity associated with the membrane pellet (bound counts/total counts x 100).

Table 2: Statistical analysis of first group of binding assay data (experimental similarities).

Strain[a]	Experiment[b]	Mean[c]	T[d]	P[e]
1953	1	0.577	2.67	0.051
1953	2	0.479		
1953	1	0.577	0.140	0.895
1953	5	0.573		
1953	5	0.573	0.573	0.598
1953	6	0.588		
1953	2	0.479	0.085	0.937
1953	4	0.476		
1953	3	0.525	2.72	0.054
1953	6	0.588		
2035	1	0.796	3.59	0.023
2035	2	0.681		
2035	1	0.796	0.401	0.707
2035	5	0.810		
2035	5	0.810	1.89	0.131
2035	6	0.752		
2035	2	0.681	0.105	0.921
2035	4	0.679		
2035	3	0.761	0.262	0.806
2035	6	0.752		

a- Strains SY1953 (ste3Δ) and SY2035 (*STE3*).
b- Experiments 1 and 2, 3 and 4, and 5 and 6 were duplicate experiments performed on three consecutive days.
c- Mean of percentage of bound counts.
d- T score (Students T Test).
e- P Score (Students T Test).

Table 3: Statistical analysis of first group of binding assay data (experimental differences).

Strain[a]	Experiment[b]	Mean[c]	T[d]	P[e]
1953	1	0.577	5.79	0.0040
2035	1	0.796		
1953	2	0.479	6.28	0.0033
2035	2	0.681		
1953	3	0.525	6.92	0.0023
2035	3	0.761		
1953	4	0.476	8.47	0.0011
2035	4	0.679		
1953	5	0.575	7.40	0.0018
2035	5	0.811		
1953	6	0.588	6.40	0.0031
2035	6	0.752		

a- Strains SY1953 (ste3Δ) and SY2035 (*STE3*).
b- Experiments 1 and 2, 3 and 4, and 5 and 6 were duplicate experiments performed on three consecutive days.
c- Mean of percentage of bound counts.
d- T score (Students T Test).
e- P score (Students T Test).

Table 4: Statistical analysis of first group of binding assay data (complete analysis).

Strain[a]	Mean[b]	T[c]	P[d]
1953	0.537	10.61	0.0001
2035	0.746		

a- Strains SY1953 (ste3Δ) and SY2035 (*STE3*).
b- Mean of percentage of bound counts.
c- T score (Students T Test).
d- P score (Students T Test).

by the large T score, 10.61). In addition, due to the large sample size, the P value can also be considered, and indeed shows that the data is reliable to a very high confidence value (0.0001). In this group of binding assays a larger percentage of counts are bound in SY2035 (which contains the *STE3* receptor) than in SY1953 (from which *STE3* has been deleted). The percentage of bound counts takes into account the counts associated with the membrane fraction divided by the total counts in the experiment (unbound and bound counts combined). As can be seen in figure 2, the average percentage of bound counts were 0.74 and 0.54% for SY2035 and SY1953, respectively. The higher percentage of binding in SY2035 was consistent throughout each of the six experiments which make up the group as can be seen by comparing SY1953 to SY2035 in each experiment (Table 1).

The second group of assays was performed with fresh preparations of membranes and radiolabeled peptide. Included with each experiment in the second set of assays was a reaction which contained heat-inactivated membranes (Table 6). Heat-inactivation of membranes should reduce the non-specific binding of **a**-factor to non-Ste3p proteins. Counts bound to membranes in this experiment could represent non-specific binding of **a**-factor to lipids in the membrane, and can then can be subtracted away from the bound counts in the normal assay. The percentage of bound counts (as seen in Table 5) was calculated exactly as the first group of assays. One difference clearly evident in the second

group of assays is a much higher number of bound counts. The higher numbers bound in this set of assays results in the significant increase in the percentage of bound counts as compared with the first group of assays. Statistical analysis was performed on this group of data as previously described (Table 7). This comparison again showed that the difference in percentage bound between SY1953 and SY2035 is statistically different (as shown by the large T score, 12.45 and a P value of 0.0001). As can be seen in figure 3, the average percentage of bound counts were 14.8 and 9.9% for SY2035 and SY1953 respectively. The differences between the two groups can be attributed to fresher preparations of both membranes and radiolabeled peptide. As shown in figures 2 and 3, there are distinct differences between the first and second set of experiments. Statistical analysis of both groups of data showed that the data in each group, though they are different, are reliable down to a confidence value (P value) of .0001 (as calculated by students T test). The second group of assays did have a slightly higher T score showing statistically that this group of binding assays had a more distinct difference between the percentage bound to each strain.

Figure 2. Graph of first group of binding assays. SY1953 contained a deletion of Ste3p, the **a**-factor receptor. SY2035 contained a fully functional Ste3p.

Figure 2: Graph of First Group - of Binding Assays.

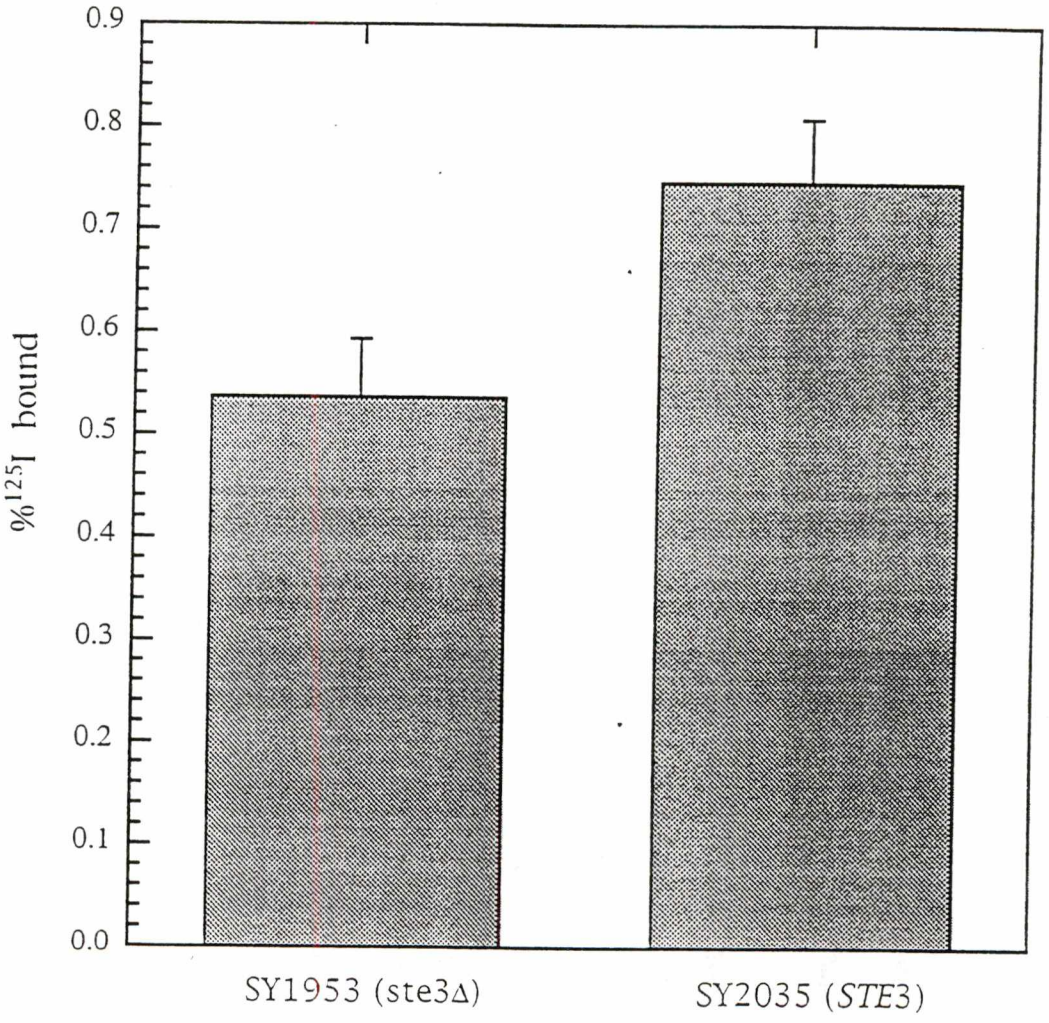


Table 5: Second group of binding assays.

Strain[a]	Expt[b]/Rxn[c]	Unbound counts[d]	Bound counts[e]	Total counts[f]	% bound[g]
SY1953	1/1	41563	4104	45089	9.10
SY1953	1/2	39132	4193	42772	9.80
SY1953	1/3	28303	3215	31086	10.34
SY1953	1/4	27846	3020	30464	9.91
SY1953	1/5	29322	3374	32278	10.45
SY2035	1/1	24512	4209	29377	14.33
SY2035	1/2	19261	3498	22481	15.56
SY2035	1/3	18064	3091	20924	14.77
SY2035	1/4	23245	3721	26737	13.92
SY2035	1/5	34822	6234	40515	15.39

a- Strains SY1953 (ste3Δ) and SY2035 (*STE3*).
b- Experiments with both strains were performed on the same day.
c- Quintuplicate reactions in each experiment.
d- Radioactivity associated with supernatant fraction.
e- Radioactivity associated with membrane pellet.
f- Total counts in binding assay (unbound + bound counts).
g- percentage of radioactivity associated with the membrane pellet (bound counts/total counts x 100).

Table 6: Binding assays of heat-inactivated membranes.

Strain[a]	Expt[b]/Rxn[c]	Unbound counts[d]	Bound counts[e]	Total counts[f]	% bound[g]
1953	1/1	41490	578	42068	1.37
1953	1/2	43608	552	44160	1.25
1953	1/3	39568	432	40000	1.08
1953	1/4	35490	402	35892	1.12
1953	1/5	39774	418	40192	1.04
2035	1/1	27822	344	28172	1.22
2035	1/2	24140	278	24418	1.14
2035	1/3	21137	231	21368	1.08
2035	1/4	21786	229	22018	1.04
2035	1/5	37583	541	38124	1.42

a- Strains SY1953 (ste3Δ) and SY2035 (*STE3*).
b- Experiments with both strains were performed on the same day.
c- Quintuplicate reactions in each experiment.
d- Radioactivity associated with supernatant fraction.
e- Radioactivity associated with membrane pellet.
f- Total counts in binding assay (unbound + bound counts).
g- percentage of radioactivity associated with the membrane pellet (bound counts/total counts x 100).

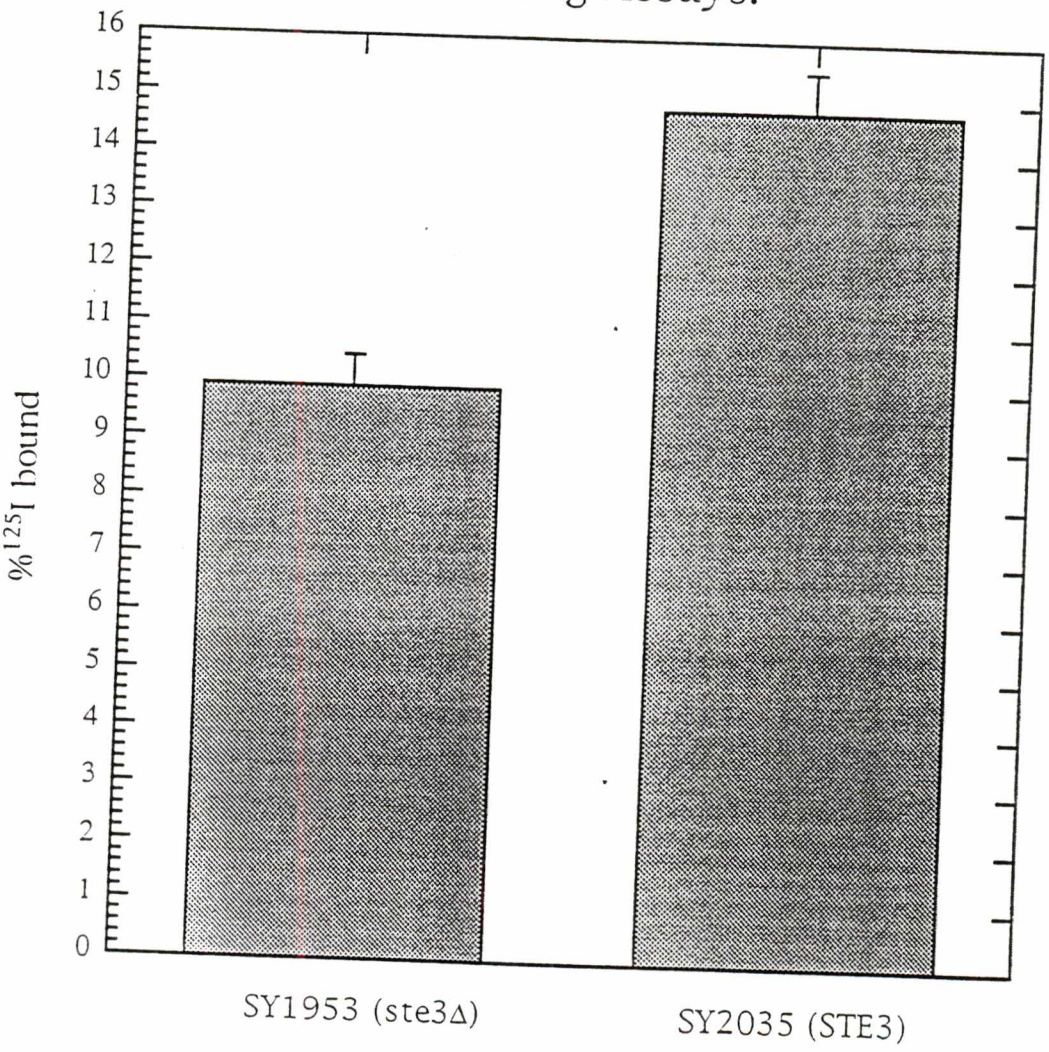
Table 7: Statistical analysis of second group of binding assay data.

Strain[a]	Mean[b]	T[c]	P[d]
1953	9.92	12.45	0.0001
2035	14.79		

a- Strains SY1953 (ste3Δ) and SY2035 (*STE3*).
b- Mean of percentage of bound counts.
c- T score (Students T Test).
d- P score (Students T Test).

Figure 3. Graph of second group of binding assays. SY1953 contained a deletion of Ste3p, the **a**-factor receptor. SY2035 contained a fully functional Ste3p.

Figure 3: Graph of Second Group of Binding Assays.



Discussion

The experimental data for this chapter comes from two separate groups of binding assay experiments performed at different times. Both groups of assays show that a higher percentage of counts are bound by the membranes which contain the Ste3p (SY2035) than by membranes which lack the receptor (SY1953). The first group of binding assays (results shown in fig. 2 and table 1) shows a difference in the percentage of bound counts between SY2035 and SY1953 with the higher percentage consistently being bound to the strain containing the receptor (SY2035). The difference in the percentage of bound counts between the two strains was validated using statistical analysis (seen in tables 2-4). The T score of 10.6106 verifies the difference between the two strains, and the P score of 0.0001 proves that this difference is reliable to a very high confidence level. The percentage of bound counts, however, is very low in both strains (approximately 0.74% in SY2035 to 0.54% in SY1953). The consistency in the higher percentage of bound counts from each experiment to the next, however, might still be attributed to some specificity in binding of the peptide to its receptor.

The second group of assays (results shown in fig. 3 and table 5) also show a clear and distinct difference in the percentage of bound counts between the two strains with the higher percentage consistently bound by the strain containing the receptor (SY2035).

As with the first group of binding assays, a statistical analysis verified the difference in percentage of bound counts (Table 7). The second group of binding assays showed a slightly higher T score of 12.4486 and a P value of 0.0001, which verifies the difference in percentage of bound counts to a high confidence value. Although the two groups of data point toward the same conclusion there are differences between the two groups of binding assays. This difference is particularly evident in the significant increase in the number of bound counts in the second group of assays. The higher number of bound counts resulted in a significant increase in the percentage of bound counts as well. The second group of assays was performed using fresh batches of both radiolabeled **a**-factor and membrane preparations. These factors alone could account for the difference between the two results. As seen in figure 3, there is a clear difference between the two strains. SY2035, which contains the Ste3p receptor, shows a much higher percentage of bound counts (14.79) than does SY1953 (9.92), from which the *STE3* gene, which encodes the Ste3p receptor, has been deleted. This difference could be attributed to the radiolabeled peptide binding specifically to either the Ste3p protein, which is present in the SY2035 strain, or to the peptide interacting with another protein. The latter interaction has not been proven, but has been speculated (Schafer and Rine 1992; Thissen and Casey 1993).

Future experiments should include competition type assays between the radiolabeled peptide and non-radiolabeled peptide. The ability of the non-radiolabeled peptide to compete out the binding of the radiolabeled peptide would show that the binding seen above was indeed due to a specific interaction with the receptor. The dissociation constant of the pheromone as well as the number of receptors per cell are occupied by the pheromone are other important information that still needs to be determined experimentally. Another series of future experiments that could further clarify the peptide interaction with the receptor should include the above assays performed with a radiolabeled **a**-factor analog in which the farnesyl moiety is replaced with a methyl group. This analog is 200- to 500-fold less active than that of WT **a**-factor. This experiment could also further clarify the role of the farnesyl moiety in the receptor/peptide interaction. A difference in percentage of bound counts in this assay could imply a specific interaction of the peptide portion of **a**-factor with the Ste3p receptor. Results that show that there is no significant difference in binding could be used to argue that the peptide is not interacting directly with the Ste3p receptor. One possible explanation for this result is that the farnesyl moiety is interacting with a farnesyl binding protein, and that this protein facilitates the receptor/peptide interaction. Though some positive direction has been made in this study, further study into this

important aspect of the signal transduction pathway in yeast still needs to be performed.

CHAPTER 3

COMPARISON AND ANALYSIS OF VARIOUS FARNESYL ANALOGS OF **a**-FACTOR VS. **a**-FACTOR IN AN AQUEOUS MATING ASSAY

Introduction

Mating between the two haploid mating types of the budding yeast *Saccharomyces cerevisiae* is mediated by two diffusible peptide pheromones, **a**-factor and α-factor (Sprague and Thorner 1992; Kurjan 1992). These peptide pheromones are functionally equivalent in their roles as ligands for specific receptors. This ligand/receptor interaction in turn activates a signal transduction cascade resulting in a number of specific cellular responses. Although α-factor and **a**-factor are functionally equivalent, they are structurally distinct. α-factor is an unmodified, 13 amino acid peptide (WHWLQLKPGQPMY) which is secreted by α-cells and is responsible for eliciting the mating response from the target **a**-cell via a specific receptor, Ste2p (Jenness *et al.* 1983). **a**-factor, by contrast, is a 12-residue lipopeptide (YIIKGVFWDPAC(farnesyl)-OCH_3), post-translationally modified by the addition of a farnesyl group and a carboxymethyl ester to its C-terminal cysteine residue (Anderegg *et al.* 1988). The target of **a**-factor is the *STE3* gene product which is a specific receptor located on the surface of the α-cell (Hagen and Sprague 1984; Bender and Sprague 1986). The binding of the pheromone

to the receptor results in the transmission of an intracellular G-protein mediated signal. This activation of the signal transduction cascade results in the subsequent physiological changes such as growth arrest at G_1 of the cell cycle and induction of specific genes whose products are required for cell and nuclear fusion, and morphological changes (such as "shmooing").

A variety of fungi communicate using a system of peptide hormones similar to that of *Saccharomyces* (Caldwell *et al.* 1995; Duntze *et al.* 1993; Bolker and Kahmann 1993). Many of these systems use modified peptides as part of the mediation of communication. The most common form of addition is the alkylation of the C-terminal cysteine by a 15-carbon unsaturated isoprene moiety: the farnesyl group. In *Saccharomyces* this alkylation is also accompanied by a carboxymethylation of the same residue.

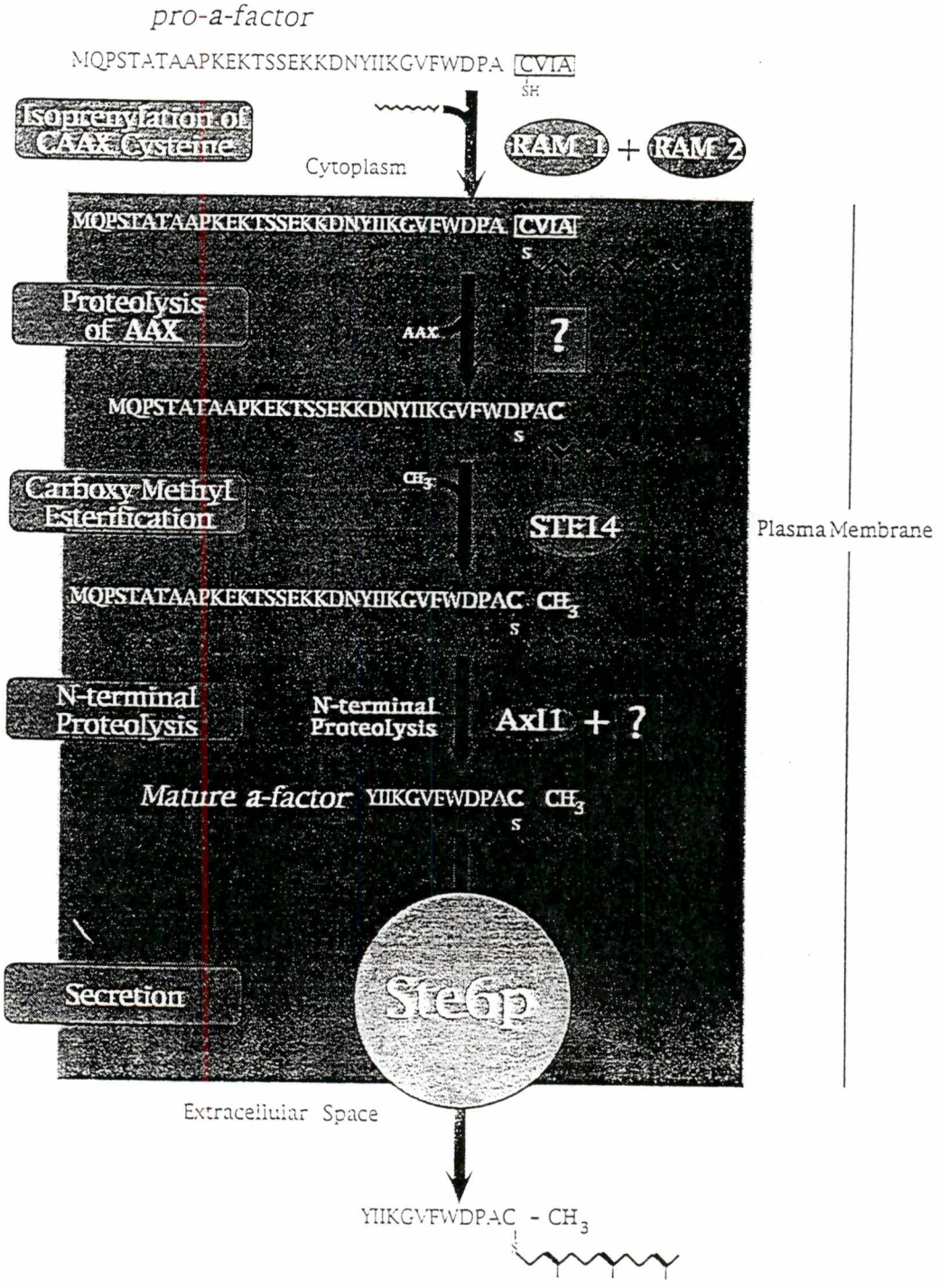
By making use of a synthetic approach, Marcus *et al.* (1991) demonstrated that both farnesylation and methylation are required for full biological activity of the **a**-factor pheromone. Non-methylated versions of **a**-factor had an activity of only 6.5%, while a non-farnesylated version had an activity of 1.6%. Removal of both the methyl and farnesyl group resulted in a pheromone that was only active at concentrations 40000 fold greater than that of naturally occurring **a**-factor. Caldwell *et al.* (1994) also noted that a geranyl-geranyl **a**-factor had an activity that was 25% as compared to the farnesylated pheromone.

In addition, the prenylation of *RAS* has been shown to be essential for its membrane localization and for its role in cellular transformation (Kato *et al.* 1992). Evidence such as this further substantiates the importance of prenyl additions such as the farnesyl group. Given the evidence mentioned above, and the fact that the target of a-factor is a membrane associated protein, the Ste3p receptor (Hagen and Sprague 1984; Bender and Sprague 1986), it is likely that a function of the farnesyl group is to aid in the localization of the pheromone to the membrane of the target cell (Sherrill *et al.* 1995). Similar conclusions have also been discussed by Black (1992), who used model peptides, and Shahinian and Silvius (1995) with peptides with double lipid modifications.

In supporting this view it is also important to consider the maturation and secretion of **a**-factor (figure 4). **a**-factor is transcribed as a 36 or 38 amino acid chain from the *MFa1* and *MFa2* gene, respectively. One copy of the mature peptide is embedded within each chain (Brake *et al.* 1985). The polypeptide is then farnesylated via the *RAM1* and *RAM2* gene products (He *et al.* 1991; Hrycyna and Clark 1990) which is then followed by membrane localization. The subsequent steps of the maturation, which includes proteolytic cleavage of the C-terminal three residues by a farnesyl-dependent membrane bound protease (Ashby *et al.* 1992; Michaelis *et al.* 1992), carboxymethylation of the now exposed cysteine, and N-terminal proteolysis of the

Figure 4. **a**-factor maturation. Pro-**a**-factor is subjected to N-terminal proteolysis. Isoprenylation of the peptide occurs at the cysteine of the CAAX (where C is cysteine, A is an aliphatic amino acid, and X is any amino acid) box. The addition of the farnesyl group is accomplished by the farnesyl transferase RAM (which was termed for Ras and **a**-factor modification). Proteolysis of three C-terminal amino acids (AAX) follows. After carboxy methyl esterification, a mature **a**-factor is achieved. The mature **a**-factor is secreted through the plasma membrane of the **a**-cell via a mechanism involving the *STE6* gene product.

Biosynthesis of **a**-factor



excess amino acids, are all believed to take place in the plasma membrane (Caldwell *et al.* 1995). In this work, we have examined more closely the role of the farnesyl group in helping to associate **a**-factor with membranes. We studied biological activities of **a**-factors containing the novel lipid substitutions of branched variations of the farnesyl moiety. Epand *et al.* (1993) previously investigated the ability of various additions which effect the association of **a**-factor with membranes. This study suggested that the level of insertion of the pheromone into an artificial lipid bilayer depended of the type of modification applied.

Our evidence indicates that there is a specific preference for a particular relationship between the membrane and the ligand with regards to bioactivity, and that this preference is mediated via the farnesyl group. Our speculation from this evidence is that modifications of this kind mediate a different interaction or association with the membrane bilayer. This interaction would effect the subsequent interaction of the peptide portion with a membrane-localized receptor. The result of this alteration of the signal which thus effects the bioactivity of the pheromone. The implication of this is that isoprenyl moieties are not simply modes of membrane localization, but that they also play a role in the presentation of a ligand to its target.

Materials and Methods

Yeast strains and Genotypes: Strain RC757 [*MATα sst2-1 rme1 his6 met 1 cam1 cyh2*] was a gift from Russell Chen (University of Cincinnati). SM1229 [*MATa Δmfa1::LEU2 Δmfa2::URA3 trp1 his4 can1*] was a gift from Susan Michaelis (Johns Hopkins University).

Mating Induction Assays: In SM1229, the structural genes for a-factor, *MFa1* and *MFa2*, have been deleted so that diploid formation is dependent of the exogenous addition of pheromone. Mating induction in an aqueous environment was analyzed by modifying the method of Dorer *et al.* (1995). The above strains were incubated for 5 hours at 30°C in a solution containing various amounts of each of the analogs. After recovering cells by centrifugation, the number of diploid colonies formed in each case was determined by plating on diploid selective medium [0.67 g/l Yeast Nitrogen Base (without amino acids, DIFCO), 2% glucose and 1.5% agar]. All peptide stocks were verified to be approximately equal in concentration by absorbance at 279nm.

Synthesis of Modified **a**-factor Analogs: The synthesis of the 5 **a**-factor analogs was performed as previously described (Dawe 1996).

Statistical Analysis:

Statistical analysis was performed as previously described.

Results

Five **a**-factor analogs which contained novel 3-methyl substitutions of increasingly bulkier moieties were synthesized (figure 5). The response of the target cells to pheromone was tested in an aqueous environment at defined molar concentrations. This mating induction assay relies on the inability of RC757 and SM1229 to mate without the addition of exogenous pheromone. This assay differs from the restoration assay in the fact that the actual number of diploid colonies that form in the presence of a given molar amount of **a**-factor is determined.

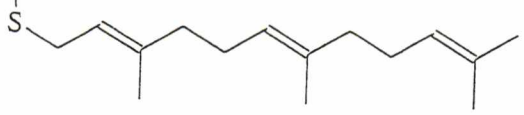
The first set of experiments involved a large range of concentrations (from 0-3000 nM concentration of **a**-factor). The purpose of these experiments was to find a concentration of **a**-factor with which to standardize the assay and serve as a control for the same experiments involving the farnesyl analogs. Figure 6 shows the results from the experiments covering the concentration range of 0-3000 nM. The activity as measured by the number of diploids per ml. in the mating reaction clearly demonstrates a plateau as early as the 20 nM concentration of **a**-factor. The same experiment was then repeated with a smaller range in concentration (0-200 nM), figure 7. The 10 nM

Figure 5. Structure of **a**-factor and lipopeptide analogs used in this study.

Figure 5. **a**-factor farnesyl analogs.

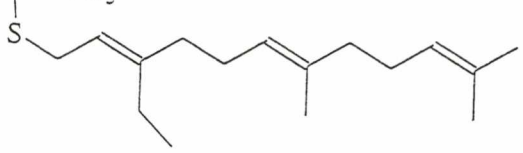
a-factor

YIIKGVFWDPAC–OCH_3



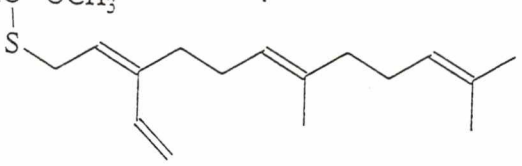
3-Ethyl-**a**-factor

YIIKGVFWDPAC–OCH_3



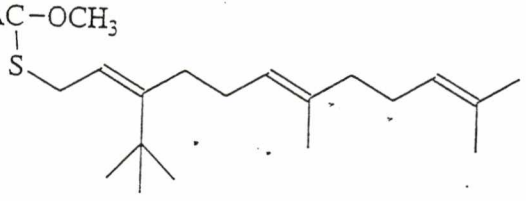
3-Vinyl-**a** factor

YIIKGVFWDPAC–OCH_3



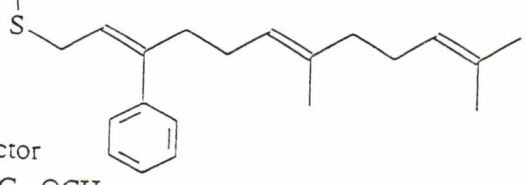
3-t-Butyl-**a**-factor

YIIKGVFWDPAC–OCH_3



3-Phenyl #1-**a**-factor

YIIKGVFWDPAC–OCH_3



3-Phenyl #2-**a**-factor

YIIKGVFWDPAC–OCH_3

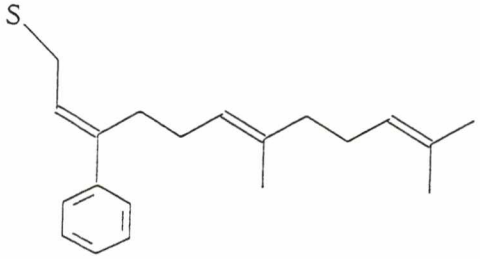
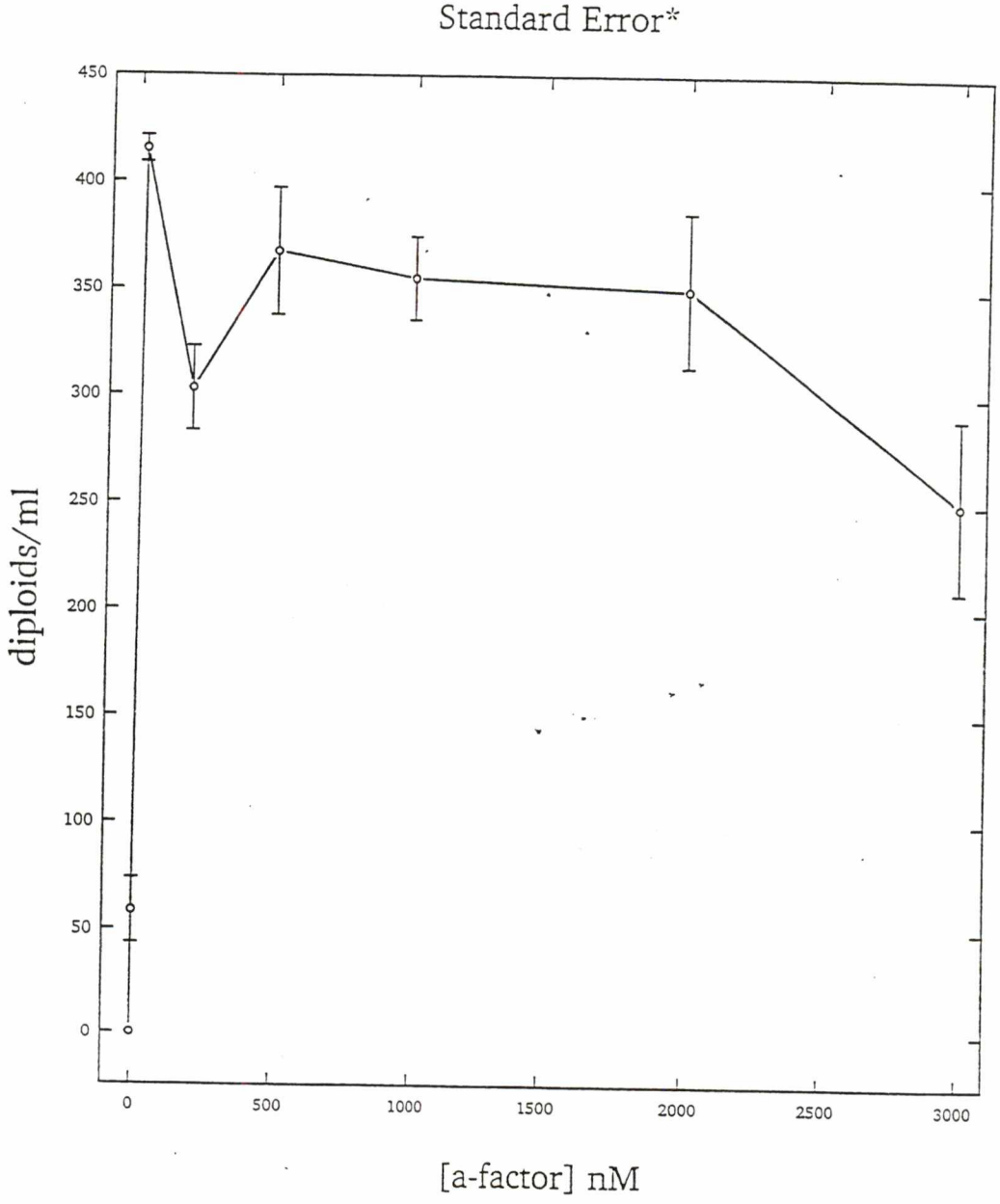


Figure 6. Mating induction assay (0-3000 nM). **a**-factor was tested for its ability to induce successful mating between strains SM1229 and RC757 over a concentration range of 0-3000nM **a**-factor. The number of diploid colonies recovered from each experiment was determined after 2 days at 30°C. Each point represents the average of three experiments.

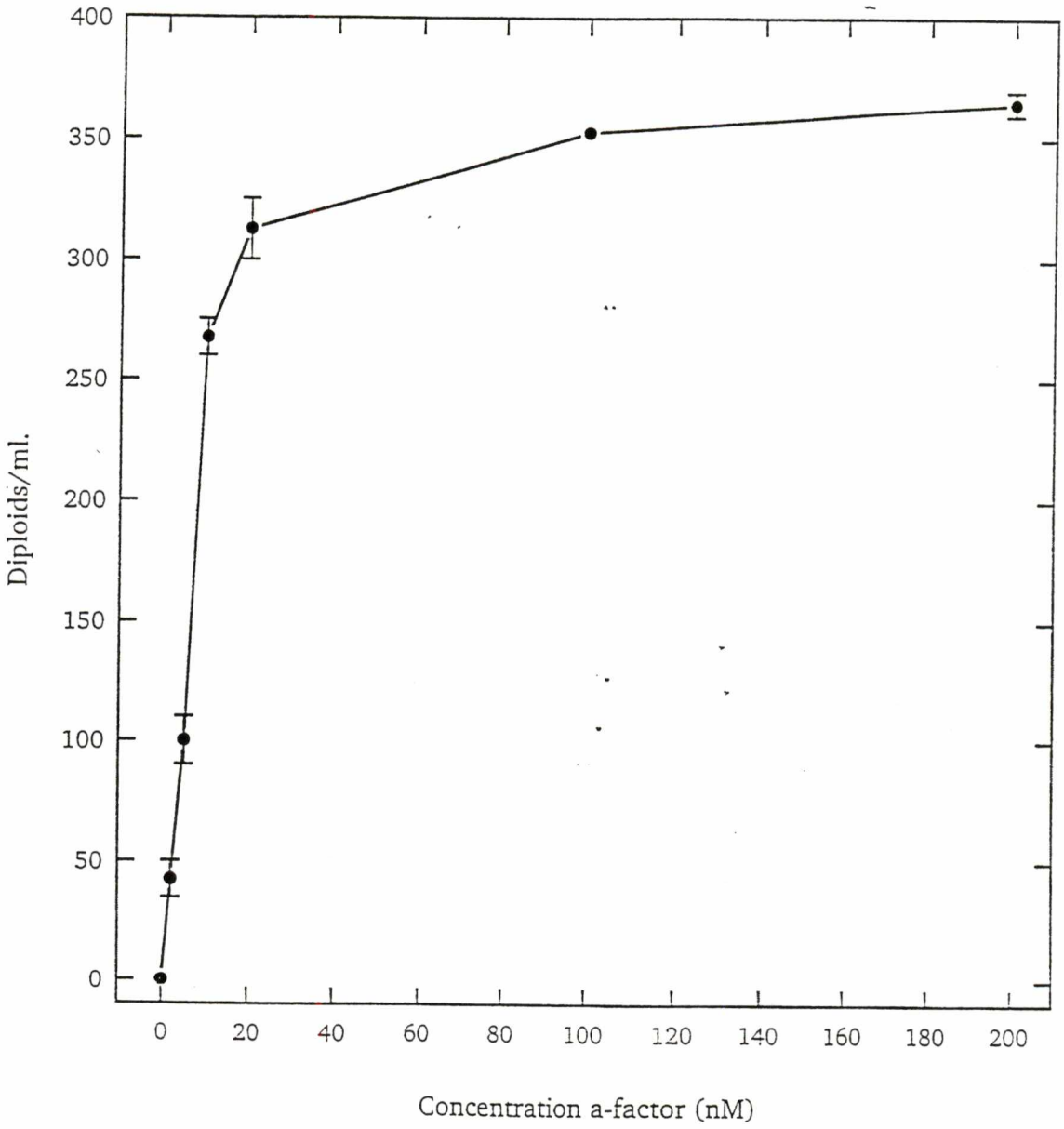
Figure 6: Graph of Diploids/ml vs. concentration (nM) of a factor.



*Error bars calculated using standard error function.

Figure 7. Mating induction assay (0-200 nM). **a**-factor was tested for its ability to induce successful mating between strains SM1229 and RC757 over a concentration range of 0-200nM **a**-factor. The number of diploid colonies recovered from each experiment was determined after 2 days at 30°C. Each point represents the average of three experiments.

Figure 7: Graph of Diploids/ml vs. concentration (nM) of a-factor.

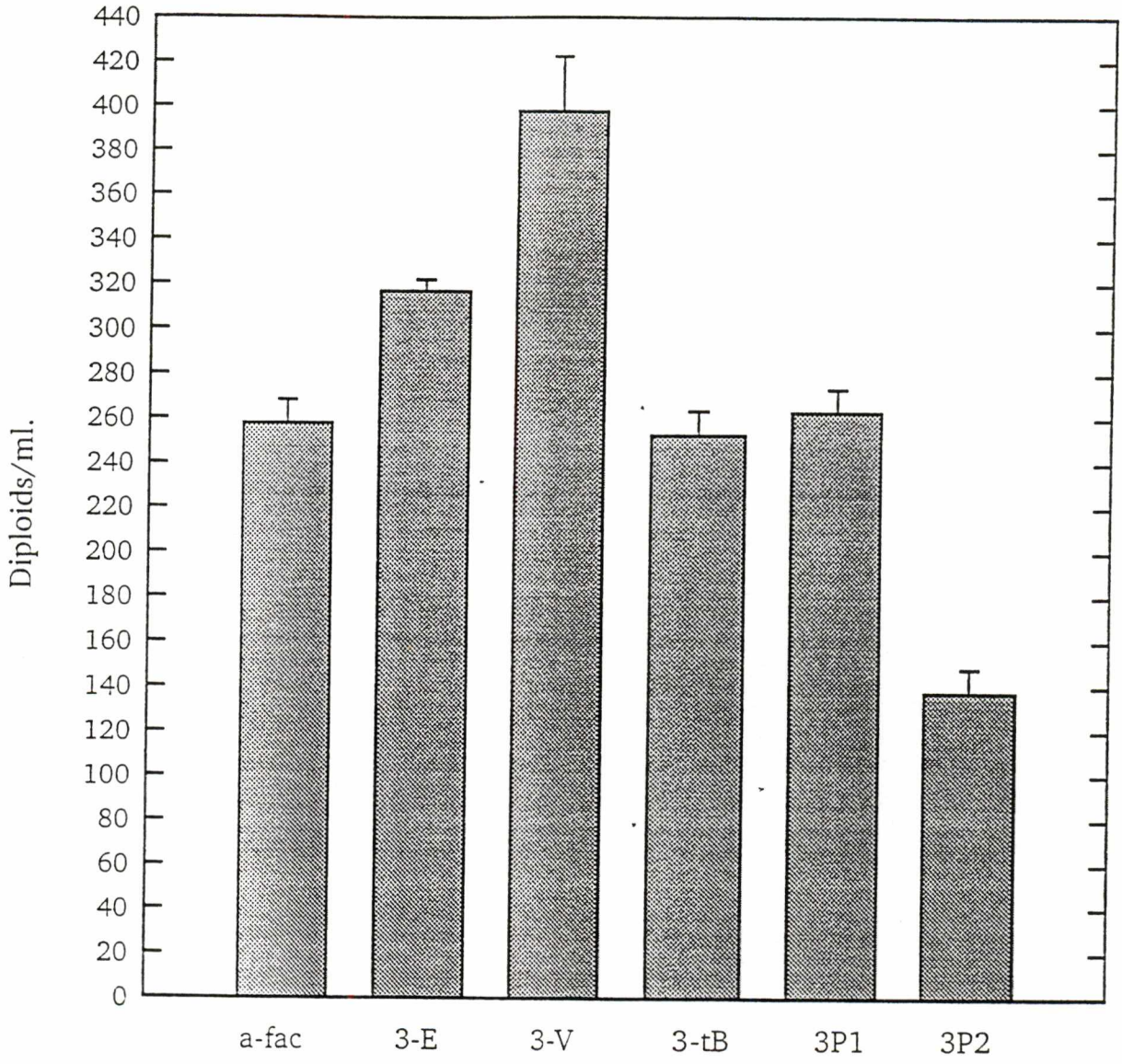


concentration of **a**-factor gave values that were on the upward portion of the curve. This concentration was chosen as the concentration with which to test all of the other analogs. All five analogs and **a**-factor as a control were then assayed at the 10 nM concentration, figure 8. The 3-ethyl- and 3-vinyl-**a**-factors both demonstrated an activity that was higher than that of normal **a**-factor. The 3-t-butyl- and 3-phenyl #1-**a**-factors both showed activity similar to that of normal **a**-factor. The 3-phenyl #2-**a**-factor showed much less activity than that of normal **a**-factor. A statistical analysis was performed to determine whether the observed differences and similarities were statistically valid (Table 8). The differences noted between the activities of **a**-factor and the 3-ethyl-, 3-vinyl-, and 3-phenyl #2-**a**-factor analogs were verified as shown by their high T scores (7.13, 7.35, and 11.31 respectively). The similarity between the activity of **a**-factor and the 3-t-butyl-, and 3-phenyl #1-**a**-factor analogs was verified as shown by there low T scores (both were .471).

Following the results of the experiment that was performed at a fixed concentration of 10 nM it was decided to perform an experiment in which each of the 5 analogs and **a**-factor were tested over the narrow concentration range of 0-50 nM (table 9). This was to confirm the above results as well as compare the activity of the analogs to **a**-factor across the range of concentrations. In agreement with the previous experiment the 3-ethyl- and 3-vinyl **a**-factors both demonstrated a higher

Figure 8. Mating induction assay (**a**-factor vs. farnesyl analogs (10 nM)). Each analog was tested for its ability to induce successful mating between strains SM1229 and RC757 at a final concentration of 10nM. The number of diploid colonies recovered from each experiment was determined after 2 days at 30°C. Each point represents the average of three experiments.

Figure 8: Graph of Diploids/ml vs. farnesyl analog at 10 nM concentration.



a-fac- a-factor
3-E- 3-Ethyl-a-factor
3-V- 3-Vinyl-a-factor
3-tB- 3-t-Butyl-a-factor
3P1- 3-Phenyl#1-a-factor
3P2- 3-Phenyl#2-a-factor

Table 8: Statistical Analysis of **a**-factor versus modified **a**-factor analogs.

Analog comparison[a]	Mean[b]	T[c]	P[d]
a-factor vs. 3-ethyl-**a**-factor	257.5 316.5	7.13	0.02
a-factor vs. 3-vinyl-**a**-factor	257.5 397.5	7.35	0.02
a-factor vs. 3-t-butyl-**a**-factor	257.5 252.5	0.47	0.68
a-factor vs. 3-phenyl #1-**a**-factor	257.5 262.5	0.47	0.68
a-factor vs. 3-phenyl #2-**a**-factor	257.5 137.5	11.31	0.01

a- Comparison of **a**-factor to modified **a**-factor analogs.
b- Mean of diploid colonies/ml. formed.
c- T score (Students T Test).
d- P score (Students T Test).

Table 9: Mating induction assay with **a**-factor and farnesyl analogs covering the concentration range from 0 to 50 nM.

Peptide[a]	Conc.[b]	Rxn 1[c]	Rxn 2[d]	Rxn 3[e]
a-factor	2	58	55	48
	5	120	122	132
	10	270	276	284
	20	340	352	364
	50	330	346	338
3-E-**a**-factor	2	60	68	58
	5	156	152	168
	10	330	328	342
	20	400	412	388
	50	380	392	398
3-V-**a**-factor	2	66	74	76
	5	170	168	182
	10	400	398	424
	20	430	452	448
	50	410	402	388
3-tB-**a**-factor	2	56	62	48
	5	105	122	118
	10	258	276	260
	20	318	332	324
	50	310	296	314
3-P1-**a**-factor	2	52	46	58
	5	128	110	116
	10	248	242	264
	20	350	374	366
	50	324	342	336
3-P2-**a**-factor	2	34	46	42
	5	70	86	74
	10	128	146	140
	20	212	230	224
	50	218	222	198

a- Peptide used in mating induction assay.
b- Concentration of peptide used in assay.
c-e- Triplicate reactions performed at each concentration of mating induction assay.

activity than normal **a**-factor at each point in the range (figure 9). The 3-t-butyl- and 3-phenyl #1-**a**-factors showed activity that was comparable to **a**-factor, confirming the results from the previous experiment. The 3-phenyl #2-**a**-factor again showed activity that was much less than that of normal **a**-factor. This difference in activity is interesting in the fact that the 3-phenyl #1- and 3-phenyl #2-**a**-factors are identical except for the presumed isomerization of the allylic double bond. This structural difference appears to have significant effect on the biological activity of these pheromones.

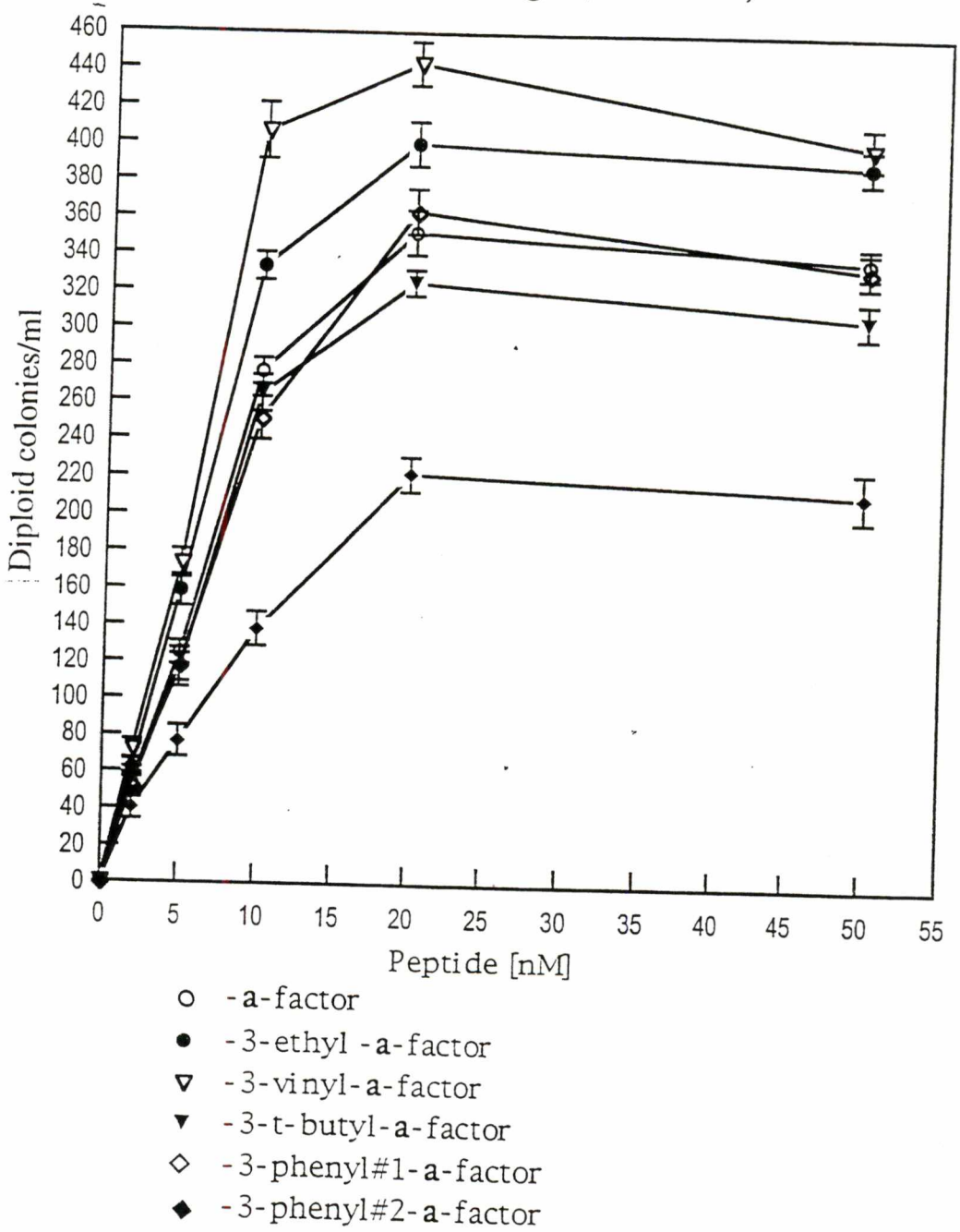
Discussion

Sherrill *et al.* (1995) have previously suggested that the principal function of lipid modifications to peptides and proteins is for membrane association. Similar conclusions were also reached by Parish and Rando (1996) following examination of the characteristics of a number of systems. Their conclusions suggested that the specificity of a receptor-ligand interactions are mediated primarily by protein-protein interactions and that lipid-protein interactions are not as likely to play a role.

Lipid-protein interactions are discussed in more detail in a review by Schafer and Rine (1992) in which mechanisms for prenyl-mediated protein targeting are discussed. One possible mechanism for prenyl-mediated protein targeting proposed by

Figure 9. Mating induction assay (**a**-factor vs. farnesyl analogs (0-50 nM)). Each analog was tested for its ability to induce successful mating between strains SM1229 and RC757 over a concentration range of 0-50nM peptide concentration. The number of diploid colonies recovered from each experiment was determined after 2 days at 30°C. Each point represents the average of three experiments.

Figure 9: Mating induction assay a-factor vs. farnesyl analogs (0-50nM)



Schafer and Rine is the association of prenylated proteins with cellular membranes by interactions between the prenyl group and membrane lipids. There are two models of lipid interaction. The first is the "dumb lipid" model, in which the prenyl group provides a non-specific lipophilic glue that provides affinity for membranes, but does not mediate targeting of the prenylated protein to a specific membrane organelle. The second model is the "smart lipid" model, in which lipid modification directs the protein to a specific locale through a nonreceptor-based mechanism. An example of this model is the localization of GPI-linked proteins to plasma membrane sites called caveolae (Anderson *et al.* 1992). The clustering of GPI-linked proteins in caveolae can be disrupted by drugs suggesting that specific lipid composition of caveolae attracts GPI-linked proteins through specific lipid interactions (Rothburg *et al.* 1992). In addition to lipid-protein interactions, Schafer and Rine (1992) also discuss a prenyl-protein receptor model. In this model a membrane-localized receptor specifically recognizes both the prenyl moiety and some specific amino acid sequence or protein structure. A fourth model proposes that prenylation may instead be a precondition for a secondary modification that actively directs protein localization (Schafer and Rine 1992).

Previous evidence from our laboratories (Caldwell *et al.* 1994) has shown that the substitution of a geranylgeranyl moiety for the farnesyl of **a**-factor results in a peptide with biological

activity and therefore implies recognition of the geranylgeranyl **a**-factor by the *STE3* receptor protein. However, in contrast to the trend previously observed in which changes from prenyl (C_5) to geranyl (C_{10}) to farnesyl (C_{15}) groups to **a**-factor increased bioactivity, the addition of the geranylgeranyl (C_{20}) to **a**-factor slightly decreased the bioactivity (25% activity as compared to wild-type **a**-factor) (Marcus *et al.* 1991, Caldwell *et al.* 1994). This decrease in activity implies that hydrophobicity itself may not be the simple characteristic required for preferred ligand binding to its receptor. The farnesyl lipid could then be recognized in a specific manner or it may provide a specific presentation of the peptidyl portion of the pheromone to the receptor.

To further address the role of the farnesyl group in the biological activity of the lipopeptide pheromone **a**-factor of *Saccharomyces cerevisiae*, a number of novel 3-methyl substitutions which contained progressively bulkier moieties were synthesized. The ability of these pheromones to induce a biological response from a population of target cells was determined using a quantitative mating assay performed in an aqueous environment. Analysis of the activities of all the analogs has shown that all were capable of eliciting a biological response. It should be noted, however, that the analogs show a variable level of activity which indicates that there is a possible preference

for the smaller additions (ethyl and vinyl) over that of the bulkier additions (t-butyl and phenyl).

The activity of each of the analogs as compared to that of **a**-factor was determined by considering the activity of **a**-factor as 1.0 in the assays, and the activities of the other analogs normalized to this value. Throughout these assays the activities of the ethyl and vinyl additions are approximately 2-fold higher than that of **a**-factor, whereas the larger modifications show levels of activity that are comparable to that of **a**-factor. One particular point of interest is the variation of activities associated with the 3-phenyl #1- and 3-phenyl #2-**a**-factor analogs, which are the isomers of from the synthesis of the phenyl-modified farnesyl group. The variation of the spatial relationship of the peptide relative to the farnesyl, which is imparted by this alteration, appears to have a significant effect on the ability of the 3-phenyl #2-**a**-factor analog to interact with the receptor. This effect is presumed to be a shift in the location of the peptide portion to one which is less favorable with respect to the binding site located on the Ste3p. In our lab Caldwell *et al.* (1994) observed results which were similar to the above results with topological variants of **a**-factor where the spatial orientation of both the farnesyl and carboxymethyl groups were altered. Together these results suggest that there is a role for the farnesyl group beyond membrane association. This role may be in aiding the interaction

of the peptide with the receptor by orientation of the peptide relative to the membrane surface.

Both the **a**-factor and α-factor pheromones of *Saccharomyces cerevisiae* have been shown to have specific structural requirements for bioactivity which are fulfilled by various portions of the peptide (Zhang *et al.* 1996; Caldwell *et al.* 1994). A specific interaction of the peptide with its receptor would be influenced by the location and orientation of the critical residues of the ligand to the receptor binding site. In the case of the **a**-factor/Ste3p interaction both ligand and receptor are associated with the membrane. This interaction could be effected by any factor which alters the membrane interface, thus also affecting the bioactivity of the compound. In this study we see a preference for small size increases of the 3-methyl group in **a**-factor, of which the 3-ethyl and 3-vinyl substitutions gave the optimal activities. The ability, however, of most of the additions to maintain a level of biological activity similar to that of **a**-factor suggests a role for the farnesyl group in presentation of the peptide in addition to a role in membrane targeting. This investigation could not, however, have been accomplished without the use of novel chemical synthesis. This powerful tool could be used to further investigate the importance of orientation of a lipid anchor to its accompanying pheromone. By expanding the studies discussed above the role of the isoprenyl additions, in terms of the

biological activity of these molecules, could be examined in greater detail in the future.

REFERENCES

References

Anderegg, R. J., Betz, R., Carr, S. A., Crabb, J. W., Duntze, W. Structure of *Saccharomyces cerevisiae* mating hormone **a**-factor. Identification of S-farnesyl cysteine as a structural component. J. Biol. Chem. **263**:18236-18242 (1988).

Anderson, R. G. W., Kamen, B. A., Rothberg, K. G., Lacey, S. W. Potocytosis: sequestration and transport of small molecules by caveolae. *Science* **255**:410-411 (1992).

Ashby, M. N., King, D., Rine, J. Endoproteolytic processing of a farnesylated peptide in vitro. *Proc. Natl. Acad. Sci. USA* **89**: 4613-4617 (1992).

Bender, A., Sprague, G. F. Yeast peptide pheromones, **a**-factor and alpha-factor, activate a common response mechanism in their target cells. *Cell* **47**: 929-937 (1986).

Black, S. Development of hydrophobicity parameters for prenylated proteins. *Biochem. Biophys. Res. Comm.* **186**:1437-1442 (1992).

Blumer, K.J., Reneke, J. E., Thorner, J. The *STE2* gene product is the ligand binding component of the α-factor

receptor of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **263**: 10836-10842 (1988)

Bolker, M., Kahmann, R. Sexual pheromones and mating responses if fungi. *Plant Cell* **5**: 1461-1469 (1993).

Botstein, D., Fink, G. Yeast: An experimental organism for modern biology. *Science* **240**: 1439-1443 (1988).

Brake, A., Brenner, C., Najarian, R., Laybourne, P., Merryweather, J. Structure of genes encodeing precursors of the yeast peptide mating pheromone **a**-factor, p. 103-108. *In* Protein transport and secretion. M. J. Gething (ed.) Cold Spring Harbor laboratory, Cold Spring Harbor, N.Y. (1985).

Burkholder, A. C., Hartwell, L. H. The yeast α-factor receptor: Structural properties deduced from the sequence of the *STE2* gene. *Nucleic Acids Res.* **13**:8463-8475 (1985).

Caldwell, G. A., Wang, S-H., Dawe, A.L., Naider, F., Becker, J.M. Identification of a hyperactive mating pheromone of *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Comm.* **197**:1173-1178 (1993).

Caldwell, G. A., Wang, S-H., Naider, F., Becker, J.M. Consequences of altered isoprenylation targets on a-factor export and bioactivity. *Proc. Natl. Acad. Sci. USA.* **91**:1275-1279 (1994).

Caldwell, G. A., Wang, S-H., Xue, C-B., Jiang, Y., Lu, H-F.,Naider, F., Becker, J.M. Molecular determinants of bioactivity of the *Saccharomyces cerevisiae* lipopeptide mating pheromone. *J. Biol. Chem.* **269**:19817-19826 (1994).

Caldwell, G. A., Naider, F., Becker, J. M. Fungal lipopeptide pheromones: a model system for the study of protein prenylation. *Microbiol. Rev.* **59**:406-422 (1995).

Dawe, A. L. Investigations of the *Saccharomyces cerevisiae* **a**-factor lipopeptide: structure-function relationships and target cell responses. Ph. D. Thesis, Univ. Tennessee (1996).

Dohlman, H. G., Thorner, J., Caron, M. G., Lefkowitz, R. J. Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* **60**:653-688 (1991).

Dorer, R., Pryciak, P. M., Hartwell, L. H. *Saccharomyces cerevisiae* cells execute a default pathway to select a mate in the absence of pheromone gradients. *J. Cell Biol.* **131**:845-861 (1995).

Duntze, W., Betz, R., Popping, B. Lipopeptide pheromones of yeast and basidiomycetes involved in mating. (1993). *In* Signal transduction: prokaryotic and simple systems. Kurjan, J., Taylor, B. L. (eds), pp. 239-254. Academic Press, Inc, San Diego

Epand, R. E., Xue, C. -.B., Wang, S. -. H., Naider, F., Becker, J. M., Epand, R. M. Role of prenylation in the interaction of the **a**-factor mating pheromone with phospholipid bilayers. *Biochemistry* **32**:8368-8373 (1993).

Eriotou-Bargiota, E., Xue, C. -. B., Naider, F., Becker, J. M. Antagonistic and synergistic peptide analogs of the tridecapeptide mating pheromone of *Saccharomyces cerevisiae*. *Biochemistry* **31**:551-557 (1992).

Fasoli, M. O. F., Kerridge, D. Uptake of pyrimidines and their derivatives into *Candida glabrata* and *Candida albicans*. *J. Gen. Microbiol.* **136**:1475-1481 (1988).

Hagen, D. C., Sprague, G. F. Induction of the yeast alpha-specific *STE3* gene by the peptide pheromone **a**-factor. *J. Mol. Biol.* **178**:835-852 (1984).

Hagen, D. C., McCaffrey, G., Sprague, G. F. Evidence the yeast *STE3* gene encodes a receptor for peptide pheromone **a**-factor: gene sequence and implications for the structure of the presumed receptor. *Proc. Natl. Acad. Sci. USA* **83**:1418-1422 (1986).

He, B., Chen, P., Chen, S. -. Y., Vancura,K.L., Michaelis, S., Powers, S. RAM2, an essential gene of yeast, and RAM1 encode the two polypeptide component of the farnesyltransferase that prenylates a-factor and Ras proteins. *Proc. Natl. Acad. Sci. USA.* **88**:11373-11377 (1991).

Hrycyna, C. A., Clarke, S. Farnesyl cystiene C-terminal transferase activity is dependent on the *STE14* gene product in *Saccharomyces cerevisiae. Mol. Cell. Biol.***10**:5071-5076 (1991).

Herskowitz, I. Life cycle of the budding yeast *Saccharomyces cerevisiae. Science* **52**:536-553 (1988).

Jenness, D. D., Burkholder, A. C., Hartwell, L. H. Binding of alpha-factor pheromone to yeast **a** cells: chemical and genetic evidence for an alpha-factor receptor. *Cell* **35**:521-529 (1983).

Kato, K., Cox, A. D., Hikasa, M. M., Graham, S. M., Buss, J. E., Der, C. J. Isoprenoid addition to Ras protein is the critical modification for its membrane association and transforming activity. *Proc. Natl. Acad. Sci. USA* **89**:6403-6407 (1992).

Kurjan, J. Pheromone response in yeast. *Ann. Rev. Biochem.* **61**:1097-1129 (1992).

Lovin, L. Structure-Function studies of yeast mating pheromones. M.S. Thesis, Univ. Tennessee (1994).

Marcus, S., Caldwell, G. A., Xue, C. -. B., Naider, F., Becker, J. M. Total in vitro maturation of the *Saccharomyces cerevisiae* lipopeptide mating pheromone. *Biochem. Biophys. Res. Comm.* **172**:1310-1316 (1990).

Marcus, S., Caldwell, G.A., Miller, D., Xue, C. -. B., Naider, F., Becker, J. M. Significance of C-terminal cysteine modifications to the biological activity of the *Saccharomyces*

cerevisiae **a**-factor mating pheromone. *Mol. Cell. Biol.* **11**:3603-3612 (1991).

Michaelis, S., Herskowitz, I. The **a**-factor pheromone of *Saccharomyces cerevisiae* is essential for mating. *Mol. Cell. Biol.* **8**:1309-1318 (1988).

Michaelis, S., Chen, P., Berkower, C., Sapperstein, S., Kistler, A. Biogenesis of yeast **a**-factor involves prenylation, methylation, and a novel export mechanism. *Antonie van Leeuwenhoek.* **61**:115-117 (1992).

Naider, F., Becker, J. M. Structure-activity relationships of the yeast α-factor. *CRC Crit. Revs. Biochem.* **21**:225-248 (1986).

Naider, F., Jelicks, L. A., Becker, J. M., Briodo, M. S. Biologically significant conformation of the *Saccharomyces cerevisiae* alpha-factor. *Biopolymers* **28**:487-497 (1989).

Nakayama, N., Miyajima, A., Arai, K. Nucleotide sequences of *STE2* and *STE3*, cell type-specific sterile genes from *Saccharomyces cerevisiae*. *EMBO J.* **5**:2643-2648 (1985).

Ott, R. Lyman. An introduction To Statistical Methods and Data Analysis, 4th Edition. Duxbury Press, Belmont, CA (1993).

Parish, C.A., Rando, R. P. Isoprenylation/methylation of proteins enhances membrane association by a hydrophobic mechanism. *Biochemisty* **35**:8473-8477 (1996).

Raths, S. K., Naider, F., Becker, J. M. Peptide analogues compete with the binding of alpha-factor to its receptor in *Saccharomyces cerevisiae. J. Biol. Chem.* **263**:17333-17341 (1988).

Rothburg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. -. S., Glenney, J. R., et al. Caveolin, a protein component of caveolae membrane coats. *Cell* **68**:673-82 (1992).

Schafer, W. R., Kim, R., Sterne, R., Thorner, J., Kim, S. -. H., Rine, J. Genetic and pharmacological suppression of oncogenic mutation in ras genes of yeast and humans. *Science* **245**:379-385 (1989).

Schafer, W. R., Trueblood, C. E., Yang, C. -. C., Mayer, M. Enzymatic coupling of cholesterol intermediates to a mating pheromone precursor and to the Ras protein. *Science* **249**:1133-1139 (1990).

Schafer, W.R., Rine, J. Protein prenylation: genes, enzymes, targets, and functions. *Annu. Rev. Genet.* **30**:209-237 (1992).

Scheckman, R. Protein localization and membrane traffic in yeast. *Annu. Rev. Cell Biol.* **1**:115-143 (1985).

Shahinian, S., Silvius, J. R. Doubly-lipid-modified protein sequence motifs exhibit long lived anchorage to lipid bilayer membranes. *Biochemistry* **34**:3813-3822 (1995).

Sherrill, S. C., Khouri, O., Zeman, S., Riose, D. Synthesis and biological activity of flourescent yeast pheromones. *Biochemistry* **34**:3553-3560 (1995).

Sprague, G. F., Thorner, J. Pheromone response and signal transduction during the mating process of *Saccharomyces cerevisiae*. (1992). *In* The molecular biology of the yeast *Saccharomyces cerevisiae*. Broach, J. R., Pringle, J. R., Jones, E. W. (eds). Cold Spring Harbor laboratory Press, cold Spring Harbor, N. Y.

Sterne, R. E., Thorner, J. Processing and secretion of a yeast peptide hormone by a novel pathway. *J. Cell Biol.* **103**:189a (1986).

Sterne, R. E. A novel pathway for peptide hormone biogenesis: processing and secretion of the mating pheromone **a**-factor by *Saccharomyces cerevisiae*. Ph.D. Thesis, Univ. California, Berkeley. (1989).

Thissen, J. A., Casey, P. J. Microsomal membranes contain a high affinity binding site for prenylated peptides. *J. Biol. Chem.* **268**:13780-13783 (1993).

Zhang, Y. L., Dawe, A. L., Jiang, Y., Becker, J. M., Naider, F. A superactive peptidomimetic analog of a farnesylated dodecapeptide yeast pheromone. *Biochem. Biophys. Res. Comm.* **224**:327-331 (1996).

Vita

John Wilgus was born in Nashville, Tennessee on March 24, 1969. He attended elementary and middle schools in Knoxville, Tennessee. In May of 1987, he graduated from Knoxville Central High School. John received his Bachelor of Science degree in Microbiology Cum Laude from the University of Tennessee in 1994. He entered graduate school in the Cellular, Molecular, and Developmental Biology department the following fall. During his time in graduate school he was a teaching assistant at both the undergraduate and graduate levels. His thesis research was performed in the laboratory of Dr. Jeffrey M. Becker. In May of 1997, he received a Master of Science in Life Sciences.