INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

U·M·I

University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600

Order Number 9108190

The immunological and molecular characterization of alpha-agglutinin from *Saccharomyces cerevisiae*

Wojciechowicz, Donald Charles, Ph.D.

City University of New York, 1990

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

A

THE IMMUNOLOGICAL AND MOLECULAR CHARACTERIZATION OF ALPHA-AGGLUTININ FROM *Saccharomyces cerevisiae*

by

Donald Wojciechowicz

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1990

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

Aug 10, 1990
date

Chairman of Examining Committee
Dr. Joseph S. Krakow, Hunter College

AUG. 10, 1990
date

Executive Officer
Dr. Peter C. Chabora

Dr. Shirley Rabs, Hunter College

Dr. Peter N. Lipke, Hunter College

Dr. Janet Kurjan, Columbia University

Dr. Reza Greene, Mt. Sinai School of Medicine

Supervisory Committee

The City University of New York

Abstract

THE IMMUNOLOGICAL AND MOLECULAR CHARACTERIZATION OF ALPHA-AGGLUTININ FROM *Saccharomyces cerevisiae*

by

Donald Wojciechowicz

Adviser:Professor Peter N. Lipke

α-agglutinin is a cell surface glycoprotein expressed on mating type α cells in the yeast *Saccharomyces cerevisiae*. The function of α-agglutinin is to bind to a-agglutinin, thereby causing agglutination of cells of opposite mating type. Agglutination between cells of opposite mating type enhances the probability of cell fusion to form a diploid zygote. Therefore, α-agglutinin is an important cell adhesion molecule which facilitates mating in *Saccharomyces cerevisiae*.

The work presented here further clarifies the structure, function and expression of α-agglutinin. An polyclonal antibody was used to study the cell surface expression of α-agglutinin. I have shown that there are 5 x 10^4 molecules of α-agglutinin constitutively expressed per α cell. Induction with a-factor causes a modest increase in α-agglutinin expression to 6.5 x 10^4 molecules per cell. The spatial distribution of α-agglutinin on the cell surface in uninduced cells is polar while induced cells express α-agglutinin more

uniformly. Buds and daughter cells do not express cell surface α-agglutinin. Lastly, α-agglutinin is expressed in the shmoo tip region of induced α cells, the location were a and α cells fuse to form a diploid zygote.

Using the antibody, I have also shown that the *AGα1* gene codes for α-agglutinin. This gene is α-specific and shown to be required for agglutinability of *MATα* cells. Notable features of *AGα1* include that the ORF 1)codes for a 70 kD protein 2)contains an acidic region within the amino half of the glycoprotein 3)contains a 15 amino acid hydrophobic carboxy terminus 4)has a putative signal secretion sequence and 5)has numerous potential N and O-linked glycosylation sites throughout the coding sequence.

Phenotypes of *AGα1* truncation mutants further elucidate the domain structure of α-agglutinin. Using molecular cloning to introduce termination codons within the ORF of *AGα1*, I have shown that 1)the carboxy hydrophobic terminus of α-agglutinin is necessary for cell wall anchorage and 2) the amino half of the glycoprotein (possibly the acidic region) contains the binding domain of α-agglutinin. Most of the O-linked sugar is associated with the carboxy half of the molecule while N-linked carbohydrate is found throughout α-agglutinin. Lastly, I show that α-agglutinin is efficiently transported through the secretory pathway.

Acknowledgements

To my Mother and Father for supporting my efforts throughout the years: "Thanks for being there, I finally made it"

To Lori, my wife and friend: "Thanks for being patient and understanding"

To Dr. Peter Lipke, my mentor: "Thanks for freeing me of my chains"

To Dr. Robert Mallon, my friend and colleague: "It was easier because of your help and support"

To Dr. Ursula Muller-Eberhard, my friend, teacher, and colleague: "All in good time"

To Dr. Shirley Raps, my teacher and professional guardian: "I'm glad I didn't destroy your office"

To my fellow laboratory workers (Hyacinth McCain, Cheryl Clarke and Jacqueline Benn) without whom the effort would have been unbearable, the proverbial: "Thanks"

Table of Contents

List of Tables

List of Illustrations

Introduction

1. CELL TYPES, LIFE CYCLE AND CELL CYCLE OF *SACCHAROMYCES CEREVISIAE*

Saccharomyces cerevisiae, known as Baker's yeast, can exist in three vegetative forms: $\underline{a}$, α and a/α (Fig. 1). $\underline{a}$ and α cells are haploid forms which can mate with each other to form diploid a/α cells. Diploids can sporulate under conditions of nitrogen starvation. Haploid cells are normally incapable of undergoing spore formation.

Sporulation results in the generation of an ascus which contains four spores. Once germinated, these spores give rise to 2 $\underline{a}$ and 2 α haploid cell types (Fig. 1).

During vegetative growth, *Saccharomyces cerevisiae* reproduces by budding. Bud formation marks the phenotypic beginning of the S phase of the cell cycle (Fig. 12). Bud enlargement continues through G2 and M phases until cytokinesis (bud separation). Cells newly formed by budding are called daughter cells.

2. MATING AND THE ROLE OF THE AGGLUTININS

An early step in mating is the binding of an α cell to an $\underline{a}$ cell. Binding of one mating type to the other is mediated by agglutinins which are expressed on the cell surface (Fig. 2). The expression of these agglutinins is mating type specific, since $\underline{a}$ cells express a-agglutinin

Figure 1. Life cycle of *Saccharomyces cerevisiae*.

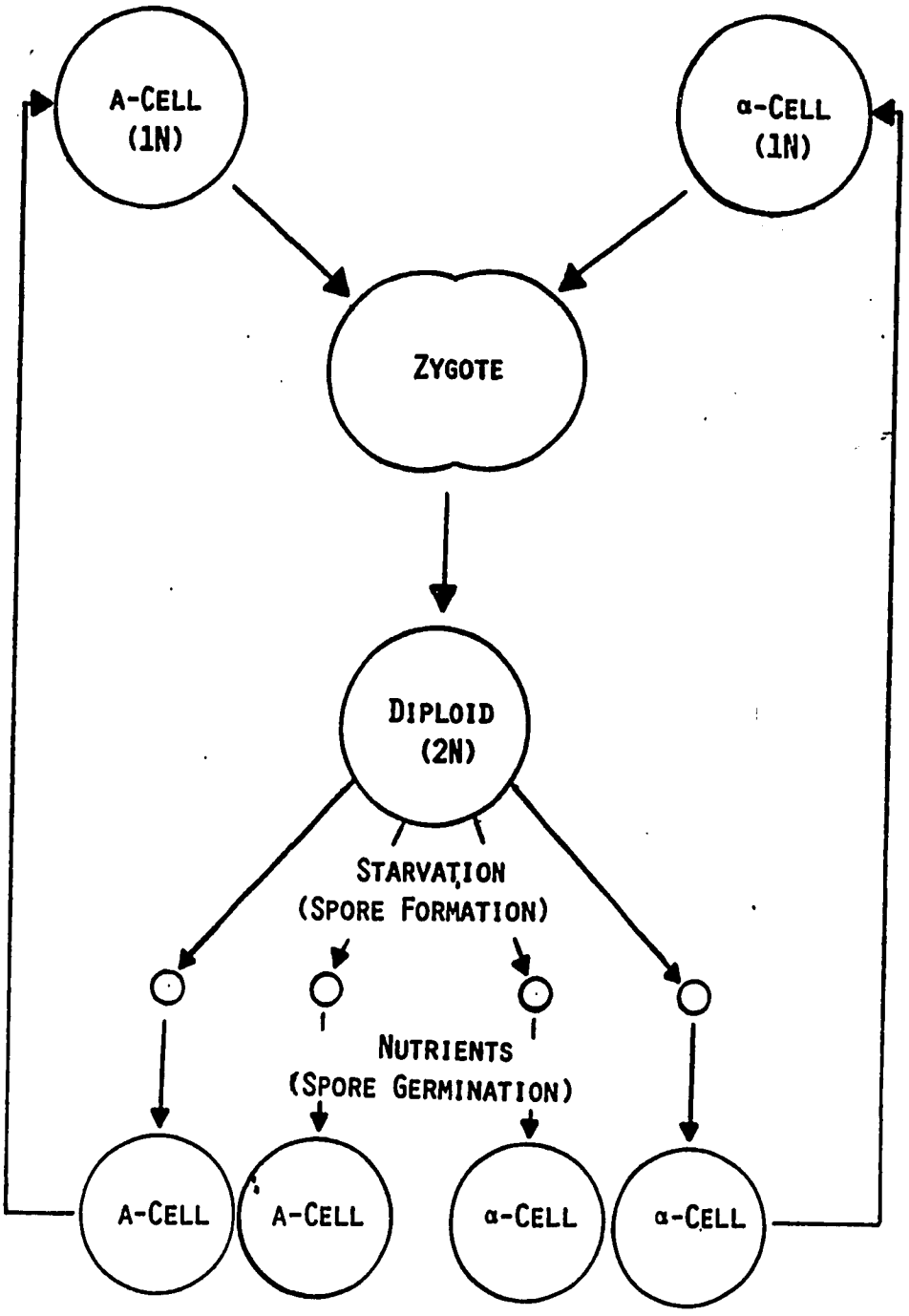
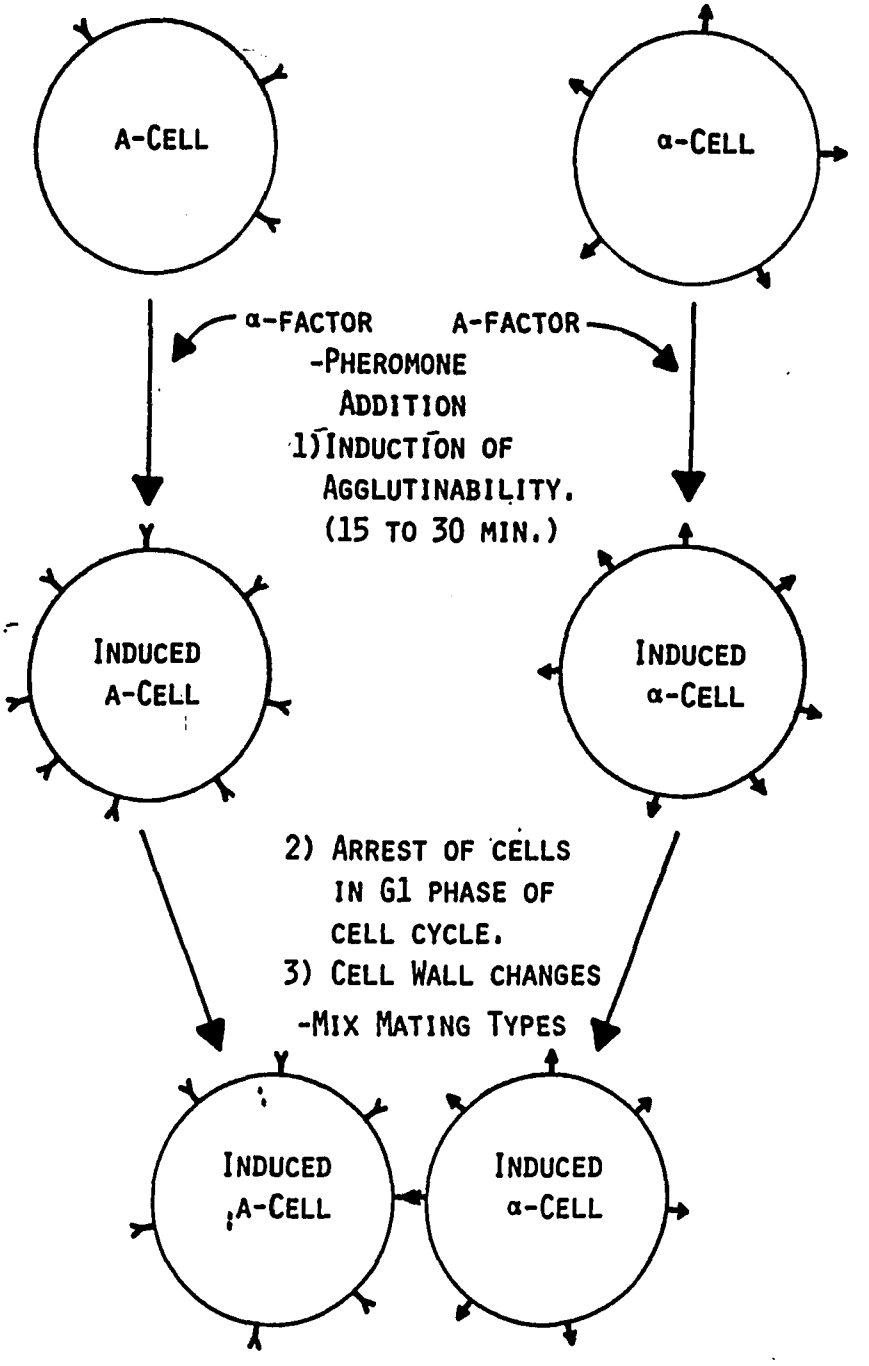

A-CELL
(1N)
α-CELL
(1N)
ZYGOTE
DIPLOID
(2N)
STARVATION
(SPORE FORMATION)
NUTRIENTS
(SPORE GERMINATION)
A-CELL
A-CELL
α-CELL
α-CELL

Figure 2. Pheromone action on haploid cells. Haploid cells exposed to pheromone from the opposite mating type undergo a series of morphological and physiological changes which include: induction of agglutinability, cell cycle arrest and cell wall changes. Haploid cells exposed to pheromone from the opposite mating type are referred to as induced cells.


A-CELL
α-CELL
α-FACTOR
-PHEROMONE
ADDITION
A-FACTOR
1) INDUCTION OF
AGGLUTINABILITY.
(15 TO 30 MIN.)
INDUCED
A-CELL
INDUCED
α-CELL
2) ARREST OF CELLS
IN G1 PHASE OF
CELL CYCLE.
3) CELL WALL CHANGES
-MIX MATING TYPES
INDUCED
A-CELL
INDUCED
α-CELL
"AGGLUTINATION"

and α cells express α-agglutinin. Agglutinin expression is constitutive but can be induced by the action of peptide pheromones secreted by the opposite mating type (Fig. 2 and see below). The modulation of agglutinin expression in response to pheromone is likely to be controlled by DNA binding proteins which regulate transcription of these genes (Fig. 3). Once stable association is formed between an a and α cell, the cells fuse to form a zygote (Fig. 1).

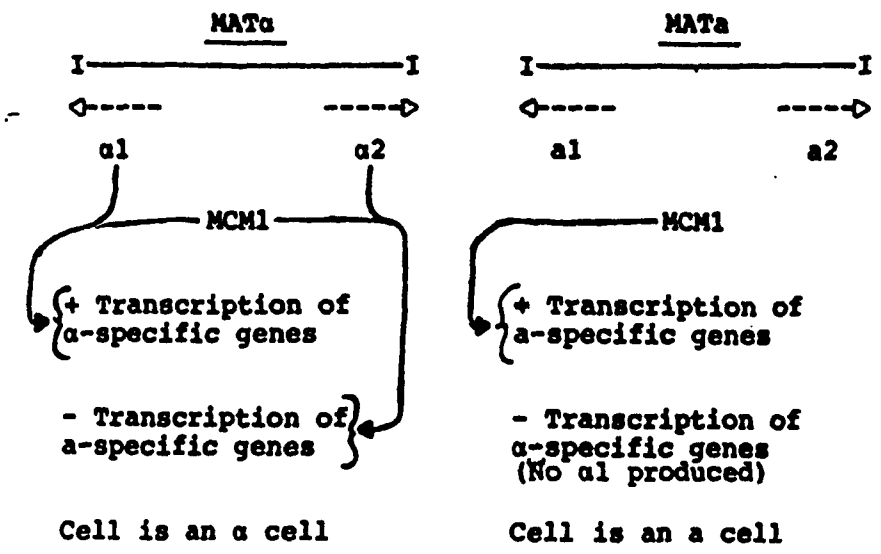
3. CONTROL OF MATING TYPE

A single locus on chromosome III determines whether a haploid cell is an a or α cell. This locus is designated *MAT* (Herskowitz and Oshima 1981), is transcriptionally active, and contains a copy of either *MAT*a or *MAT*α.

Two other loci; on chromosome III, *HML* and *HMR*, also have a copy of either *MAT*a or *MAT*α. However, unlike *MAT*, these loci are transcriptionally inactive and play no direct role in determining the mating type of the cell. Instead, *HML* and *HMR* are storage loci which allow the cell to maintain a copy of *MAT*a and *MAT*α so the cell has the potential to switch mating type from α to a or a to α.

The two alternative *MAT* alleles *MAT*α and *MAT*a control the mating type of the cell because these loci code for proteins which (in association with

Figure 3. Control of mating-type-specific genes in *Saccharomyces cerevisiae*.


MATα
I
I
α1
α2
MCM1
+ Transcription of α-specific genes
- Transcription of a-specific genes
Cell is an α cell
MATa
I
I
a1
a2
MCM1
+ Transcription of a-specific genes
- Transcription of α-specific genes (No α1 produced)
Cell is an a cell

other proteins) either suppress or induce the expression of mating type specific genes such as pheromones and agglutinins (Fig. 3) (Nakagawa and Yanagishima 1982, Manney et al. 1983).

4. TRANSCRIPTIONAL CONTROL OF MATING TYPE GENES

Part of the *MAT* locus differs in two alleles containing segments of *MATa* and *MATα*. The *MATα* segment encodes for two DNA binding proteins: α1 and α2 (Fig.3). α1 is a positive regulator of α-specific gene expression. α1 binds (in association with MCM1 protein) to a control region upstream of α-specific genes to promote transcription. Specifically, α1 binds to a 10 bp sequence, known as the Q box, with the aid of MCM1. MCM1 (also known as PRTF) binds to a 16 bp near palindromic sequence (known as the P box) which is adjacent to the Q box (Bender and Sprague 1987, Jarvis et al. 1988, Tan et al. 1988). α1 and MCM1 function in a cooperative fashion to bind to their respective palindromic sequences. Both proteins are necessary to promote transcription of α-specific genes (Bender and Sprague 1987). When a perfect palindromic sequence of the P box is placed upstream of a reporter gene, transcription occurs without α1. This result indicates that MCM1 is the transcriptional activator and that α1 aids only in the binding of MCM1 to the near palindromic sequence found in α-specific genes (Jarvis et al. 1988).

The protein product of *MAT*α2, on the other hand, is a suppressor of a-specific gene expression because it binds (again in conjunction with MCM1 protein) to a sequence upstream of a-specific genes and inhibits transcription (Johnson and Herskowitz 1985). α2 may inhibit transcription by preventing MCM1 from making proper contact with the P box of a-specific genes (Jarvis et al. 1988). In summary, α1 is a transcriptional activator of α-specific genes while α2 is a repressor of a-specific genes. Thus a cell which expresses *MAT*α is functionally an α cell.

*MAT*a does not produce either α1 or α2 (Fig. 3). In the absence of α1, α-specific genes are not transcribed. In the absence of α2, repression of a-specific genes is relieved. Transcription of a-specific genes occurs because MCM1 protein is still produced and functions as a transcriptional activator (Jarvis, Hagen and Sprague, 1988; Tan et al. 1988). Thus, such a cell is an a cell.

The *MAT*a segment codes for two possible products: a1 and a2 (Fig. 3). However, neither of these genes have been shown to play a role in the transcription of mating type-specific genes in haploid cells. In diploid cells, a1 acts in concert with α2 to repress transcription of the *RME* gene (a negative regulator of meiosis) (Mitchell and Herskowitz 1986), and possibly (in cooperation with the *AAR1* gene product) inhibits

transcription of α1. (Harashima et al. 1989). Although it has been widely believed that a1-α2 represses expression of haploid-specific genes (Herskowitz 1989), there is new evidence suggesting that the product of the *AAR1* gene is involved in regulating haploid-specific gene expression (Harashima et al. 1989).

5. PHEROMONES

Haploid cells secrete peptide pheromones known as sex factors (Duntze et al. 1970). α-factor, which is 13 amino acids in length (Stotzler et al. 1976), is produced by α cells and binds to a receptor located on the plasma membrane in a/a cells. The receptor is the product of the *STE2* gene (Burkholder and Hartwell 1985) and is expressed only on a/a cells. Conversely, a-factor is 12 amino acids in length (Betz et al. 1987), and has a farnesyl group attached to its carboxy terminus cysteine residue (Anderegg et al. 1988). a-Factor is produced by a/a cells and binds to a receptor (product of the *STE3* gene, Hagen et al. 1986) located on the plasma membrane in α cells.

The pheromones are made as larger precursors (Kurjan and Herskowitz 1982, Brake et al. 1985). In the case of α-factor, the precursor is cleaved by the action of proteases to generate smaller, mature, biologically active peptides. Products of the *KEX2* (Fuller et al. 1985), *STE13* (Julius et al. 1984) and *KEX1*

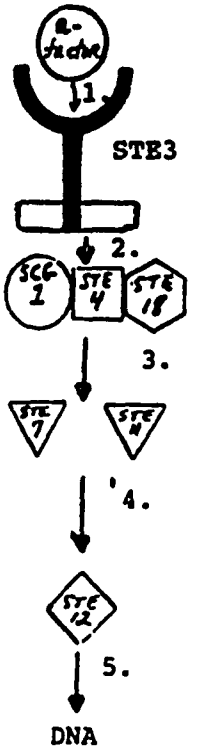
(Dmochowska et al. 1987) genes process precursors of α-factor. Similarly, it is likely that the products of the *STE6*, *STE14* and *STE16* genes process precursors of a-factor (Cross 1988). The expression of pheromone is constitutive but their expression increases if cells are induced with pheromone from the opposite mating type (Strazdis and MacKay 1983, Achstetter 1989).

Once pheromone has bound to receptor, the cell undergoes morphological and physiological changes which prepare haploid cells of one mating type to mate with cells of the opposite mating type (Fig. 2). Changes induced by pheromone include: cell cycle arrest in the G1 phase of the cell cycle (Duntze et al. 1970), cell wall changes (Lipke et al. 1976) and an increase in the agglutinability and expression of agglutinin (Betz et al. 1978; Terrance and Lipke 1981, 1987; Watzele et al. 1988; Wojciechowicz and Lipke 1989).

6. MECHANISM OF PHEROMONE ACTION (Fig. 4)

Upon α-factor binding to receptor, the receptor-ligand complex is internalized (Chvatchko et al. 1985) and it appears that both ligand and receptor are degraded (Jenness and Spatrick 1986). Pheromone receptors possess seven potential transmembrane spanning domains. In this, they are structurally similar to other receptors (rhodopsin and beta-adrenergic receptor) which transduce their signal through the action of G proteins. The

Figure 4. Hypothetical pathway of intracellular signalling by pheromone.



1. a-factor binds to receptor (STE3).

2. factor binding causes signal to be transmitted to G protein: SCG1, STE4 and STE18 peptides comprise G protein.

3. G protein effects a change in suspected protein kinases STE7 and STE11.

4. Change in STE7 and/or STE11 catalytic specificity/activity causes phosphorylation of STE12, a putative DNA binding protein.

5. Phosphorylation of STE12 by STE7 or STE11 causes a change in the specificity of binding of STE12 to DNA.

SCG1 gene (*GPA1*), which shows homology to the α subunit of mammalian G proteins, has been cloned and shown to be necessary for pheromone mediated responses (Dietzel and Kurjan 1987, Miyajima et al. 1987). Additionally, the *STE4* gene shows homology to the β subunit and the *STE18* gene exhibits weak homology to the γ subunit of mammalian G proteins (Whiteway et al. 1989). Null mutations in either *STE4* or *STE18* supress mutations in *SCG1*. A Null mutation in *SCG1* is haploid specific. Such a mutant is large and grows slowly. This phenotype resembles those of cell-cycle "start" mutants and suggests that *SCG1* is involved in the pheromone response pathway (Dietzel and Kurjan 1987). The observation that null mutations of *STE4* and *STE18* suppress a null mutation in *SCG1* suggests that *STE4* and *STE18* are also involved in the pheromone response pathway (Fig. 4) (Whiteway et al. 1989).

Products of the *STE7* (Teague et al. 1986) and *STE11* (Herskowitz 1989) genes (suspected protein kinases), appear to play a role in pheromone mediated responses downstream of G protein involvement (Fig. 4).

There is evidence suggesting that the product of the *STE12* gene, a putative DNA binding protein (Dolan et al. 1989) is involved in setting the constitutive level of expression of mating type-specific genes (Fields and Herskowitz 1985, Fields et al. 1988). STE12 protein has

also been shown to bind to an upstream regulatory sequence necessary for the induction of gene expression in response to pheromone (Dolan et al. 1989). This sequence has been referred to as the pheromone response element (Kronstad et al. 1987).

The observation that the *STE12* product binds to the pheromone response element suggests that *STE12* may also play a role in modulating the expression of mating type specific genes in response to pheromone. It is thought that binding to this sequence by the STE12 protein increases the transcriptional rate of pheromone inducible genes (which may account for the inducible expression of a and α-agglutinin) (Dolan et al. 1989). It has been shown that the *STE12* product interacts with the MCM1 product to bind to an upstream region of a mating type a-specific gene (Herskowitz 1989). This finding further complicates the role of *STE12* in regulating the expression of mating type-specific genes. It is possible that STE12 protein interacts with one or more proteins to modulate the constitutive level of transcription and interacts with a second set of proteins to modulate the inducible level of expression of the same gene during pheromone induction.

Null mutations in *STE7*, *STE11* or *STE12* decrease the transcriptional rate of mating type specific genes (Fields et al. 1988). Null mutations in all three genes show the same decrease in the level of

transcription of a mating type specific gene as a single null mutation in any one of the three genes. This data suggests that the dose effect on the level of transcription by the three genes is not additive. This implies that the three genes may act through a single pathway. This raises the possibility that *STE7* and *STE11* products may act by phosphorylating STE12 protein (Dolan et al. 1989). It is speculated that phosphorylation of STE12 protein by *STE7* or *STE11* product increases the ability of STE12 protein to activate transcription. In summary, G proteins, protein kinases and DNA binding proteins have been implicated in modulating the expression of mating type specific genes in the pheromone response pathway (Fig. 4).

7. STRUCTURE AND FUNCTION OF THE AGGLUTININS

A number of agglutinins from various species of yeast have been biochemically characterized. These include: a and α-agglutinin from *Saccharomyces cerevisiae* (Yoshida et al. 1976, Terrance et al. 1987, and Watzele et al. 1988), the 16-cell agglutinin (a-agglutinin) and the 17-cell agglutinin (α-agglutinin) from *Saccharomyces kluyveri* (Pierce and Ballou 1983, Weinstock and Ballou 1986, and Lasky and Ballou 1988), the 5-cell agglutinin (a-agglutinin) and the 21-cell agglutinin (α-agglutinin) from *Hansenula wingei* (Burke et al. 1980) and the a and α-agglutinins from

Pichia amethionina (Mendonca-Previato et al. 1982). Due to differences in nomenclature, the agglutinins from *Pichia amethionina* are of the opposite designation as that found in *Saccharomyces cerevisiae*.

7A. Biochemical Characterization of α-Agglutinins

a. *Saccharomyces cerevisiae*

α-agglutinin from *Saccharomyces cerevisiae* has been isolated (Yamaguchi et al. 1982, Terrance et al. 1987). The glycoprotein is approximately 130 to 200 kD in size (as determined by gel electrophoresis) with 50 to 75 % of the total mass comprised of carbohydrate (both N and O linked). Thus, the size of the peptide portion of α-agglutinin is approximately 50 to 100 kD. Yamaguchi et al. found an intracellular form of agglutinin (200 kD) which has a higher molecular weight than agglutinin isolated from the cell wall (130 kD). They ascribe the difference in size to differences in glycosylation. Deglycosylation of agglutinin with endo H produces multiple species of peptide: 38, 72, 105, 145 and 160 kD. All these species are capable of binding to a cells (Terrance et al. 1987). Assuming that all N-linked sites are accessable to endo H, this result indicates that N-linked carbohydrate is not involved in binding.

Maximum association of α-agglutinin with a-agglutinin occurs at a pH of 5.5. (Terrance and Lipke 1981). Binding of α-agglutinin to a-agglutinin is reversible at pH 9.0 (Terrance and Lipke 1981). Binding is perturbed by ionic detergents (0.05 % SDS) or salt (5 M NaCl) but not by non-ionic detergents (1% Triton X-100). These results suggest that ionic interactions may play an important role in α-agglutinin function. The binding activity of the glycoprotein is sensitive to heat and proteases but unaffected by treatment with 2-mercaptoethanol and periodate (Terrance and Lipke 1981, Yamaguchi et al. 1982).

α-agglutinin binding is monovalent (has only one binding site per molecule) (Terrance and Lipke 1981, Yamaguchi et al. 1982). α-agglutinin exhibits two states of binding: a weak and strong state (Lipke et al. 1987). The association constant for the strong state is 10^9 liters/mol. Glycoprotein components responsible for the two states of binding are identical since weakly bound material can be dissociated from a cells and found to exhibit strong binding when reassociated with a cells.

b. *Saccharomyces kluyveri*

α-agglutinin from *Saccharomyces kluyveri* is similar to that of *Saccharomyces cerevisiae* (Pierce and Ballou 1983, Weinstock and Ballou 1986, Lasky and Ballou 1988). α-agglutinin extracted by treatment of

whole cells with glucanase has a molecular weight of approximately 400 kD. Agglutinin extracted from cells with Zymolase (glucanase contaminated with protease) results in the isolation of several forms of agglutinin having molecular weights of 40, 60 and 134 kD. When the 400 kD form is exposed to *Oerskovia xanthineolytica* protease, species of 60 and 150 kD are generated. This result suggests that the smaller peptides are proteolytic fragments of the larger species. The glycoprotein contains both N and O-linked sugar. The 400 kD species is 84 to 90 % carbohydrate by weight. The 134 kD species is 75 % carbohydrate and the 60 kD species is 46% carohydrate by weight. The peptide molecular weight of the 60 kD species is therefore 32 kD. The apparent molecular weight of the 60 kD species is not affected by treatment with endo H. This result indicates that this species lacks N-linked carbohydrate. This finding also suggests that N-linked carbohydrate does not play a role in binding because the 60 kD species possesses binding activity.

All the isolated forms of *Saccharomyces kluyveri* agglutinin have binding activity. The binding of the glycoprotein is monovalent and sensitive to heat (60°C for 30 min.) and proteases (trypsin and pronase) but resistant to mannosidases. Optimum pH for binding of $\underline{a}$ and α cells is pH 4.5. Binding of the two mating types is not inhibited by Triton X-100 (10%) or

salt (2 M NaCl). Amino acid analysis of the 60 kD active fragment reveals that it contains large amounts of serine and threonine and is acidic in nature. There are 5 x 10^5 molecules of agglutinin expressed per α-cell. The association constant of the glycprotein is 1 x 10^9.

c. *Pichia amethionina*

The a-factor-Z from *Pichia amethionina* (Burke et al. 1980, Mendonca-Previato et al. 1982) is highly glycosylated (about 90% carbohydrate by weight) and is greater than 200 kD in size. Upon subtilisin treatment a 27 kD active peptide is generated. Amino acid analysis of the active peptide reveals that it is rich in serine and threonine and is acidic in nature. The agglutinin is sensitive to heat (100°C for 40 minutes).

d. *Hansenula wingei*

Agglutinin from *Hansenula wingei* 21-cells (Burke et al. 1980) is similiar to a-factor-Z agglutinin isolated from *Pichia amethionina* in that an active 27 kD peptide which has binding activity has been purified. The peptide is purified from cells treated with trypsin and is therefore probably a proteolytic fragment of a larger form. This is substantiated by the observation that cells treated with Zymolase release an active species with a higher molecular weight.

The 27 kD peptide contains little carbohydrate (5% mannose). Amino acid analysis of the 27 kD peptide indicates a high proportion of serine and threonine residues. The peptide is also acidic and its binding to *H. wingei* 5-cells is pH dependent. This suggests that the acidic residues on α-agglutinin may be critical to function. The binding activity of α-agglutinin is sensitive to heat but resistant to reduction.

e. Summary

Active peptides of α-agglutinin isolated from the various species have been shown to be heterogenous in molecular weight and carbohydrate content (Table 1). However, comparative examination of the agglutinins reveals certain similarities: 1) α-agglutinin from each species is generally heat labile, sensitive to proteolysis and resistant to treatment with periodate (carbohydrate oxidizing agents) and 2-mercaptoethanol (reducing agents)(Table 1) 2) Active peptides from these species tend to be rich in serine and threonine and acidic in nature and 3) The binding activity of α-agglutinin appears to be optimum at an acidic pH (4.5 to 5.5), suggesting that charged amino acids (possibly acidic residues) on α-agglutinin may be important in binding to a-agglutinin.

7B. Biochemical Characterization of a-Agglutinins

Table 1

Comparison of Biochemical Characteristics of the α-Agglutinins

	Saccharomyces cerevisiae	*Saccharomyces kluyveri*	*Hansenula wingei(1)*	*Pichia amethionina*
Molecular weight (kD):	150-200	400	27 (Fragment)	>200
Carbohydrate Content (%):	50-75	84-90	5% (Fragment)	90
N-linked:	YES	YES	nd	Likely
O-linked:	YES	YES	Likely	Likely
Binding Sites:	1	1	1	1
Sensitivity to 1) Reducing Agents:	NO	NO	NO	NO
2) Periodate:	NO	nd(2)	nd	nd
3) Proteases:	YES	YES	nd	Likely
4) Heat:	YES	YES	YES	YES

nd= Not Determined

(1)= The 27 kD peptide from *H. wingei* probably represents a proteolytic fragment of a higher molecular weight form of agglutinin.

(2)= Not determined but probably resistant to periodate since α-mannosidases to not diminish binding activity.

a. *Saccharomyces cerevisiae*

a-agglutinin has been isolated from from *Saccharomyces cerevisiae* (Yosida et al. 1976, Yamaguchi et al. 1984, Watzele et al. 1988). When a-agglutinin is extracted from cells by reducing agents an active peptide of 23 kD is generated (Orlean et al. 1986). This peptide contains 29% O-linked carbohydrate by weight. Treatment of the peptide with hydrogen fluoride generates a 13 kD deglycosylated species (Watzele et al. 1988).

The binding of the peptide is monovalent and its activity resistant to heat and endo H but sensitive to treatment with periodate. This suggests that O-linked carbohydrate on the peptide may be important for function.

The 23 kD peptide represents the binding fragment of a-agglutinin since it has been demonstrated that in other yeasts, especially *Saccharomyces kluyveri* and *Hansenula wingei* (see below), treatment with reducing agents releases small peptides which have binding activity. The observation that mutants in a-agglutinin expression fall into at least two complementation groups implies that a-agglutinin may be composed of at least two non-identical subunits (Roy et al. 1990). It is believed that one subunit is the binding fragment and the other is the core peptide which anchors the binding peptide to the

cell surface. Because the binding peptide can be released from the cell surface by reducing agents, it is thought that the binding peptide is bound to the core peptide by a disulfide linkage. Amino acid sequencing of the 23 kD fragment shows that the molecule contains a single cysteine residue.

Yamaguchi et al. isolated an intracellular form of a-agglutinin which is approximately 130 kD. During isolation they did not use reducing agents which would dissociate the binding fragment from the core fragment. Therefore, it is possible that this large molecular weight form of a-agglutinin is a complex which contains both the core and binding fragment.

A polyclonal antibody to the binding fragment was produced (Watzele et al. 1988). Using this antibody; it has been shown that a-agglutinin expression is induced in response to α-factor. This results in a significant increase in cell surface expression of the binding fragment of a-agglutinin (as detemined by immunofluorescence). Upon induction of cells with α-factor, the binding fragment of a-agglutinin is first expressed on the cell surface in the region of bud formation. The binding fragment is expressed first on the growing bud because this is the site where new cell wall synthesis occurs.

b. *Saccharomyces kluyveri*

a-agglutinin from *Saccharomyces kluyveri* (Pierce and Ballou 1983) is similar to a-agglutinin from *Saccharomyces cerevisiae*. a-agglutinin extracted from cells with Zymolase has a molecular weight of 500 kD and is 95% carbohydrate by weight. Treatment of this glycoprotein with reducing agents releases a smaller binding fragment which has binding activity. A binding fragment can be liberated from a cells by reducing agents which has a molecular weight of 35 kD. A *mnn1* mutant of a cell (a mutant defective in terminal glycosylation) liberates an active fragment of 25 kD. *mnn1* mutants glycosylate cell wall mannoprotein aberrantly. Instead of attaching a maximum of eight side chain mannose residues, the mutant can only attach a maximum of three. The 25 kD fragment is 30 % carbohydrate. Treatment of the 25 kD peptide with trypsin reduces its molecular weight to 16 kd without loss of binding activity.

The binding activity of this peptide is heat stable (100°C for 1 hour) but labile to α-mannosidases and treatment with periodate. The peptide binding activity is monovalent. The susceptability of the peptide suggests that, like the a-agglutinin from *Saccharomyces cerevisiae*, it is the carbohydrate portion of the molecule which confers activity. The activity of a-agglutinin isolated from the *mnn1* mutant suggests that there can be variation in the carbohydrate content without loss of binding activity.

c. *Pichia amethionina*

Agglutinin from *Pichia amethionina* (designated α-agglutinin by the authors, Mendonca-Previato et al. 1982) is isolated as a large molecular weight species which is comprised of 80% carbohydrate. It is rich in serine and threonine residues (over 50%). Unlike the *Saccharomyces* agglutinins, the binding activity of this agglutinin is multivalent. The agglutinin is sensitive to treatment with 2-mercaptoethanol. This result suggests that the agglutinin is comprised of a binding fragment and core fragment which are associated through a disulfide linkage. The binding activity of the agglutinin is heat stable.

d. *Hansenula wingei*

Agglutinin from Type 5 cell of *Hansenula wingei* (Taylor et al. 1968; Taylor and Orton 1971; Yen and Ballou 1974) is a large molecular complex. Upon treatment with reducing agents the complex separates into a large molecular weight and numerous small molecular weight species. The smaller molecular weight species exhibit activity but the larger component is relatively inactive. It is likely that the small molecular weight species of about 12 kD represents the binding fragment and that the larger fragment represents the core fragment. The small fragments are made of predominantly carbohydate with a

small peptide backbone. Amino acid analysis reveals that the active form is comprised of peptide which is over 60% serine and threonine.

The binding activity of the intact species (unreduced) is heat stable (100°C for 30 min.) but sensitive to exo-α-mannanase. The association constant for binding is 4 x 10^8 liters/mol. Again, this data suggests that it is the carbohydrate portion of the molecule which confers binding activity.

e. Summary

It appears that a-agglutinin is comprised of a binding fragment and a core fragment. The binding fragment is associated with the core fragment through a disulfide linkage. In the genus *Saccharomyces*, a-agglutinin activity is monovalent while in *Hansenula* and *Pichia*, a-agglutinin activity is multivalent (Table 2). In all cases, there is evidence to suggest that the carbohydrate portion of the molecule is important for function.

8. MECHANISM OF AGGLUTINATION

The similarities seen in the susceptibilities of each type of agglutinin to physical and chemical agents suggest a molecular mechanism by which the agglutinins function. The observation that a-agglutinin loses its binding ability upon treatment with periodate suggests

Table 2

Comparison of Biochemical Characteristics of Binding Peptides from the a-Agglutinins*

	Saccharomyces cerevisiae	*Saccharomyces kluyveri*	*Hansenula wingei*	*Pichia amethionina*
Molecular weight (kD):	23	35	12	nd
Carbohydrate Content (%):	30	30-40	50	nd
N-linked:	NO	Not likely	Not likely	nd
O-linked:	YES	Likely	Likely	Likely
Binding Sites:	1	1	1(1)	1(1)
Sensitivity to 1) Reducing Agents:	YES	YES	YES	YES
2) Periodate:	YES	YES	nd	nd
3) Proteases:	Generally resistant to protease treatment			
4) Heat:	NO	NO	NO	NO

nd= Not Determined

*In all cases, the binding fragment is liberated from a larger molecular weight complex by treatment with reducing agents. The complex contains the binding fragment plus core fragment (see text for details). The complex is large (has a molecular weight greater than 400 kD) and is highly glycosylated (generally greater than 80 % carbohydrate by weight).

(1)= In these species, the complex (binding plus core fragment) is multivalent.

that carbohydrate integrity is important for function. Conversely, the binding activity of α-agglutinin is lost if exposed to protease but not altered by treatment with periodate or endo H. These results suggests that the peptide portion of the molecule is important for activity and that the carbohydrate portion is not essential for function. Collectively, this data suggests that α-agglutinin is a lectin for a-agglutinin.

Active peptides of α-agglutinin from *Hansenula wingei* (Burke et al. 1980), *Saccharomyces kluyveri* (Pierce and Ballou, 1983) and *Pichia amethionina* (Mendonca-Previato et al. 1980) contain a significant number of acidic residues. This coupled with the observation that the binding of α-agglutinin in *Saccharomyces cerevisiae* is optimum at an acidic pH (Terrance and Lipke 1981) suggest that acidic residues on α-agglutinin play an important role in binding to a-agglutinin.

Materials and Methods

Part 1

STRAINS AND GROWTH: X2180-1A (a) and X2180-1B (α) were grown in minimal medium (Yeast Nitrogen Base with glucose) (YNB) to log phase at room temperature and induced with a or α-factor for 60 minutes as described previously (Terrance and Lipke 1981). Strain W303-1B (α) was grown in enriched yeast extract-peptone-glucose medium (YEPD). W303-1B was induced by adding an equal amount of spent a cell enriched medium (source of a-factor) which had been buffered to pH 4.25 with 1M sodium citrate. Cells were harvested by centrifugation at 1,000 x g for 5 minutes and stored at 4°C in 100 mM sodium acetate, pH 5.5 with 10 ug/ml cycloheximide and (buffer A) at a cell density of 1x 10^8 cells per ml.

GENERAL METHODS: α-agglutinin was isolated as described (Terrance et al. 1987). Treatment of purified α-agglutinin with'endo-N-acetyl glucosaminidase H (endo H) employed 0.001 U of enzyme for 16 hours at 25°C in 10 mM sodium acetate pH 5.5 with 1mM phenyl methyl sulfonyl fluoride (PMSF)(Trimble and Maley 1984). Quantitative agglutination assays were performed as previously described except cells were assayed using 1 x 10^7 cells/ml (Terrance and Lipke 1981). The ability

of cells to agglutinate is defined in terms of their Agglutination Index (AI). AI values vary from 0 to 1 with higher values indicating greater agglutinability (Terrance and Lipke 1981). SDS-Polyacrylamide gel electrophoresis (Laemmli 1970) (SDS-PAGE) was performed employing 7.5% polyacrylamide gels.

PRODUCTION AND ADSORPTION OF ANTI-α-AGGLUTININ: Antibodies to α-agglutinin were produced by a primary subcutaneous injection of 100 ug of endo H treated α-agglutinin in Freund's complete adjuvant followed by booster intravenous injections of 100 ug of treated α-agglutinin in phosphate buffered saline. The antigen contained peptide plus cleaved N-linked carbohydrate. Antiserum was adsorbed against an equal volume of $\underline{a}$ or α cells which were grown in YNB to late log phase, washed in 140 mM NaCl, 10 mM Tris-HCl pH 7.5 and 5 mM EDTA pH 8.0, and heat killed at 60°C for 1 hour. Following incubation at 60°C, cells were washed three times with the same buffer and then incubated with antiserum. The antiserum was adsorbed twice for a total of 12 hours, retrieved by centrifugation, and stored at -20°C.

IMMUNODETECTION OF α-AGGLUTININ BY WESTERN BLOTTING: Transfer of protein from polyacrylamide gels to nitrocellulose membranes was carried out in a buffer consisting of 25 mM Tris, 192 mM glycine and 20% methanol

pH 8.3 as described by Towbin et al. (1979). Transfer was for 1 hour at 100 volts using a Trans-Blot Cell (Bio-Rad, Richmond, Ca.) in which 1.5 liters of buffer was added. The transfer of protein to nitrocellulose membrane (pore size 0.45 um) was monitored by including Coomassie stained molecular weight standards when performing SDS-PAGE. Antibody dilutions and washings employed 140 mM NaCl, 10mM Tris, pH, 7.4 (buffer B). All incubations and washings were performed on a platform rotator at room temperature. Blots were first incubated in buffer B with 3% (w/v) bovine serum albumin (BSA) for 30 minutes followed by incubation in anti-α-agglutinin (diluted 1:100) for 2 hours. The blots were washed and incubated in goat anti-rabbit IgG (diluted 1:100) for 30 minutes followed by washing and incubation in rabbit peroxidase-anti-peroxidase (PAP) (diluted 1:1000) for 45 minutes. Blots were washed and placed in substrate to visualize bound antibody. Substrate was made by first placing 10 mg of 4-chloro-1-napthol in 5 ml of cold methanol. This mixture was then added to 15 ml of buffer B to which 600 ul of 3% (v/v) hydrogen peroxide had been added.

ANTIBODY INHIBITION ASSAY: Cells were equilibrated in 140 mM NaCl, 10 mM Tris, pH 7.4 with 10 ug/ml cycloheximide (buffer C). 100 ul of α cell suspension was centrifuged (1000 x g for 5 minutes) in 13 x 100 mm glass tubes and

the cells were resuspended in 200 ul of antiserum diluted in buffer C. This mixture was incubated for 90 minutes at room temperature on a platform rotator. The cells were centrifuged and the spent antiserum removed. The cells were washed once in 500 ul of buffer C and resuspended in 2.9 ml of buffer A. a Cell suspension (100 ul) was then added. The cells were mixed and centrifuged (1,000 x g for 5 minutes). Cells were resuspended using a constant speed mixing device and left undisturbed for 20 minutes. The turbidity of the mixture was determined using a Spectronic 21 spectrophotometer at 660 nM (Terrance and Lipke 1981). To demonstrate competition for antibody inhibition by purified α-agglutinin, increasing amounts of α-agglutinin (equilibrated in buffer C) were added simultaneously to α cells and antibody prior to incubation. The remainder of the inhibition assay was carried out as described above.

INDIRECT FLUORESCENT ANTIBODY ASSAY: Cells from 25 ul of suspension were washed in buffer C and incubated in anti-α-agglutinin.(diluted 1:20) in 100 ul of 140 mM NaCl, 30 mM Tris, pH 7.4, 10 ug/ml cycloheximide with 1 % gelatin (w/v) (buffer D). After a 90 minute incubation on a platform rotator, the cells were washed 3 times in 500 ul of buffer D and incubated for 45 minutes in 50 ul of anti-rabbit IgG conjugated with fluorescein (diluted 1:10). The cells were washed 3 times in 500 ul of buffer

D and resuspended in deionized water. The cells were then added to microscope slides coated with 1% (w/v) polylysine (70 kD) and allowed to adhere for 10 minutes. Slides were then washed with deionized water to remove unbound cells. Cells were observed and photographed (2 minute exposure, 800 ASA) using Tri-X-pan 400 film (Kodak) employing a UV microscope (Zeiss). To demonstrate antibody specificity with adsorbed antibody, α-agglutinin adsorbed antibody was prepared by incubating 5 ul of a-cell-adsorbed anti-α-agglutinin with 270 ng (50 ul) of purified α-agglutinin (equilibrated in buffer C) overnight at 4°C. This mixture was then used directly in the indirect fluorescence antibody assay.

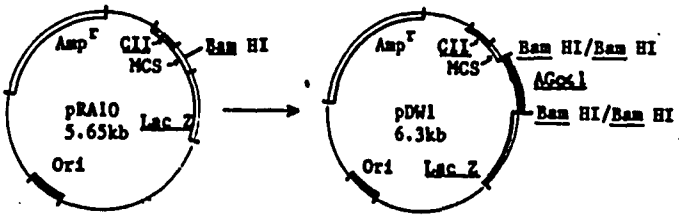
ENZYME IMMUNOASSAY: Antibody incubations in 13 x 100 mm tubes were identical to that described in the flourescent antibody assay except that the labelled second antibody was a 1:250 dilution of anti-rabbit IgG conjugated to alkaline phosphatase. Upon completion of the second antibody incubation, the cells were washed 3 times with 500 ul of buffer D and placed in 800 ul of substrate solution (0.1 M glycine, pH 10.4, 0.01 mM $CaCl_2$, 0.01 mM Mg_2SO_4, and 1 mg/ml of p-nitrophenyl phosphate). The mixture was then placed at 37°C for 1 hour. NaOH (1M, 200ul) was added followed by 2 ml deionized water. The cells were centrifuged (1000 x g for

5 minutes) and the absorbance of the supernatant was determined at 405 nm. Antibody concentrations used were in 5-fold excess over the amount of antigen being assayed.

Part 2

CONSTRUCTION OF PLASMID CODING FOR FUSION PROTEIN: A 670 bp *Bam* HI fragment encoding 226 amino acids from within the *AGα1* open reading frame (ORF) was isolated by GeneClean (Bio 101, La Jolla, Ca.) and subcloned into the *Bam* HI site of pRA10 to create pDW1. The region of the ORF subcloned and expressed in pDW1 is shown in Figure 22. pRA10 is an *E. coli* expression vector which uses the lambda promoter left (pL) for expression of cloned genes (Mallon et al. 1986). To construct pDW1, pRA10 was linearized by digestion with *Bam* HI and its termini dephosphorylated by the addition of calf intestine alkaline phosphatase for 15 minutes at room temperature. Dephosphorylation prevented vector from ligating on itself. The plasmid was then electrophoresed on a 0.8% agarose gel and the band excised and the DNA isolated using GeneClean (Bio 101, La Jolla, Ca.). The 670 bp *Bam* HI fragment of *AGα1* was isolated from pH27 using the same methodology except the dephosphorylation step was omitted. Vector and insert were ligated overnight at 8°C in a 30 ul reaction mixture containing 25 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$,10 mM DTT, 3mM ATP to which 1 unit of T4 DNA ligase had been added. The ligated mixture was then transformed into *E. coli* strain MZ1, which contains a temperature sensitive (ts) cI repressor which allows

expression of fusion protein under the control of pL when the temperature of the culture is raised from 32 to 42°C. pDW2 was constructed by digesting pH27 with *Eco* RI and *Hpa* II and the resulting 1500 bp fragment ligated into pRA10 which had been cut with *Eco* RI and *Nar* I. pDW3 was constructed by digesting pH27 with *Nhe* I and *Hpa* II and the resulting 800 bp fragment ligated into pRA 10 which had been digested with *Xba* I and *Nar* I. Clones harboring plasmid were selected by plating the mixture on Luria agar plates supplemented with 100 ug/ml of ampicillin.



Construction of pDW1 from pRA10

PRODUCTION OF FUSION PROTEIN: Transformants were picked and grown in Luria Broth (LB) with 100 ug/ml ampicillin to an $O.D._{660}$ of 0.2 at which time they were transferred to a 42°C water bath for 1.5 hours to induce the expression of fusion protein. Following induction, cells were centrifuged at 10,000 x g for 5 minutes and the cell pellet stored at -20°C.

ISOLATION OF FUSION PROTEIN: Induced cells from 75 ml of culture were thawed and resuspended in 10 ml of 50 mM EDTA, 1% glucose, 25 mM Tris-HCl pH 7.5, 10 ug/ml lysozyme, 1% Triton-X100 and incubated for 10 minutes at 4°C. This mixture was then centrifuged at 10,000 x g for 5 minutes at room temperature. The gelatinous pellet was then solubilized in 2.5% SDS and equal portions electrophoresed on a 10 % SDS-PAGE gel. Upon completion of electrophoresis, the gel was placed for 30 seconds in Coomassie stain (0.025% (w/v) Coomassie brilliant blue, 50 % methanol, 5% acetic acid). The gel was then washed briefly in water and the prominently staining band, representing fusion protein, was excised from the gel. Gel slices from 2 gels were then cut into 1 mm squares and placed in 2 ml of 150 mM NaCl, 10 mM Tris pH 7.5, 1mM EDTA, 5 mM DTT and 0.01 % (w/v) SDS. This mixture was incubated overnight at room temperature on a platform rotator to facilitate passive elution of fusion protein. Typically, the amount of protein isolated from 2 gels equalled 50 ug.

PRECIPITATION OF PURIFIED FUSION PROTEIN FOR ANTIBODY PRODUCTION: Protein eluted from gel slices was precipitated using ion-pair extraction (Konigsberg and Henderson 1983). 50 ul of eluted protein was precipitated by adding 950 ul of 85% (v/v) acetone, 5% (v/v)

triethylamine and 5% (v/v) acetic acid. This mixture was left for 15 minutes at 0°C. The mixture was then centrifuged at 10,000 x g for 5 minutes at room temperature. The protein pellet was washed once with 500 ul of acetone and air dried at room temperature.

PRODUCTION OF ANTIBODY TO FUSION PROTEIN: Antibodies were raised in female New Zealand White rabbits. 100 ug of precipitated fusion protein was emulsified in 1 ml of Freund's complete adjuvant and injected subcutaneously. This was followed by injection of 100 ug of protein emulsified in Freund's incomplete adjuvant at 10 day intervals. This was repeated three times so that each rabbit received a total of four injections over a period of 40 days. Rabbits were bled weekly and the antiserum stored at -20°C .

PRODUCTION OF CELL EXTRACTS AND WESTERN BLOTTING: Cell extracts of W303-1B and *agα1::LEU2* mutant (*agα1*$^-$) were prepared as follows. Cells from 1 liter of culture in YEPD ($O.D._{660}$ 0.8) were centrifuged at 1,000 x g for 5 minutes and resuspended in 50 ml of 100 mM sodium acetate, 0.03 % Triton X-100 and 1mM p-chloro mercuribenzoate. The suspended cells were added to 10 ml volume of glass beads and disrupted by placing this mixture on a bead beater (Terrance et al. 1987) till 90 % of the cells were broken as determined by

phase contrast microscopy. The mixture was then poured off from the glass beads and then centrifuged at 28,000 x g for 10 minutes. The supernatant (crude cell extract) was collected and stored at -20°C till assayed. Protein quantitation of crude extract was determined using the BCA Protein Assay (Pierce Chemical Co., Rockford, Ill.). For Western blots, 5 ug of cell extract was incubated with 0.001 U of endo H as described in Part 1. Western Blotting was performed as described in Part 1 except that: 1) the primary antibody incubation (with anti-α-agglutinin or antibody to fusion protein) was overnight at room temperature and 2) nitrocellulose was incubated with 3% (w/v) gelatin instead of BSA to prevent non-specific binding by antibody.

INHIBITION OF AGGLUTINATION ASSAY EMPLOYING FUSION PROTEIN: Assay was performed as in Part 1 except that increasing amounts of purified fusion protein were incubated with 15 ul of anti-α-agglutinin for 1.5 hours prior to assay.

ADSORPTION OF ANTI-α-AGGLUTININ FOR WESTERN BLOTTING: 100 ul of α-agglutinin (540 ng) was incubated with 0.003 U of endo H overnight at room temperature as described in Part 1. The following reagents were added to the reaction mixture to the concentrations indicated: EDTA 1mM, pH 8.0, Tris-HCl 10 mM, pH 7.5, NaCl 150 mM. To this final

mixture, 30 ul of anti-α-agglutinin was added and incubated overnight at 4°C. The adsorbed antiserum was used to determine whether purified α-agglutinin could neutralize binding of antibody to fusion protein as determined by Western blotting.

Part 3

PREPARATION OF MINI-PREPARATIONS FOR SCREENING TRANSFORMANTS: Because most of the restriction sites used for the fill-in reactions are recognized by enzymes which do not function well in the presence of contaminants (eg. tRNA), a procedure was developed to isolate plasmid DNA from a small scale culture which yields plasmid DNA which is relatively free of such contaminants. This is a modification of a protocol communicated by Dr. Robert Mallon of Schering-Plough Corporation. 1 ml of an overnight culture was centrifuged at 10,000 x g for 3 minutes and the supernatant discarded. The cell pellet was resuspended in 100 ul of a solution containing 1 % (w/v) glucose, 25 mM Tris-HCl, pH 7.5, EDTA 50 mM, pH 8.0. To this mixture, 200 ul of a solution containing 1% (w/v) SDS and 0.2 N NaOH was added and mixed gently. The mixture was then placed on ice for 10 minutes and 150 ul of 5 M potassium acetate, pH 5.0 was added, mixed gently and the mixture once again placed on ice for 10 minutes. The mixture was then centrifuged for 5 minutes at 10,000 x g and the supernatant (400 ul) transferred to a new tube. 800 ul of cold 95% ethanol was added and the mixture placed on ice for 20 minutes. A centrifugation at 10,000 xg for 5 minutes followed. The supernatant was removed and the pellet dried by placing the tube in a 45°C water bath. The pellet was then dissolved in

250 ul of 50 mM NaCl, 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA, pH 8.0. 200 units (2 ul) of RNase T1 (U.S. Biochemical, Cleveland, Ohio) was then added and the mixture incubated at 37°C for 1 hour. Next, 60 ul of 2 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0 was added followed by the addition of 300 ul of a 50/50 mixture of phenol/chloroform. The mixture was vortexed for 20 seconds and then centrifuged for 5 minutes at 10,000 x g. 250 ul of the top aqueous phase was then removed to a new tube and to this 500 ul of cold 95% ethanol was added. The mixture was placed on ice for 20 minutes followed by centrifugation at 10,000 x g for 5 minutes. The supernatant was discarded and the pellet washed with 500 ul of 70 % ethanol. The pellet was harvested by a brief centrifugation (10,000 x g for 5 seconds). The supernatant was discarded and the pellet was dried by placing the tube in a 45°C water bath for 5 minutes. The pellet was dissolved in 30 ul of water. Typically, 4 to 6 ul of DNA was used for each restriction enzyme digest.

LARGE SCALE PREPARATION OF DNA: This procedure was communicated by Dr. Robert Mallon of Schering-Plough Corporation. Cells were grown in LB with ampicillin overnight (100 to 200 ml cultures) and centrifuged at 5,000 x g for 10 minutes. The pellet was resuspended in 10 ml of 25 mM Tris-HCl, pH 7.5, 50 mM EDTA, pH 8.0 and 1

% (w/v) glucose. To this, 20 ml of 1% (w/v) SDS, 0.2 N NaOH was added and gently mixed. The mixture was then placed on ice for 10 minutes, after which 15 ml of cold 5 M potassium acetate (pH 5.0) was added and gently mixed. The mixture was then placed on ice for 10 minutes followed by centrifugation at 5,000 x g for 5 minutes. To the supernatant (40 ml), 80 ml of cold 95 % ethanol was added and placed on ice for 20 minutes. The mixture was centrifuged at 5,000 x g for 10 minutes and the pellet dissolved in 20 ml of 10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0. Ammonium sulfate, 3.8 g, was then dissolved in the mixture. The mixture was placed on ice for 20 minutes followed by centrifugation at 5,000 x g for 10 minutes. The resulting pellet contained high molecular weight RNA which was discarded. The supernatant was placed in two volumes of cold 95 % ethanol and placed on ice for 20 minutes. The mixture was centrifuged at 5,000 x g for 10 minutes and the pellet dried in a 45°C water bath. The pellet was then dissolved in 2 ml of 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0. To this 500 U (5 ul) of RNase T1 was added and the mixture placed at 37°C for 1 hour. 600 ul of 2 M NaCl, 10 mM Tris-HCl, pH 7.5, 1mM EDTA, pH 8.0 was then added followed by 2.5 ml of a 50/50 mixture of phenol/chloroform. The mixture was then vortexed for 30 seconds and centrifuged at 5,000 x g for 5 minutes. 2 ml of the aqueous phase was removed and placed in 4 ml of cold 95% ethanol. The mixture was

then placed on ice for 20 minutes and centrifuged at 5,000 x g for 5 minutes. The resulting pellet was washed once with 1 ml of 70 % ethanol, centrifuged briefly and air dried in a 45°C water bath. The pellet (plasmid DNA) was then dissolved in 200 ul of sterile deionized water.

TRANSFORMATION OF DNA INTO *E. COLI*: This protocol was communicated by Dr. Robert Mallon of Schering-Plough Corporation. Strain DC646 was made competent by the following procedure. Cells were grown in LB broth (250 ml culture) overnight to an $O.D._{660}$ of 0.4 at which time they were centrifuged at 4,000 x g for 10 minutes. The cells were resuspended gently in 5 ml of 100 mM $CaCl_2$ and placed on ice for 5 minutes. The cells were then centrifuged at 1,000 x g for 5 minutes at 4°C. The cells were then placed in 5 ml of 100 mM $CaCl_2$ overnight at 4°C. 500 ul of sterile glycerol was added and mixed very gently into the cell suspension. 200 ul aliqots were then prepared and frozen at -70°C.

A 200 ul aliquot of competent cells was quickly defrosted and placed on ice. DNA to be transformed was placed in 100 ul of 100 mM $CaCl_2$. To this 100 ul of competent cells was added. The cell suspension was placed on ice for 45 minutes then heat shocked at 42°C for 2 minutes. 1 ml of LB was added and the mixture placed at

37°C for 1.5 hours after which a portion (usually 100 ul) was plated. YEp351 and pRA10 transformants were selected on LB agar plates containing 100 ug/ml ampicillin. pH27 containing transformants were selected on LB agar plates with 100 ug/ml kanamycin.

TRANSFORMATION OF DNA INTO *SACCHAROMYCES CEREVISIAE*: A lithium acetate procedure was used to introduce plasmid DNA (YEp351-*AGc.1*) into *Saccharomyces cerevisiae* (Johnston 1988). Cells (Lc.21) were grown in YEPD (100 ml culture) overnight at room temperature to an $O.D._{660}$ of 0.2. The cells were centrifuged at 4,000 x g for 5 minutes and resuspended in 10 ml of 10 mM Tris-HCl pH 7.5 and 1mM EDTA pH 8.0 and centrifuged at 200 x g for 5 minutes. The cells were resuspended in 1.5 ml of 100 mM lithium acetate, 10 mM Tris-HCl pH 7.5 and 1 mM EDTA pH 8.0. The cell suspension was incubated at 30°C for 1 hour. 200 ul of cell suspension was removed and 2 to 5 ug of plasmid DNA added. The cells were incubated at 30°C for 30 minutes. 1.2 ml of 50% polyethylene glycol was added and mixed gently but thoroughly into the cell suspension. The suspension was then incubated at 30°C for 1 hour. The cell suspension was then heat shocked at 42°C for 5 minutes. The cells were spun down at 10,000 x g for 3 to 5 seconds and the polyethylene glycol removed. Cells were washed twice, each time with 1 ml water to remove traces of

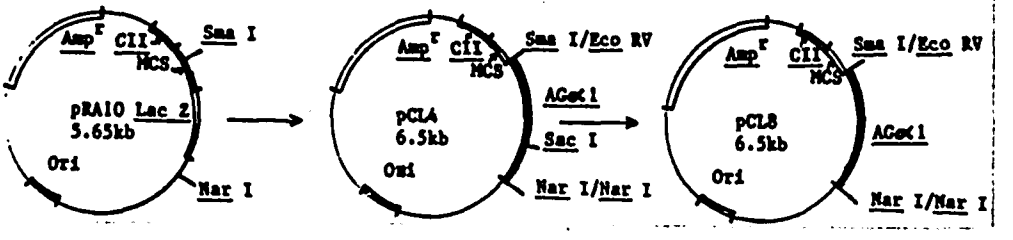
polyethylene glycol. Cells were resuspended in 200 ul water. Each plate received 100 ul of resuspened cells. Cells transformed with YEp351 were selected on YNB plates supplemented with amino acids minus leucine. Cells were plated and transformants detected 2 to 3 days thereafter. Typically, 50 to 300 transformants would result from a transformation employing 5 ug of DNA.

RESTRICTION SITE FILL-IN REACTIONS IN *AGα1*: In most cases pRA10 was used as a shuttle vector to construct specific deletions of restriction sites in the ORF of *AGα1*. Once a restriction site was modified, DNA coding the ORF was cut out of pRA10 and ligated into YEp351-*AGα1* which contains a 4.5 kb *Xba* I-*Hind* III fragment of *AGα1*. This 4.5 kb fragment contains the entire *AGα1* gene and essential upstream sequences. Both pRA10 and YEp351 have ampicillin resistance markers so that transformants containing these plasmids were selected on LB agar with 100 ug/ml ampicillin. All transformations of *E. coli* employed strain DC646, a derivative of HB101.

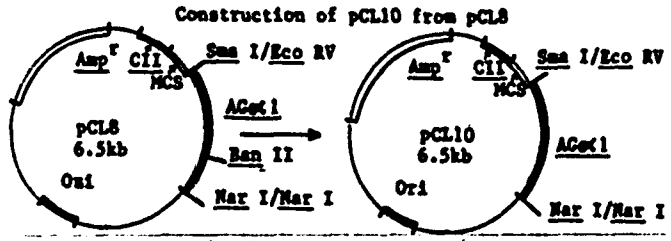
To create pCL4 which contains most of the ORF of *AGα1*, pRA10 was cut with *Sma* I and *Nar* I and YEp 351-*AGα1* was cut with *Nar* I and *Eco* RV. Vector and insert were ligated. pCL4 was used to make the *Sac* I deletion by cutting the plasmid with *Sac* I and filling in the 5' termini with T4 DNA polymerase

under the following conditions: DNA (approximately 500 ng) in 33 mM Tris-acetate, pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 2 mM dNTP's, 1 mM DTT and 2 units of T4 DNA polymerase. The mixture was incubated for 30 minutes at 37°C in a water bath. The DNA was isolated by using GeneClean and then ligated. Transformants were selected and mini-preparations of plasmid DNA prepared to identify a clone which had the *Sac* I site eliminated. pCL8 was identified as being identical to pCL4 except that the *Sac* I site was now missing.

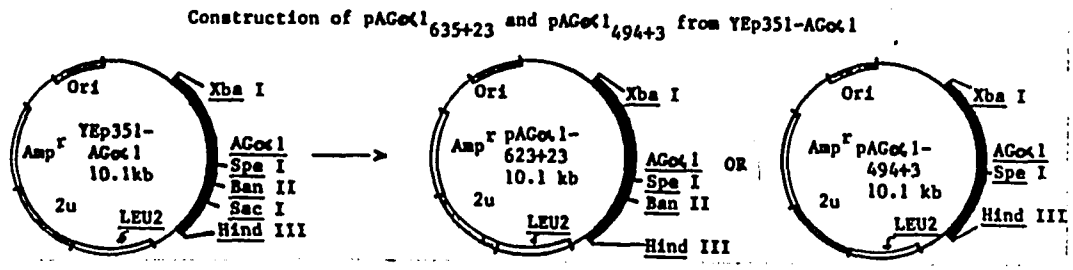
Construction of pCL4 and pCL8 from pRA10



A *Ban* II site was eliminated in the same way as that for the *Sac* I site except that pCL8 was employed. The reason for using this plasmid is that there were originally 2 *Ban* II sites in pCL4 and destroying the *Sac* I site simultaneously eliminated a *Ban* II site. Thus, pCL8 had only one *Ban* II site remaining, the one to be modified. pCL8 was cut with *Ban* II, incubated with T4 DNA polymerase and ligated as described above. Transformants were selected and a transformant identified which had a plasmid with both *Ban* II sites eliminated. This plasmid was designated pCL10.



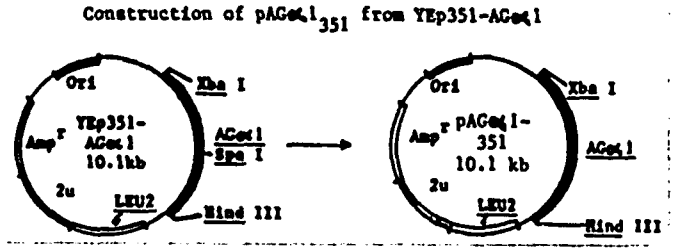
Fragments containing the ORF from pCL8 and pCL10 were subcloned into YEp351-*AGα1* by cutting with *Spe* I and *Hind* III liberating 1445 bp from pCL4 and pCL8. YEP351-*AGα1* was cut with the same enzymes. Fragments containing the deleted restriction sites were then ligated into YEP351-*AGα1*. These constructs were identified: pAGα1$_{635+23}$ (*Sac* I deletion) and pAGα1$_{494+3}$ (*Ban* II deletion).



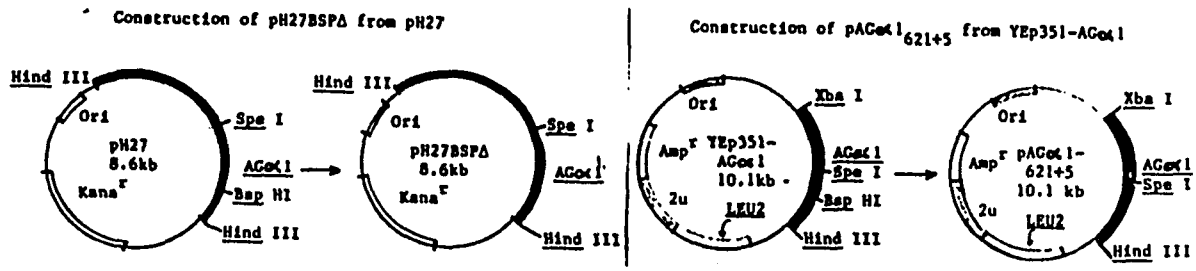
For uniformity and clarity, the final constructs (modified restriction site subcloned into YEp351) were designated pAGα1$_{x1+x2}$ where X1 is the number of amino acids coded by *AGα1* and X2 is the number of residues coded by out of frame sequence.

The *Spe* I fill-in was constructed by cutting YEp351-*AGα1* with *Spe* I and filling in the 3' termini with Klenow fragment of DNA polymerase I under the following conditions: DNA (approximately 500 ng), 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 2 mM dNTP's, and 5 units of Klenow enzyme. The reaction mixture was incubated for 30 minutes at 23°C. DNA

was isolated by GeneClean and ligated. Transformants were screened for the proper construct: pAGα1$_{351}$ by demonstrating that the *Spe* I site was eliminated.

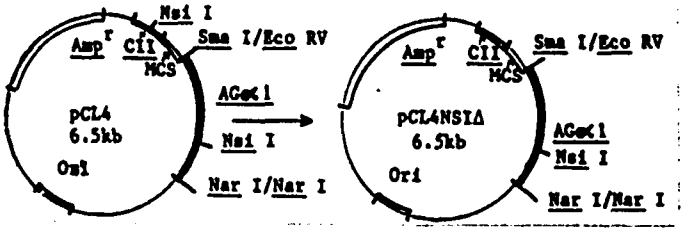


The *Bsp* HI fill-in was constructed by digesting pH27 with *Bsp* HI and filling-in the ends with Klenow fragment as described above. The plasmid was ligated and a transformant selected which carried pH27 which had the *Bsp* HI site replaced with an *Nsi* I site (pH27BSPΔ). The presence of an *Nsi* I site indicated a proper fill-in with Klenow enzyme. pH27BSPΔ was digested with *Spe* I and *Hind* III and ligated into YEp351-*AGα1*. This construct was designated pAGα1$_{621+5}$.



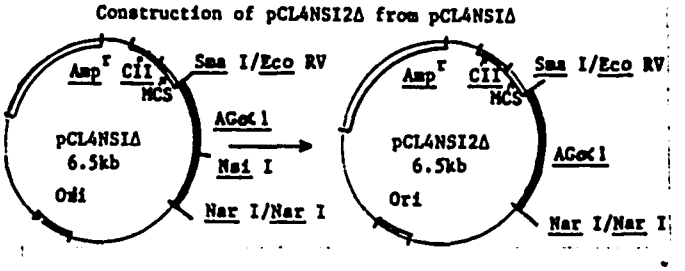
To construct pNSIIΔ, pCL4 was digested partially with *Nsi* I, incubated with T4 DNA polymerase, ligated, transformed and a clone selected which had one of the two *Nsi* I sites in pCL4 eliminated. Unfortunately, the clone identified: pCL4NSIΔ, had the *Nsi* I site within vector sequence eliminated.

Construction of pCL4NSIΔ from pCL4



pCL4NSIΔ, was redigested with *Nsi* I so that the only other site in the plasmid, the one within the ORF, would be cleaved. Once digested with *Nsi* I, pCL4NSIΔ was incubated with T4 DNA polymerase, ligated, transformed and a clone selected which both *Nsi* I sites eliminated. This clone was designated pCL4NSI2Δ.

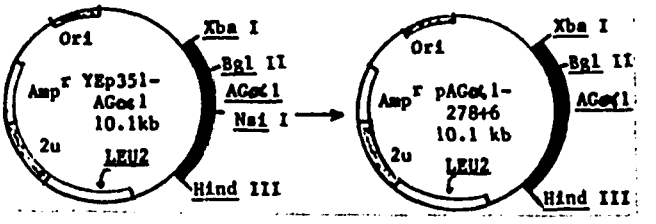
Construction of pCL4NSI2Δ from pCL4NSIΔ



YEp351-*AGα1* was digested with *Xba* I and *Eco* RI. pCL4NSI2Δ was cut with the same enzymes. A 1060 bp fragment (containing a *Bgl* II site) was isolated from a YEp351-*AGα1* digest and subcloned into pCL4NSI2Δ. This plasmid was designated pCL4'NSI2Δ. This construct was made so that the restriction site *Bgl* II would be present upstream of the modified *Nsi* I site to allow easy subcloning of the modified *Nsi* I site into YEp351-*AGα1*. Subsequent digestion of pCL4'NSI2Δ with *Bgl* II and *Hind* III liberated a 3485 bp fragment containing the deleted *Nsi* I site in the ORF of *AGα1*. This fragment was then ligated into YEp351-*AGα1* which had also been

cut with *Bgl* II and *Hind* III. The resulting plasmid was designated pAGα1$_{278+6}$.

Construction of pAGα1$_{278+6}$ from YEp351-AGα1



ISOLATION OF α-AGGLUTININ FROM CULTURE MEDIUM: Transformed clones of Lα21 were grown in 200 ml of YNB with leucine deficient synthetic medium at room temperature overnight to stationary phase. The medium was collected by centrifugation at 3,000 x g for 5 minutes to pellet the cells. The supernatant was dialyzed overnight against 4 liters of 10 mM sodium acetate, pH 5.5 at 4°C. The dialyzed culture medium was processed 100 ml at a time. A 1 ml (bed volume) column of DEAE Sephadex (equilibrated in 10 mM sodium acetate) was made using a 3 ml syringe. The dialyzed medium was placed over the column and allowed to flow through by gravity. The column was then washed with 3 ml of 10 mM sodium acetate and the column eluted with 2 ml of 250 mM NaCl, 10 mM sodium acetate (pH 5.5). All the activity attributed to α-agglutinin (see Results, Part 3) could be isolated from the medium in this fashion. Typically, 600 to 1000 units of activity was isolated from 100 ml of culture. The isolated material was then dialyzed overnight against 500 ml of 10 mM sodium acetate (pH 5.5) to remove NaCl. The dialysis tubing used to dialyze culture media and

isolated α-agglutinin was pretreated with 0.1 % (w/v) BSA to prevent loss of agglutinin due to non-specific adsorption to tubing.

WESTERN BLOTTING OF ISOLATED α-AGGLUTININ: α-agglutinin was deglycosylated with endo H as described in Part 1. Typically 3 to 15 units of activity was incubated overnight at room temperature with 0.001 unit of endo H. Western blotting was performed as described in Part 2.

CELL FRACTIONATION: Cells were grown to log phase in YNB with leucine deficient synthetic medium. Cells from 20 ml of culture were harvested at 1,000 x g for 5 minutes. Cells were washed once in 5 ml of 30 mM Tris, pH 7.5 and resuspended in 500 ul of 30 mM Tris, pH 7.5 with 1mM PMSF and 5% (v/v) 2-mercaptoethanol. The cell suspension was incubated at 37°C for 30 minutes and the cells harvested by centrifugation at 1,000 x g for 5 minutes. The supernatant was dialyzed overnight in 10 mM sodium acetate, pH 5.5 to remove 2-mercaptoethanol. The dialyzed material was labelled the periplasmic fraction. The cells were then resuspended in 500 ul of 1% Triton X-100, 1mM PMSF, 10 mM Tris, pH 7.5. An equal volume of glass beads were added and the mixture vortexed for 1 minute (4 times)(Conzelmann et al. 1990). This treatment resulted in 65% of the cells being lysed (as determined by phase contrast microscopy). The mixture was then centrifuged at

10,000 x g for 5 minutes. The supernatant was labelled cytoplasmic fraction.

DNA SEQUENCING: Filled-in restriction sites were sequenced by the dideoxynucleotide chain-terminating method (Sanger et al. 1977) using doubled stranded DNA as template. Sequence was derived using either DNA polymerase I (Klenow fragment) (Mierendorf and Pfeffer 1987) or T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.) (Tabor and Richardson 1987). Label was ^{35}S dATP. Sequencing using Klenow enzyme was carried out as described (Mierendorf and Pfeffer 1987). Sequencing using Sequenase was done as outlined in instructions supplied with the enzyme. Mn^{+2} was added to the reaction buffer to facilitate reading sequences close to the primer. 18 mer oligonucleotide primers were made by the Hunter College Sequence and Synthesis Facility. The primer sequences were approximately 40 bp upstream of sequences to be determined.

RESULTS AND DISCUSSION

Part 1

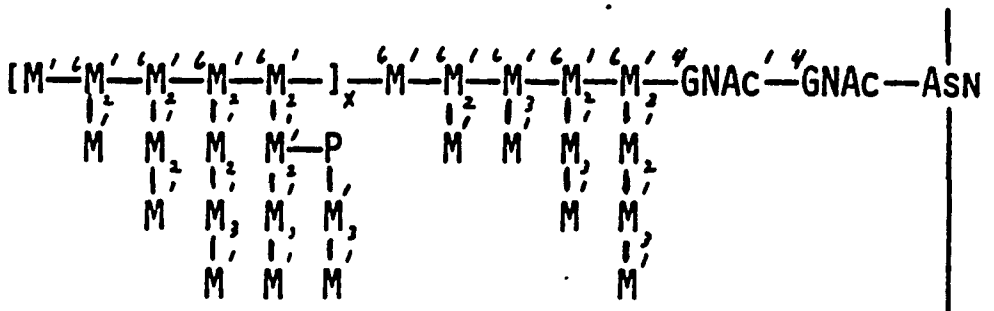
1. ANTIBODY SPECIFICITY AND REACTIVITY

1a. Introduction

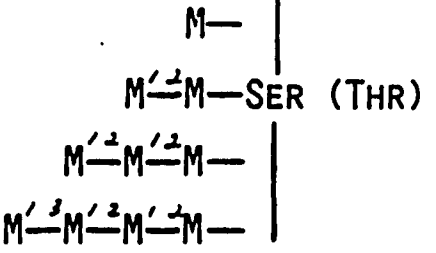
An antibody was made to study the cell surface expression of α-agglutinin. The antiserum was produced in rabbits against purified α-agglutinin which had been pre-treated with endo H to cleave N-linked carbohydrate. When the antiserum was tested by Western blotting of crude cell extracts from a and α cells, the pattern of immunostaining was identical for both extracts. In addition, the antiserum caused both a and α cells to immunostain in an indirect immunofluorescence assay. These results indicated that the antiserum contained antibodies which recognized epitopes common to both cell types. It was reasoned that antibodies might have been produced against carbohydrate epitopes since 1) the α-agglutinin preparation used to inject rabbits still contained soluble N-linked sugar which had been cleaved from the protein and 2) the α 1-3 mannose linkage present in N-linked carbohydrate (Fig. 5) is an immunodominant epitope in rabbits and goats (Ballou 1970, 1982). To test this hypothesis, the antiserum was absorbed once with heat-killed a cells (strain X2180-1A). This strain is isogenic to α strain (X2180-1B) used in this study. These two strains of opposite mating types

Figure 5. Glycosylation in *Saccharomyces cerevisiae*. Moieties present in N-linked (A) and O-linked (B) carbohydrate. Each N-linked glycosylation site may contain from 50 to 100 mannose residues which contribute significantly to the molecular weight of glycoproteins in *S. cerevisiae*. 1B) Short, O-linked carbohydrate contains from 1 to 4 mannose residues. C) Major immunodominant epitope in mannan from strain X2180 (Ballou 1982).

4A)



4B)



4C)

express identical forms of O and N-linked carbohydrate. Adsorption of antiserum with X2180-1A therefore results in the elimination of antibodies which recognize epitopes on O and N-linked carbohydrate.

1b. Results

After adsorption, the antiserum was tested by indirect immunofluorescence to monitor the efficiency of adsorption. The absorbed antiserum still caused a weak fluorescence of $\underline{\underline{a}}$ cells, indicating that a single adsorption was not adequate in removing contaminating antibodies. After the antiserum was adsorbed a second time, it no longer reacted with $\underline{\underline{a}}$ cells but still caused the fluorescence of α cells (Fig. 6). This result indicated that a population of antibody remained in the antiserum that recognized at least one antigenic determinant which was specific to α cells. To determine the nature of immunofluoresence seen in α cells, the adsorbed antiserum was incubated with purified α-agglutinin prior to assay. The preincubated antiserum did not bind to α cells in indirect fluorescent antibody assays. Therefore, the antibody which bound to α cells was directed against α-agglutinin-specific antigenic determinants. Western blotting (Fig. 7) indicated that the adsorbed antiserum was able to recognize native and all the major forms of endo H treated α-agglutinin (i.e.

Figure 6. Specificity of antibody and cell surface distribution of α-agglutinin. Phase micrographs (Top) and matching fluorescent micrographs (Bottom). Uninduced α cells express α-agglutinin in a polar fashion while induced cells express α-agglutinin more evenly. a Cells; which do not express α-agglutinin, are a negative control.

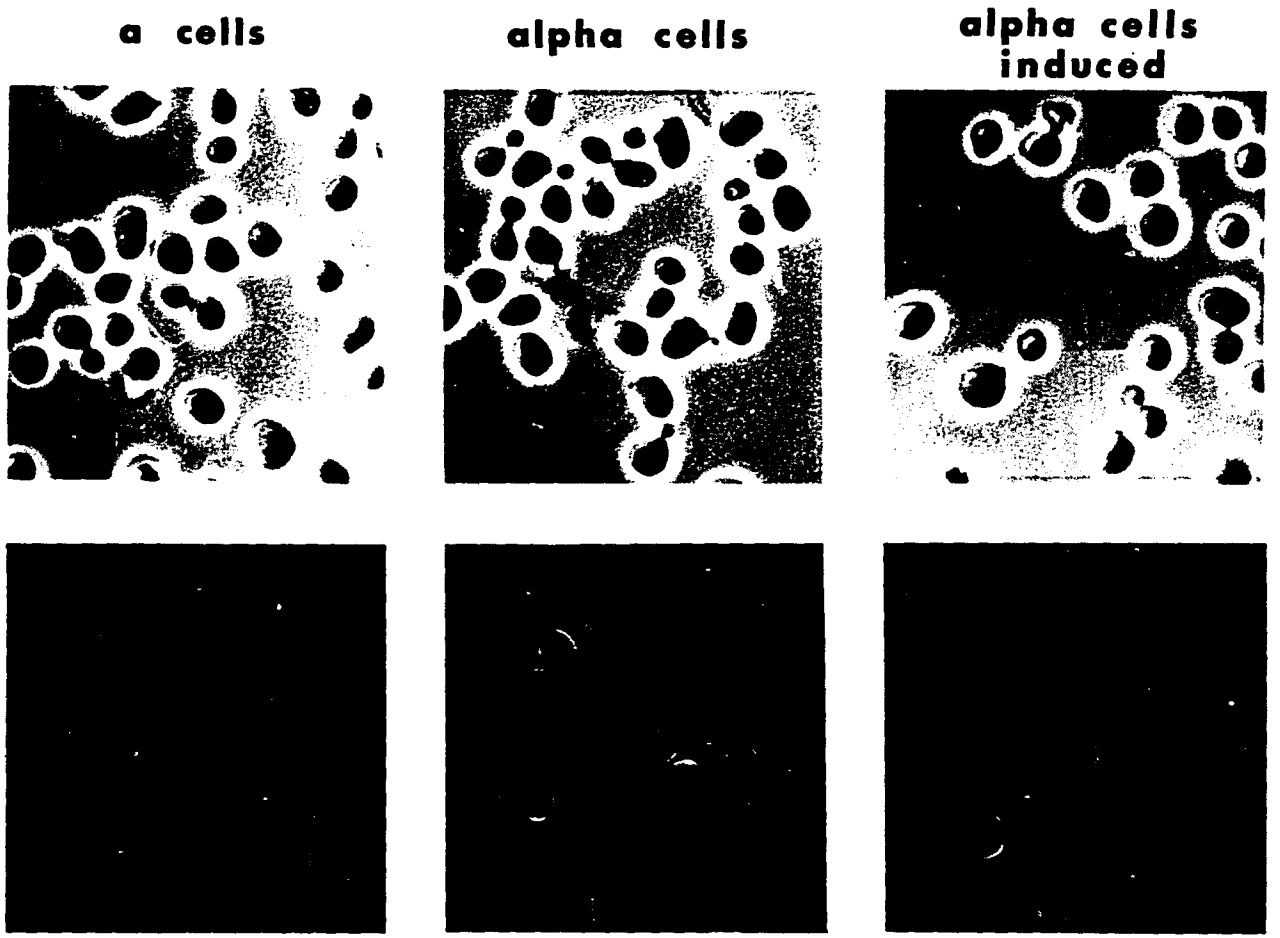
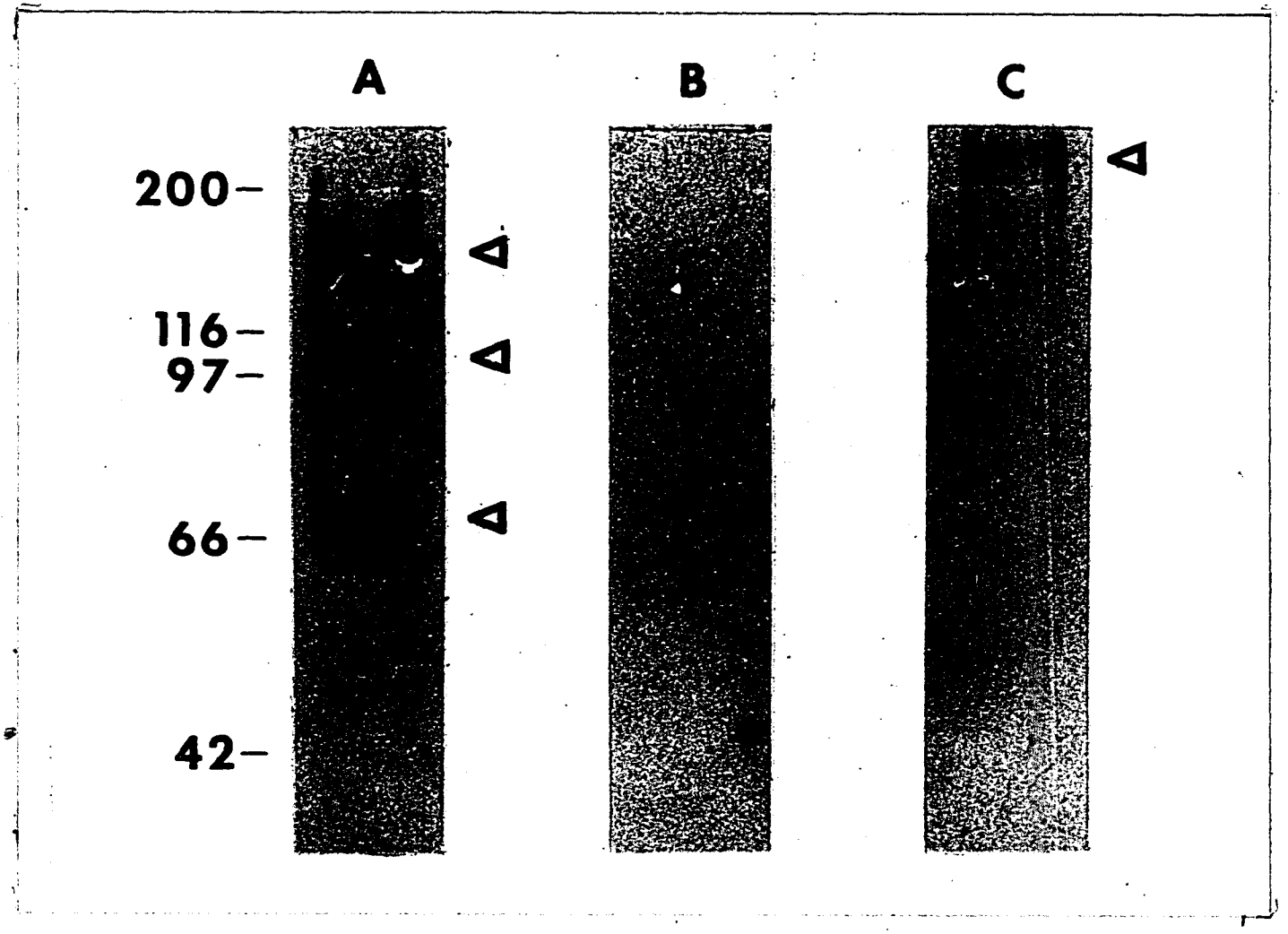

a cells
alpha cells
alpha cells induced

Figure 7. a cell adsorbed antiserum recognizes purified α-agglutinin by Western blotting. Lane A, 250 ng of endo H treated α-agglutinin. Lane C, 250 ng native α-agglutinin. Lane B, endo H alone. Arrowheads identify α-agglutinin peptides. Numbers indicate kD.


A
B
C
200–
116–
97–
66–
42–

72, 105, 145, 160 kD species). All these species are capable of binding to $\underline{\underline{a}}$ cells (Terrance et al. 1987).

The absorbed antibody was tested for its ability to inhibit the binding of α cells to $\underline{\underline{a}}$ cells. Antiserum inhibited the agglutinin-mediated binding of the two mating types when it was preincubated with α cells (Fig. 8) but not if the preincubation was with $\underline{\underline{a}}$ cells. Neither preimmune serum (data not shown) nor antiserum preadsorbed with α cells inhibited agglutination (Fig. 8). Inhibition effected by antibody was neutralized by adding purified α-agglutinin to compete with cell-bound α-agglutinin for antibody. Neutralization of antibody inhibition was found to be dependent upon the amount of purified α-agglutinin added to the mixture (Fig. 9). These results demonstrate that the a-agglutinin binding site on α-agglutinin is at least partially blocked by these α-agglutinin-specific antibodies.

2. QUALITATIVE EXPRESSION OF α-AGGLUTININ

The spatial distribution of α-agglutinin on the cell surface was determined by indirect immunofluorescence. Strain X2180-1B exhibited poor fluorescence when grown at 30°C (data not shown). These cells had an agglutination index of 0.5-0.6. When X2180-1B was grown at 22°C, cells stained much more intensely (indicating a higher level of α-agglutinin

Figure 8. Inhibition of agglutination by anti-α-agglutinin. Increasing amounts of a-cell-adsorbed antiserum (●) or α-cell-adsorbed antiserum (▲) were incubated with α cells. The cells were washed and a cells added. The agglutinability of the cell suspension was then assayed. Error bars (range for n=2) not visible are smaller than symbols.

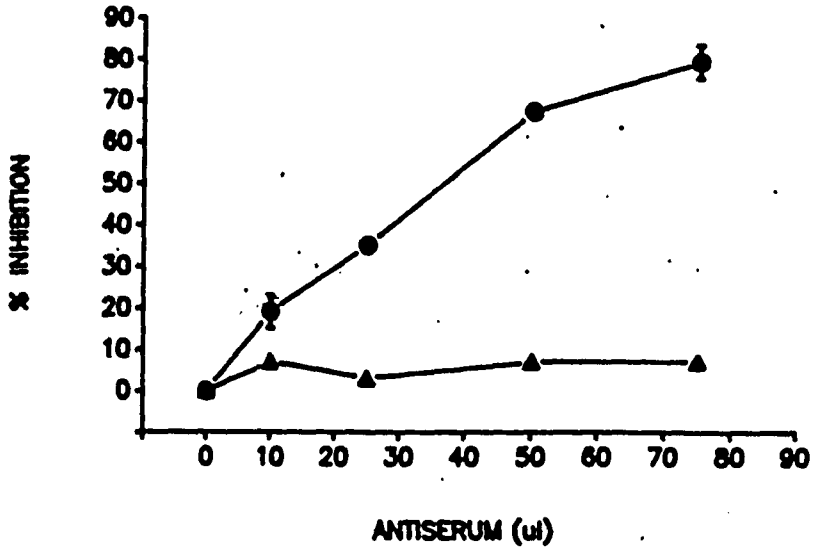
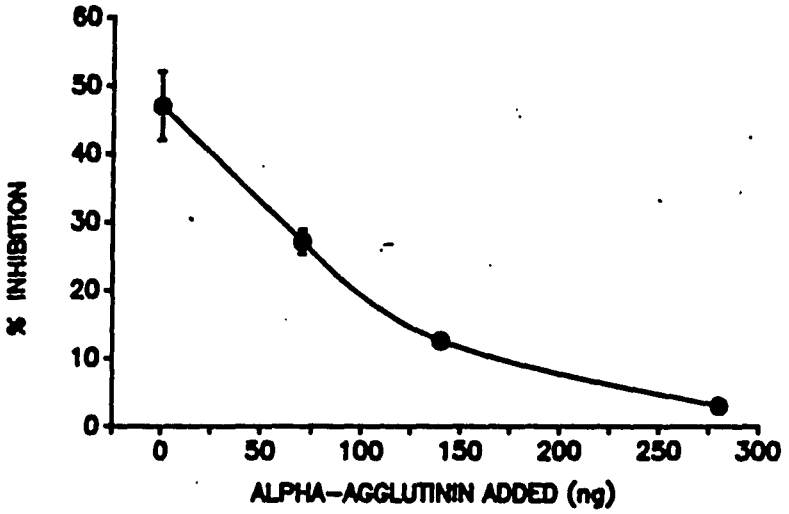

% INHIBITION
90
80
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70 80 90
ANTISERUM (ul)

Figure 9. Competition of antibody inhibition by purified α-agglutinin. Increasing amounts of purified α-agglutinin were incubated with α cells and antiserum. The cells were then washed and a cells added. The agglutinability of the mixture was then assayed. Error bars (range for n=2) not visible are smaller than symbols.


60
50
40
30
20
10
0
% INHIBITION
0
50
100
150
200
250
300
ALPHA-AGGLUTININ ADDED (ng)

expression) and had a higher agglutination index (0.75-0.82). These results suggested that at the lower temperature, there was a higher level of constitutive expression of α-agglutinin (see Discussion). Because cells grown at 30°C did not stain intensely enough to obtain suitable micrographs, cells were grown at 22°C for all the assays described below (unless otherwise noted). Non-induced α cells expressed α-agglutinin in a polar fashion (Fig. 6) while induced α cells (cells incubated with a-factor) expressed α-agglutinin more evenly.

A second feature of α-agglutinin expression was that the buds of α cells did not express α-agglutinin (Fig. 10) (5 immunostained buds out of 50). The fraction of buds which expressed α-agglutinin did not appreciably change upon exposure to the pheromone a-factor (4 out of 35). Smaller cells within the population also failed to express α-agglutinin (Fig. 10). In contrast to α-agglutinin, a-agglutinin is expressed first on buds of a cells exposed to α-factor (Watzele et al. 1988).

Strain W303-1B was treated with a-factor for 5 hours at 30°C. Some cells exposed to pheromone showed cell wall changes which result in an aberrant morphology (shmoos). Cells producing shmoos expressed α-agglutinin in the region of the shmoo tip (Fig. 11). a-agglutinin is also expressed in the region of the shmoo tip (Watzele et al. 1988). Some of the cells not producing shmoos formed

Figure 10. α-agglutinin expression in uninduced α cells. Phase micrographs (Left) and matching fluorescent micrographs (Right) of log phase α cells. Buds (large arrows) and smaller cells (small arrows) which do not express cell surface α-agglutinin are identified.

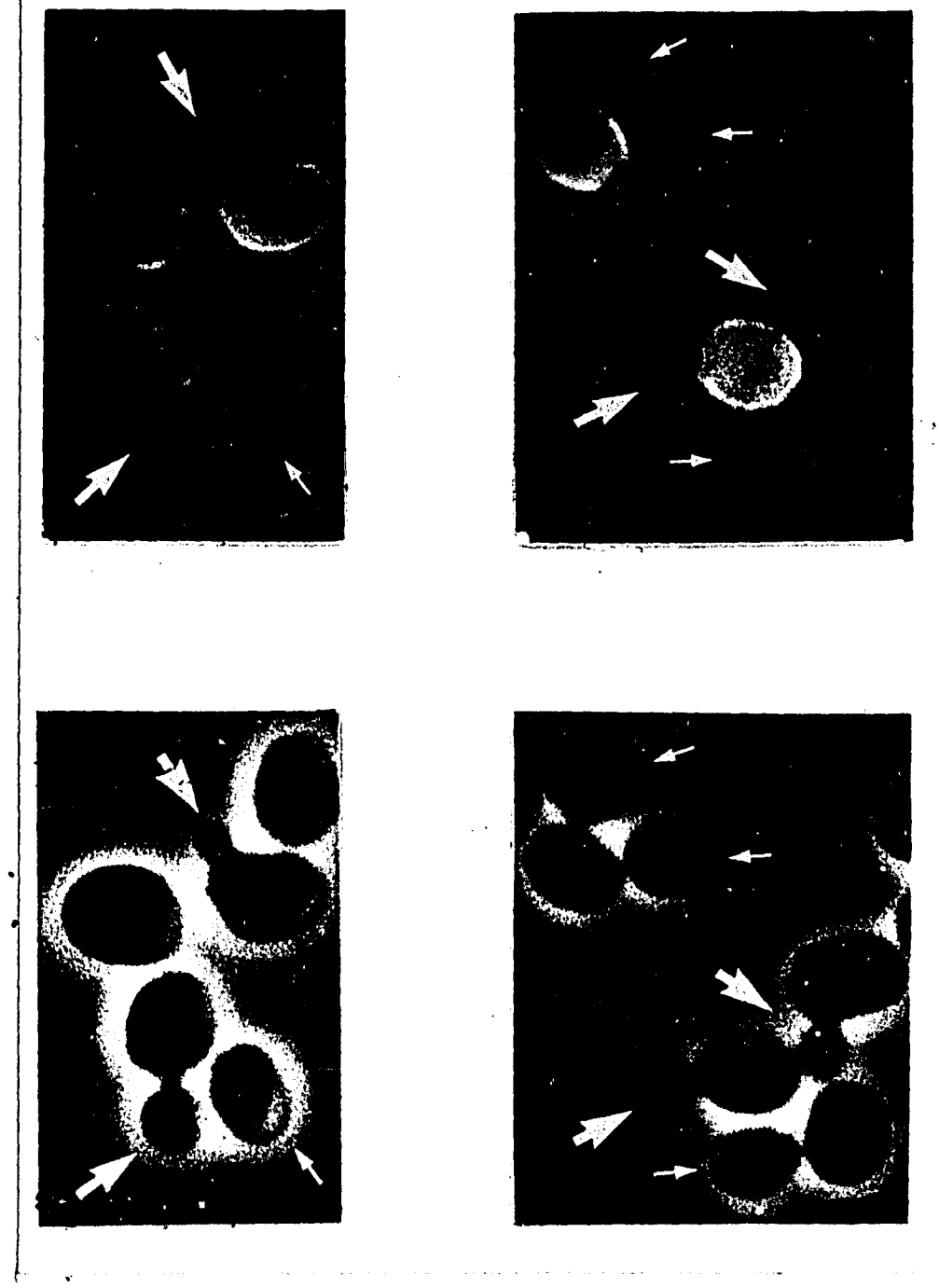
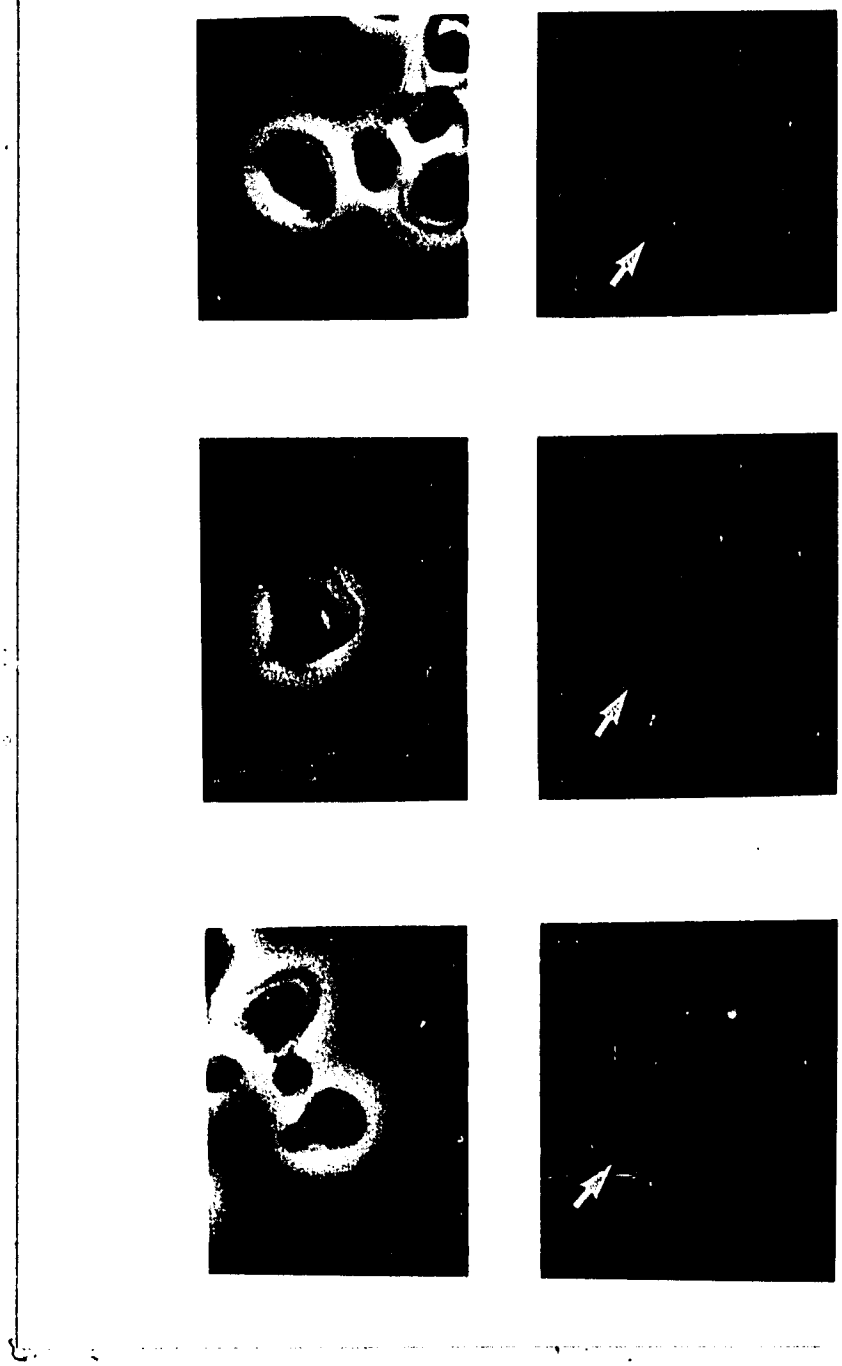


Figure 11. α-agglutinin expression in shmoo tip region of induced α cells. Phase micrographs (Top) and flurescent micrographs (Bottom). Cells induced with a-factor were assayed for α-agglutinin expression. Shmoo tips (arrows) immunostain indicating the presence of α-agglutinin.



buds which did not express α-agglutinin.

3. QUANTITATIVE EXPRESSION OF α-AGGLUTININ

3a. Relative Changes in Expression in Response to the a-factor Pheromone

It has been shown that exposure of a cells to α-factor results in an increase in the cell surface expression of a-agglutinin (Terrance and Lipke 1987, Watzele et al. 1988). Similarly, an increase in the expression of α-agglutinin might occur in α cells exposed to a-factor (Yanagishima et al. 1976, Betz et al. 1978, Terrance and Lipke 1981). To investigate this possibility, relative changes in the cell surface expression of α-agglutinin in response to pheromone were studied.

Changes in the cell surface expression of α-agglutinin were determined using an enzyme immunoassay (EIA). Strains X2180-1B (grown at 22°C) and W303-1B (grown at 30°C) were exposed to a-factor and the cells assayed for expression of cell surface α-agglutinin. As seen in Table 3, W303-1B exhibited a 2.4 fold increase in agglutinability and the expression of α-agglutinin increased 6.7 fold. For strain X2180-1B, the agglutinability increased 1.1 fold and the expression of α-agglutinin 1.3 fold. The increase in α-agglutinin

expression may be responsible for both the increases seen in the agglutinability of α cells upon treatment with pheromone (Yanagishima et al. 1976) and the uniform cell surface expression of α-agglutinin in induced cells (Fig. 6).

3b. Constitutive Expression: Determination of Site Number

The results in Figure 9 were used to calculate the number of α-agglutinin molecules constitutively expressed. Because antibody is titrated by soluble α-agglutinin, the assay is analogous to a competitive binding radioimmunoassay (Fahey et al. 1963) where soluble unlabelled antigen competes with labelled antigen for antibody. If one assumes that 1) antibody binding is proportional to a decrease in agglutinability and 2) that antibody can bind equally to both cell-bound and soluble α-agglutinin, then one can calculate the number of α-agglutinin molecules constitutively expressed in α cells. Given the peptide molecular weight of α-agglutinin to be approximately 70 kD (Terrance et al. 1987), the number of α-agglutinin molecules expressed per α cell is 5×10^4. This value is the same as that calculated by Watzele et al. using labelled a-agglutinin to determine the number of α-agglutinin molecules expressed on α cells.

Discussion

1. QUALITATIVE EXPRESSION OF α-AGGLUTININ

1a. Introduction

α-agglutinin is presumed to be anchored to the cell surface by attachment to the cell wall. The yeast cell wall is composed of an outer layer of mannan (Lipke et al. 1976), which contains 7 to 10 % protein by weight (Falcone and Nickerson 1956); a glucan layer, which forms a fibrillar network which is believed to give the cell wall its mechanical strength (Matile 1969); and chitin, which is involved in bud formation (Roberts et al. 1983). It is speculated that α-agglutinin is anchored to the wall by the mannoprotein component. If this assumption is correct, then the spatial expression of α-agglutinin may be dependent on the cell surface expression of other mannoproteins.

1b. Expression of mannan and its relationship to α-agglutinin expression

FITC-conjugated Concanavalin A, which binds specifically to α mannosides and α glucosides, was used to study the spatial distribution of mannan on the yeast cell surface by Tkacz and MacKay (1979). The probe was

found to be specific for α-mannan; since labelling could be inhibited by methyl-α-D-mannopyranoside (Tkacz et al. 1971). Uninduced α cells (strain X2180-1B) express mannan uniformly over the entire cell surface. The observation that cells grown after exposure to FITC-Con A produce buds which do not stain suggests that there is no detectable relocalization of existing mannan on the cell surface (Tkacz and Lampen 1972). Also, pulse-chase cell surface labelling experiments suggest strongly that newly synthesized mannan is deposited in the growing bud only (Tkacz and Lampen 1972). Similarly, cells labelled with ^{14}C D-mannose show that the label is stable within the cell wall for a number of cell divisions suggesting that there is little turnover of the mannoprotein component (Pastor et al. 1984).

The above results indicate that 1) the mannan component of the cell wall is a stable structure which is expressed evenly over the surface of uninduced α cells 2) newly synthesized mannan is deposited in the bud of growing cells and 3) there is little relocalization or turnover of existing mannan on the cell surface.

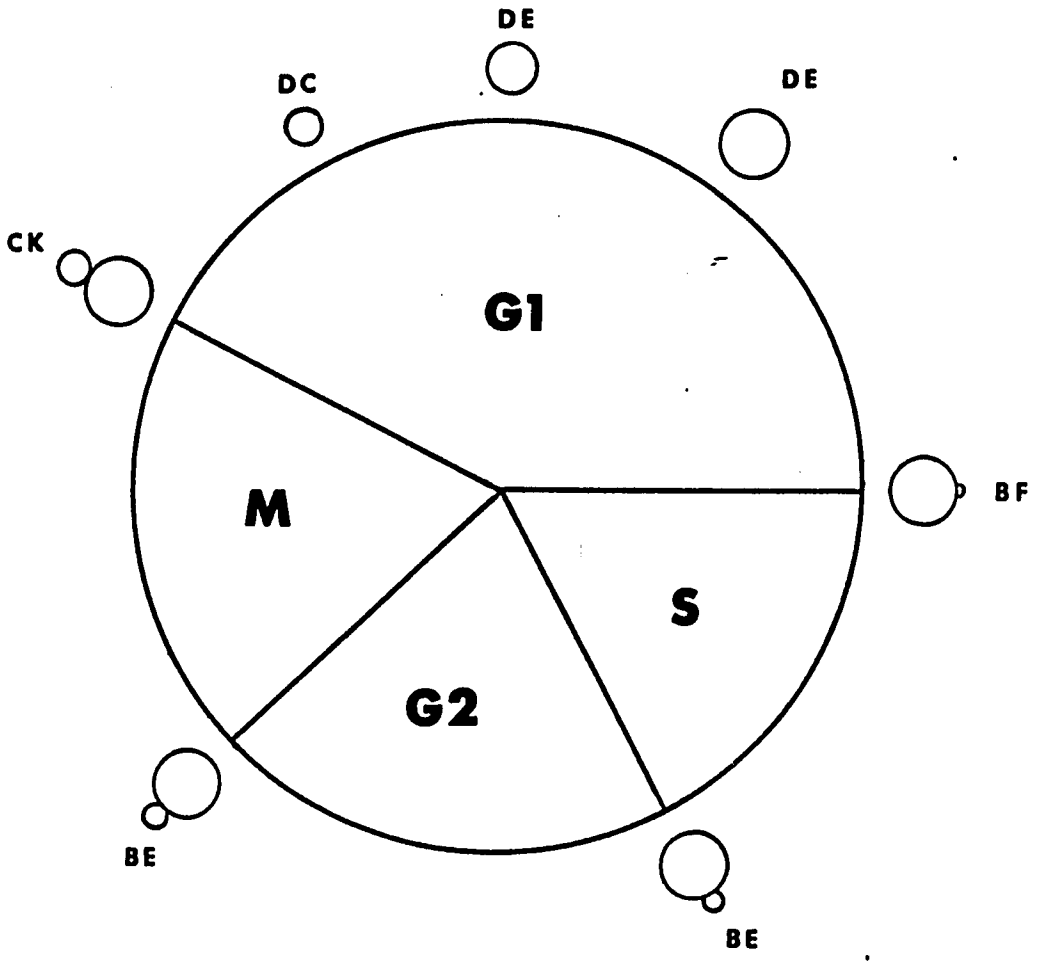
As seen in Figure 6, α-agglutinin is expressed in a polar fashion on uninduced α cells. This is in contrast to mannan which is expressed uniformly over the entire cell surface. Additionally, in buds (where new mannan is deposited) α-agglutinin is not expressed in detectable amounts (Fig. 10).

These results suggest that α-agglutinin and bulk mannan do not necessarily co-localize to the same area on the cell surface. However, there is so little known about the molecular architecture of the cell wall that the possibility of other cell wall components regulating the spatial distribution of α-agglutinin cannot be dismissed.

1c. The spatial distribution of α-agglutinin suggests models for regulating expression

Since α-agglutinin is not expressed in the bud region of cells, it is speculated that α-agglutinin expression may be restricted to the G1 phase of the cell cycle. *Saccharomyces cerevisiae* goes through a cell cycle similar to that seen in most eucaryotic cells (Cross et al. 1989). Bud formation occurs during the S phase of the cell cycle and proceeds through G2 until bud separation which marks the end of the M phase of the cell cycle (Fig. 12). Newly synthesized mannan is deposited in the bud region of cells (see above) during S, G2, and M phases of the cell cycle. In contrast, the observation that α-agglutinin is not detected in the bud region suggests that the glycoprotein is not expressed during these phases of the cell cycle. Therefore, it is possible that the expression of α-agglutinin is either cell cycle or mother/daughter regulated. Regulation of α-agglutinin

Figure 12. Cell cycle of *Saccharomyces cerevisiae*. Bud formation (BF) starts the S phase of the cell cycle. Bud enlargement (BE) occurs throughout the G2 and M phases of the cell cycle until cytokenesis (CK); which marks the end of the M phase. As a result of cytokenesis, a daughter cell (DC) is formed. Daughter cell enlargement (DE) occurs during G1 until a critical size is reached at which time the daughter cell starts DNA replication and bud formation (S phase). The cell cycle is then repeated.


DE
DC
DE
CK
G1
M
BF
S
G2
BE
BE

expression by either model would explain why both buds and small cells (daughter cells) fail to express α-agglutinin (Fig. 10).

The G1 phase encompasses about 35 to 45 % of the cell cycle (Johnston et al. 1980). From the study of *CDC* mutants, the G1 phase can be divided up into at least two subphases; a pre-START and a START phase (Pringle and Hartwell 1982).

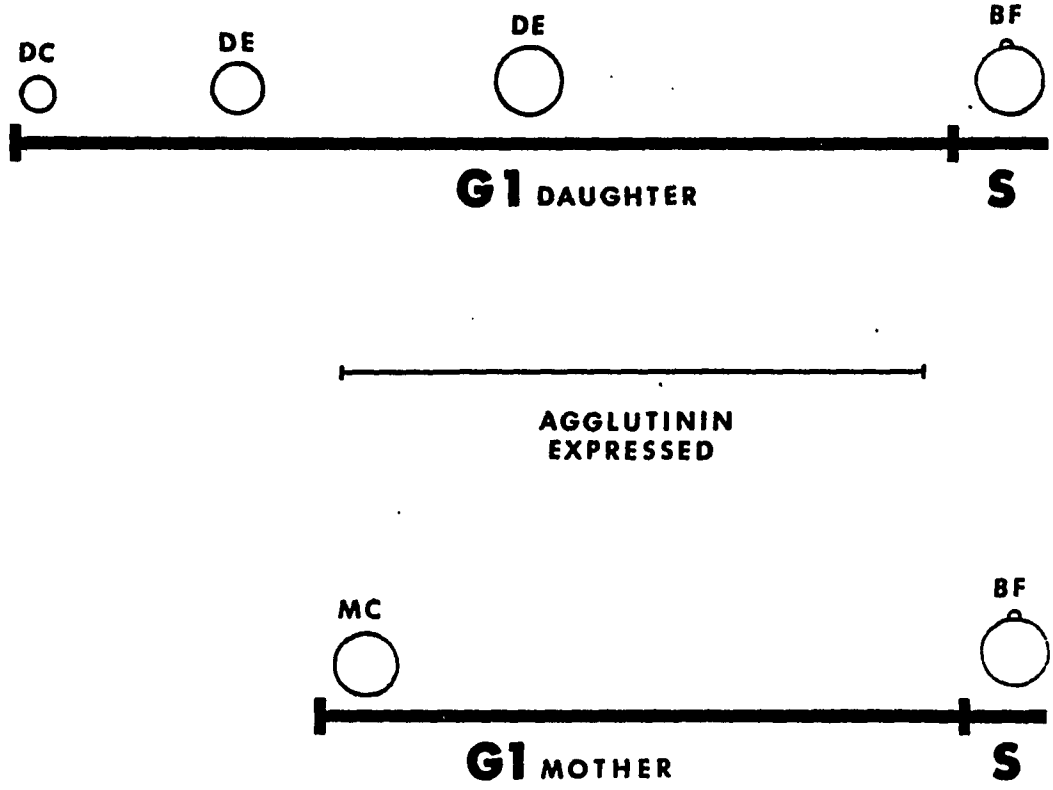
Indirect biochemical evidence suggests that α-agglutinin may be preferentially expressed during the G1 phase. It has been observed that maximum constitutive agglutinability (agglutinin expression) occurs when α cells are grown under conditions which support a prolonged generation time (reduced temperature) (Doi and Yoshimura 1977, 1978, 1985, Tohoyama et al. 1979, Wojciechowicz, Castro and Lipke, unpublished observation). It has also been shown that cells grown under conditions which limit growth and increase the generation time have relatively constant S, G2 and M phases but longer G1 phases (Carter and Jagadish 1978, Pringle and Hartwell 1982). It is likely that cells grown under reduced temperature spend more time in the G1 phase than when grown under conditions which favor a shorter generation time. Therefore, a positive correlation between increased agglutinability and growth conditions which favor a prolonged G1 phase suggests that α-agglutinin may be preferentially expressed during the

G1 phase of the cell cycle.

When *Saccharomyces cerevisiae* divides, the daughter cell which is formed is smaller than the mother cell (Hartwell and Unger 1977). Consequently, daughter cells must increase in size before they can divide (Johnston et al. 1979). Daughter cell enlargement occurs during the G1 phase of the cell cycle (Hartwell and Unger 1977, Johnston et al. 1979). This results in a daughter cell having a longer G1 phase than mother cells (cells which have divided at least once) (Fig. 13). Because small daughter cells fail to express α-agglutinin, it is the time after the cell reaches a specified size but prior to S phase (bud emergence) in which α-agglutinin might be expressed. It has been observed that up to 82% of small unbudded cells in a growing population can be daughter cells (Hartwell and Unger 1977). This latter result is consistent with the model proposed for α-agglutinin expression, since most small cells in the population (which are presumed to represent daughter cells) fail to express cell surface α-agglutinin.

That small cells do not express α-agglutinin may be a mechanism the species has evolved to prevent inappropriate mating. It is possible that small α cells are incapable of mating. Thus, the absence of α-agglutinin would be a mechanism which prevents interaction between an immature α cell with a mature a cell. Although it is not known whether daughter cells are

Figure 13. Model of α-agglutinin expression. If α-agglutinin expression is regulated, its expression (light line) is most likely restricted to the G1 phase (dark lines) from a point after daughter cell enlargment (DE) to a point in G1 just prior to bud formation (BF, S phase). The G1 phase of a daughter cell is longer than that of a mother cell because it is not necessary for a mother cell to substantially increase in size before it undergoes cell division.


DC
DE
DE
BF
G1 DAUGHTER
S
AGGLUTININ
EXPRESSED
MC
BF
G1 MOTHER
S

capable of mating before they have reached a critical size, evidence suggests that there is a relationship between cell size and the ability to mate. A dominant mutation in *DAF1* (*WHI1*), the product of which is a putative cyclin (Cross 1988, Nash et al. 1988) results in haploid cells which are phenotypically smaller than the parental strain. These cells can divide and produce pheromone, but are unresponsive to pheromone arrest in G1 and are unable to mate efficiently. These results suggest that these cells pass through G1 quickly and start mitotic cell division because of an altered critical size requirement. Cells under such cell cycle restraints would be expected to be less responsive to pheromone and inefficient in mating because the interval between budding and initiation of DNA synthesis (the G1 phase) is small. It would be interesting to determine whether these cells express normal levels of α-agglutinin. If α-agglutinin expression were restricted to the G1 phase, these cells would be expected to express little α-agglutinin on their cell surface.

Alternatively, the expression of α-agglutinin may be under mother/daughter control (*SWI5* control). It has been demonstrated that mother cells can switch mating type but daughter cells cannot (Strathern and Herskowitz 1979). Mating-type switching is controlled, in part, by the product of the *HO* gene which codes for an endonuclease necessary for mating-type switching

(Kostriken et al. 1983). The *SWI5* gene is a transcriptional activator of the *HO* gene and its expression appears to be cell-cycle regulated (Nasmyth et al. 1987). The cell-cycle regulated expression of *SWI5* causes a transient expression of *HO* during G1 (Nasmyth et al. 1987). It is speculated that daughter cells do not switch mating type because they do not have a sufficient amount of *SWI5* to initiate transcription of the *HO* gene (Nasmyth et al. 1987). Why daughter cells do not have a sufficient amount of *SWI5* may be due to proteolysis (Herskowitz 1989) or asymmetric distribution of *SWI5* during cell division (Nasmyth et al. 1987).

If α-agglutinin were under *SWI5* control, the expression of α-agglutinin would likely parellel the expression of *HO*. This would mean that α-agglutinin expression would also be restricted to the G1 phase of the cell cycle in mother cells and not expressed in daughter cells (due to an insufficient amount of *SWI5*).

1d. Possible mechanisms behind the control of α-agglutinin expression

If the level of α-agglutinin expression is regulated, it is most likely to be controlled at 1) the level of transcription; similar to that seen for the

HO gene (cell-cycle or *SWI5* controlled) (Nasmyth 1985, Nasmyth et al. 1987) or 2) the level of protein stability; as that seen for cyclins (Murray et al. 1989, Murray and Kirschner 1989).

The *HO* gene in *Saccharomyces cerevisiae* is transcribed predominantly during G1 and the DNA sequence CACGAAAA has been shown to be sufficient to confer transcriptional control which is cell-cycle regulated (Nasmyth 1985). There are ten such "cell-cycle control boxes" upstream of the *HO* gene open reading frame. It is not known whether this sequence is the binding site for *SWI5* (see above). Examination of the α-agglutinin gene (*AGα1*) upstream regulatory sequence (Lipke et al. 1989) reveals a similar sequence to the cell-cycle control consensus sequence found in the *HO* gene. The sequence AACGAAAT is found at position -183 in *AGα1*. This sequence falls within a 13 nucleotide imperfect inverted repeat (ATCATGTAACGAA/ATGCAATCTTCTA). It is interesting to note that sequences similar to the inverted repeat found in *AGα1* also appear at approximately the same position in *DAF1* (a putative cyclin) implicated in G1 arrest at START (Cross 1988) and *FUS3*, a suspected protein kinase also implicated in G1 modulation (Elion et al. 1990) (Fig. 14). The inverted repeat sequence was not found in the 5' region of *AGa1* (Roy et al. 1990), *FUS1* (Truehart et al. 1987), *STE2*, *STE3*,

Figure 14. Comparison of upstream sequences of *AGα1*, *DAF1* and *FUS3*. Dash after 13th nucleotide in sequence denotes middle of imperfect tandem repeat. Asterisk denotes nucleotides in *DAF1* and *FUS3* which show identity with that found in *AGα1*. Underlined sequence indicates region which shows similarity to "Cell-cycle box" consensus sequence which is shown at bottom of figure. Position indicates number of nucleotides upstream of the open reading frame that the sequence starts. *DAF1* and *AGα1* sequences show 53% identity and *FUS3* and *AGα1* show 62 % identity.

GENE	SEQUENCE	POSITION
DAF1	TACTAGCATCCAA-AAGCAAGCATCCG	-167
	* * * * ** * **** * **	
AGα1	ATCATGTAACGAA-ATGCAATCTTCTA	-168
	* ** * *** * **** * ***	
FUS3	AACAATTGCAGAA-AAGCAACATACTA	-128
HO	CACGAA AA "CELL-CYCLE BOX"	

STE12 (Dolan et al. 1989) or *CDC28* (Lorincz et al. 1984) (a protein kinase also involved in G1 control). The sequence A(A/T)C(G/C)AAA(T/A)GCAA, a stretch of nucleotides within the inverted repeat which shows identity between *DAF1* and *AGα1* was used to search the GenBank for other genes which contain the same sequence. No matches to this sequence were found when over 900 genes from *Saccharomyces cerevisiae* were examined. The significance of the inverted repeat in *AGα1*, *FUS3* and *DAF1* is unknown. It is tempting to speculate that it might represent an additional upstream regulatory region.

There is genetic evidence to suggest that the expression of α-agglutinin is under an additional control (other than through the pheromone response pathway) which regulates the constitutive level of expression. Two temperature sensitive mutants in α-agglutinin expression have been isolated and characterized (Doi and Yoshimura 1985). These mutants constitutively express high levels of α-agglutinin at elevated temperatures (36°C) whereas the wild type parental strains do not (Doi and Yoshimura 1979). The mutations were found to be recessive and α cell-specific. One mutation (*CAG1*) maps very close to the *MAT* locus while the other (*CAG2*) is unlinked to *MAT*. Cells carrying this mutations can still mate, respond to pheromone (arrest in G1), and produce their

own pheromone. These results suggest that the *CAG* genes code for products which are capable of specifically regulating α-agglutinin expression. Other mutants have been isolated which express agglutinins (a and α) at 22°C but not at 28°C (Yanagishima and Nakagawa 1980). These mutants responded to pheromone but the mutations were shown not to be mating type specific. From these studies it is clear that α-agglutinin expression can be temperature dependent depending upon the genotype of the strain. It is possible that the upstream region of *AGα1* contains sequences (possibly the imperfect tandem repeat) which are binding sites for the temperature sensitive products of these mutations.

If α-agglutinin is not under cell-cycle or mother/daughter transcriptional control, then an alternate possibility is that α-agglutinin is susceptible to proteolytic action by an inactivator whose activity is cell-cycle regulated. This mechanism has been postulated to explain the role of cyclin in regulating the cell cycle. Evidence suggests that cyclin accumulates during G1 to a critical level and then induces mitosis. Once mitosis has started, cyclin is rapidly degraded (Murray et al. 1989). The observation that α-agglutinin peptide which contains its carboxy terminus is susceptible to proteolytic processing in vivo (see Part 3) suggests that the mature form of α-agglutinin is susceptible to proteolysis. If the expression of

α-agglutinin were regulated by such a mechanism, α-agglutinin would be produced throughout the cell cycle but degraded by a proteolytic action which is cell-cycle regulated. The proteolytic action would be minimal or absent during the G1 phase of the cell cycle and increase at the start of mitosis (S phase). In this model, the susceptibility of α-agglutinin to proteolysis would be temporally similar to that seen for the cyclins. At present, however, there is no direct evidence supporting such a model.

In summary, the data on the spatial expression of α-agglutinin suggests that α-agglutinin cell surface expression is regulated and that α-agglutinin is expressed preferentially during the G1 phase of the cell cycle; during or after cell enlargement but prior to S phase. The mechanism by which α-agglutinin expression is modulated is not known but is speculated to occur at the level of transcription or peptide stability.

2. QUANTITATIVE EXPRESSION OF α-AGGLUTININ

2a. Constitutive and induced levels of α-agglutinin in strain X2180-1B

a cells (strain X2180-1A) express low levels of a-agglutinin (Doi et al. 1979, Watzele et al. 1988). Upon exposure to α-factor, there is a significant induction

of a-agglutinin expression (about 20-fold) (Watzele et al 1988). This induction results in the increased agglutinability of $\underline{a}$ cells (Doi et al. 1979).

In contrast, the constitutive expression of α-agglutinin in strain X2180-1B (an isogenic strain of X2180-1A) is high, since exposure of this strain to a-factor does not appreciably change its agglutinability (Yanagishima et al. 1976). This suggests that there is little induction of α-agglutinin expression. To determine whether this is the case, relative levels of cell surface α-agglutinin were measured in X2180-1B before and after treatment with a-factor.

First, uninduced and induced cells were examined by indirect immunoflurescence to determine the relative differences in the expression of α-agglutinin (Fig. 6). The expression of α-agglutinin in uninduced and induced cells was not markedly different. However, induced cells expressed α-agglutinin more uniformly than uninduced cells. This suggests that a-factor effected either 1) a redistribution of existing cell surface α-agglutinin or 2) induced the expression of additional agglutinin. Since there is no evidence suggesting that α-agglutinin is redistributed on the cell surface (see above), it was reasoned that a-factor induced a modest increase in the cell surface expression of α-agglutinin.

To confirm this, quantitative analysis of α-agglutinin expression was measured in uninduced and

induced cells by enzyme immunoassay. Upon exposure to a-factor, there is a 1.3 fold increase in the cell surface expression of α-agglutinin in induced cells of strain X2180-1B. Additionally, these cells show a 1.1 fold increase in their agglutinability, which is consistent with the results of others (Yanagishima et al. 1976). If there are 5 x 10^4 molecules/cell of α-agglutinin consitutively expressed on X2180-1B (see results), then an estimated 6.5 x 10^4 molecules/cell are expressed on cells induced with a-factor.

2b. Effect of growth temperature on constitutive expression of α-agglutinin.

When grown and induced with a-factor at 30°C, there is a 6.7 fold increase in the cell surface expression of α-agglutinin in strain W303-1B (Table 3). This result is consistent with the 20-fold increase seen in the transcription rate of α-agglutinin at 30°C when exposed to a-factor (Lipke et al. 1989). It is also observed that the constitutive level of expression of α-agglutinin is low in this strain at 30°C (Tables 3 and 4). Conversely, when strain W303-1B is grown at 22°C, the agglutinability index of these cells increases to 0.6 (from 0.3 at 30°C) with an accompanying increase in the intensity of immunostaining (Table 4). When strain X2180-1B is grown at 30°C,

Table 3

Comparison of Cell Agglutinability and Cell Surface Expression of α-agglutinin

Strain	Enzyme Immunoassay Substrate hydrolyzed (10^{-5}uMol/min)*	Agglutination Index
X2180-1B		
uninduced	5.24	0.81
induced	6.42	0.89
W303-1B		
uninduced	0.37	0.33
induced	2.45	0.79

* Values are means for triplicate determinations after subtraction of a cell controls.

Table 4

Variability in Constitutive Levels of Agglutinability and Immunostaining in Strains Grown at Different Temperatures

Temperature (°C)	Strain	Agglutination Index	Immunostain Intensity
30	X2180-1B	0.62	++
	W303-1B	0.30	+/-
22	X2180-1B	0.81	++++
	W303-1B	0.60	++

the agglutinability index of these cells decreases to 0.50-0.60 (from 0.75-0.81 at 22°C) and they immunostain with only moderate intensity (Table 4).

This data suggests that when grown at 30°C, the constitutive level of c.-agglutinin is lower in strains W303-1B and X2180-1B (as compared to growth at 22°C). This phenomenon has been reported by others (Doi et al. 1977, 1978, 1985; Tohoyama et al. 1979) who demonstrate that the induction of α-agglutinin expression by a-factor is most pronounced at elevated temperatures where the constitutive level of expression of the glycoprotein is lower. Doi, like others (Yanagishima et al. 1976, Tohoyama et al. 1979) find little induction in α-agglutinin expression when cells are grown at temperatures below 30°C. Lastly, the level of agglutinin expression varies from strain to strain (compare X2180-1B and W303-1B). This has been observed previously (Manney and Meade 1977). These authors found that X2180-1B agglutinates slightly better than other α cell strains tested. This result infers that X2180-1B expresses more agglutinin than other strains (ie. XT1219-18A and XP300-26C).

In summary, there is an increase in the cell surface expression of α-agglutinin upon exposure to a-factor. The amount of induction is dependent upon the constitutive level of expression. The constitutive level of expression is influenced by the temperature of growth.

At higher growth temperatures less α-agglutinin is constitutively expressed.

3.SUMMARY

A polyclonal antibody was used to study the cell surface expression of α-agglutinin. Strain X2180-1B was used to study the cell surface expression of this glycoprotein. The spatial distribution of α-agglutinin in uninduced cells is polar; the significance of which is unknown. Upon induction with a-factor, α-agglutinin becomes uniformly distributed on the cell surface due to a modest increase in α-agglutinin expression. The constitutive level of α-agglutinin expression is 5 x 10^4 molecules/cell and induction with a-factor causes a modest increase in the expression of α-agglutinin (to 6.5 x 10^4 molecules /cell).

The basal level of α-agglutinin expression has been shown to be dependent upon the temperature of growth. Cells grown at lower temperatures (i.e. 22°C) constitutively express more α-agglutinin than cells grown at 30°C. This suggests that cells which spend more time in G1 express more α-agglutinin. This observation in conjunction with a result which demonstrates that α-agglutinin is not expressed on buds and newly formed daughter cells in strain X2180-1B suggests that α-agglutinin expression may be cell cycle restricted; possibly to the G1 phase of the cell cycle.

Part 2

1. INTRODUCTION

To clone the α-agglutinin gene, mutants in α-agglutinin expression were made by Lipke and Kurjan by first mutagenizing strain W303-1B with ethyl methanesulfonate followed by selecting for agglutination minus clones. Agglutination defective clones were selected by incubating mutagenized cells with a cells and isolating those cells which did not agglutinate with a cells. Agglutination defective mutants were assayed for pheromone production and mating. Mutants which showed diminished capacity to produce pheromone were removed from further study. The remaining mutants were crossed with a cells and tetrad analysis performed. Most segregants showed defects in both a and α-agglutinin expression. This result indicated that the mutations were not α cell-specific and were therefore eliminated from further study. The remaining 5 mutants showed mating-type-specific defects in agglutination. These clones were crossed (by spheroplast fusion) and shown not to complement each other. This result indicated that the mutants fell into a single complementation group. Fusion of the mutants with the parental clone showed that the mutation was recessive. A yeast genomic library in YEp13 was used to transform two of the five mutants.

Transformants were assayed for their ability to agglutinate with a cells. A single plasmid, pLα21, was shown to complement mutants in α-agglutinin expression. Partial digestion of pLα21 with *Hind* III revealed that a 6.1 kb fragment could complement mutants in α-agglutinin expression.

DNA sequencing of the 6.1 kb fragment revealed the presence of an open reading frame (ORF) of 2 kb. Numerous observations suggested strongly that the product of the ORF is α-agglutinin. First, the ORF codes for a protein of 70kD. Second, the ORF contains a putative secretion sequence at the amino terminus. Third, the ORF contains 12 potential N-linked glycosylation sites. Fourth, the ORF has a high proportion of serines and threonines. Lastly, the ORF has possible upstream regulatory sequences similar to others found under *MATα1* control. These characteristics were consistent with the ORF encoding for α-agglutinin. However, biochemical evidence was lacking to support this contention.

2.RESULTS

A first approach to determining whether the ORF coded for α-agglutinin was to demonstrate that protein sequence from biochemically purified α-agglutinin matched the peptide sequence of the ORF. Approximately 1

mg of α-agglutinin was isolated as described (Terrance et al. 1987) and deglycoslyated with hydrogen fluoride (Mort and Lamport 1977). Following deglycosylation, α-agglutinin peptide was subjected to SDS-PAGE and transferred to Immobilon membrane (Matsudaira 1987). Molecular weight species of 63 and 55 kd were detected by Coomassie staining of membrane (Fig. 15). Membrane slices containing these peptides were then subjected to amino acid sequencing by Edman degradation employing an Applied Biosystems gas phase sequenator. Unfortunately a sequence could not be determined from these peptides. Failure to derive a sequence could have been due to 1) insufficent peptide 2) a blocked amino terminus or 3) incomplete deglycosylation, resulting in the presence of residual carbohydrate which interfered with sequence analysis. It was therefore necessary to try an alternative approach to identifying the gene.

A clone of strain W303-1B (α cell) was made which had a disruption of the gene in question (Lipke et al. 1989). Immunoblotting was performed on crude extracts from this clone using anti-α-agglutinin to determine whether α-agglutinin peptides could be detected. Disruption of the gene resulted in the dissappearence of α-agglutinin peptides from crude cell extracts of this clone (Fig. 16). Although this result suggested that the gene codes for α-agglutinin, the disappearance of α-agglutinin peptides from this clone could also have

Figure 15. Isolation and deglycosylation of α-agglutinin for sequence analysis. α-Agglutinin was isolated as described previously (Terrance et al. 1987) and deglycosylated with hydrogen fluoride. Lanes A and B: Samples of hydrogen fluoride treated α-agglutinin after Western blotting. Lane C: Molecular weight standards. Membrane stained with Coomassie blue. Arrows indicate major species of peptide identified. Numbers indicate kD.

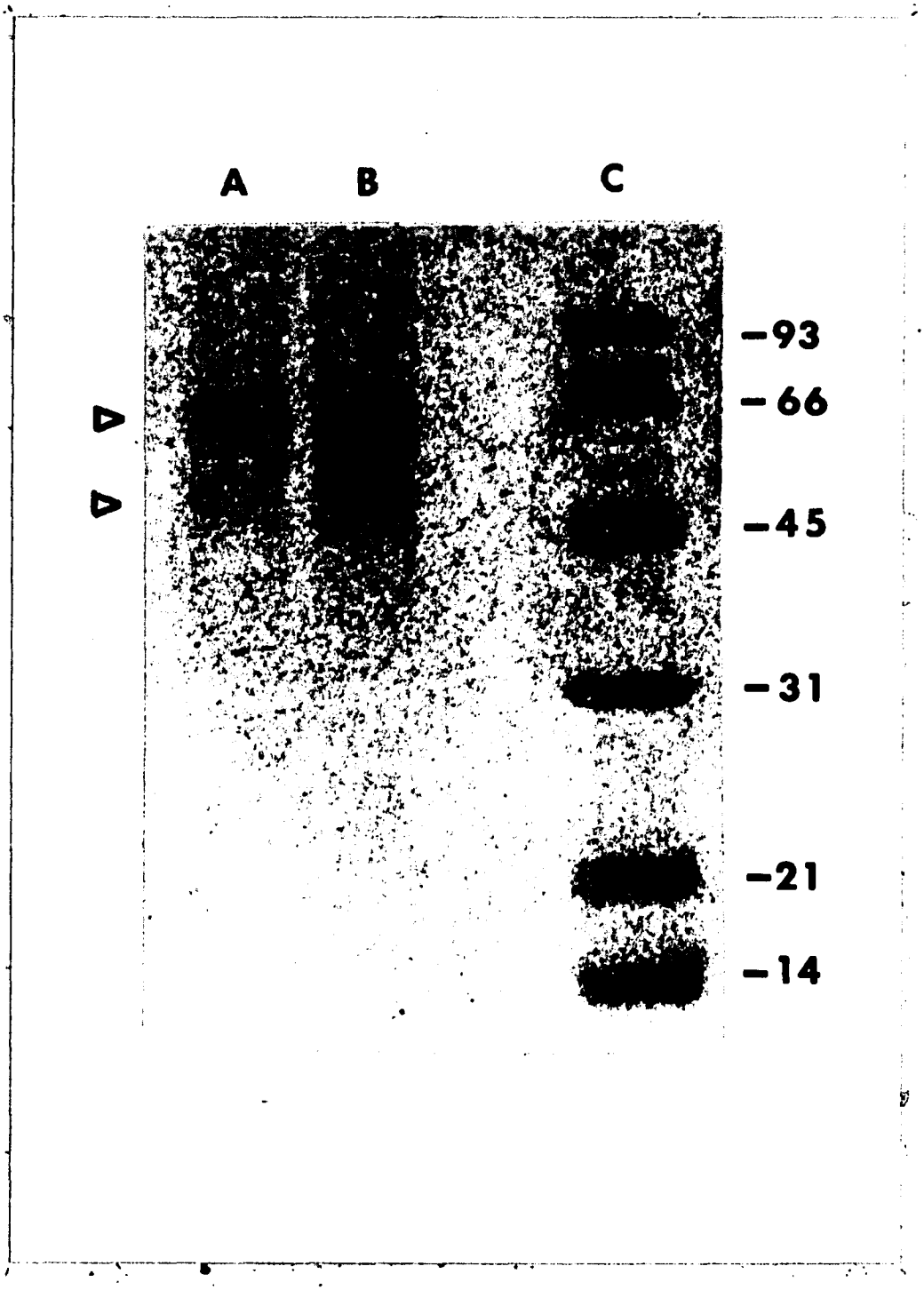
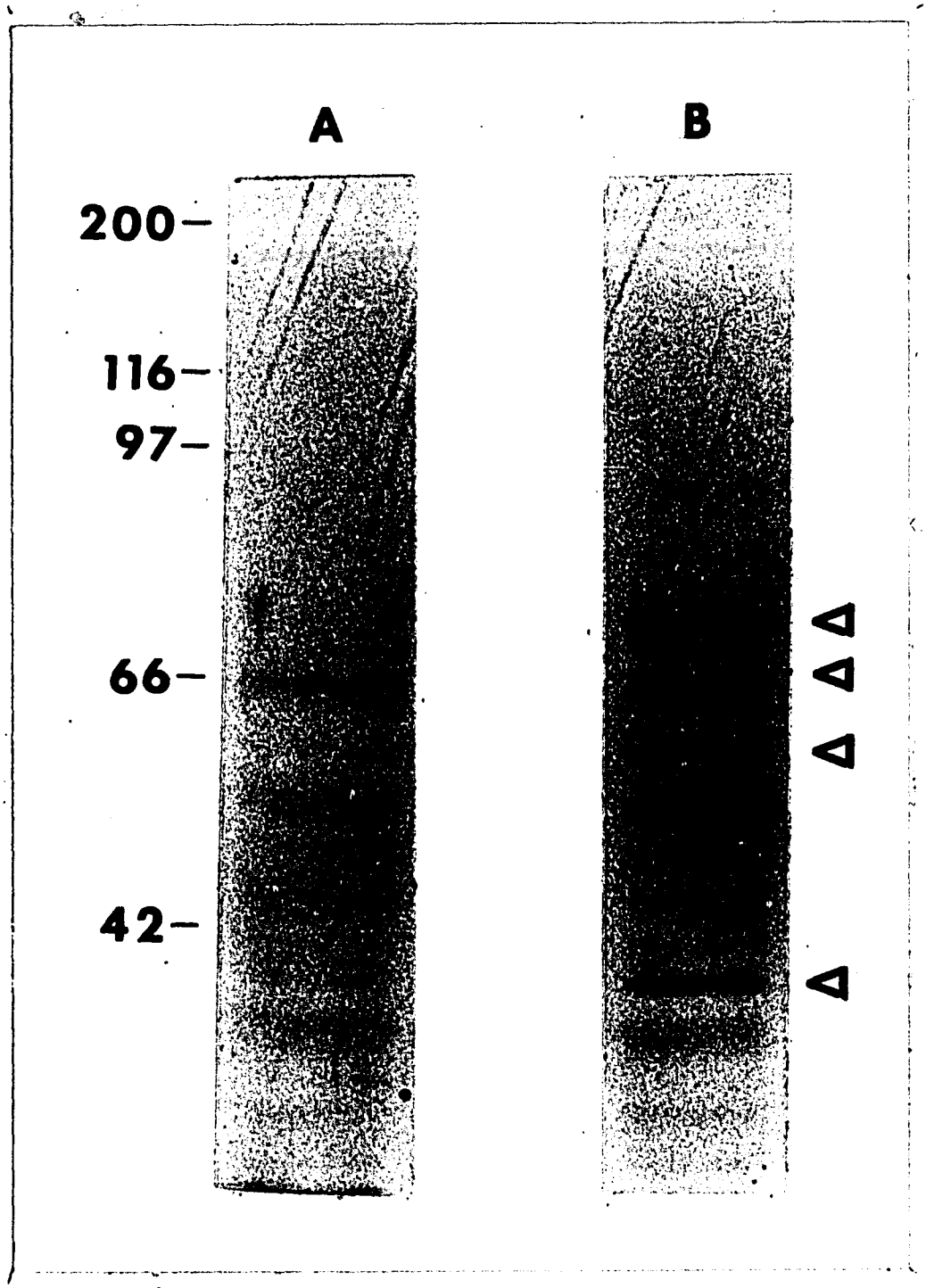

A
B
C
-93
-66
-45
-31
-21
-14

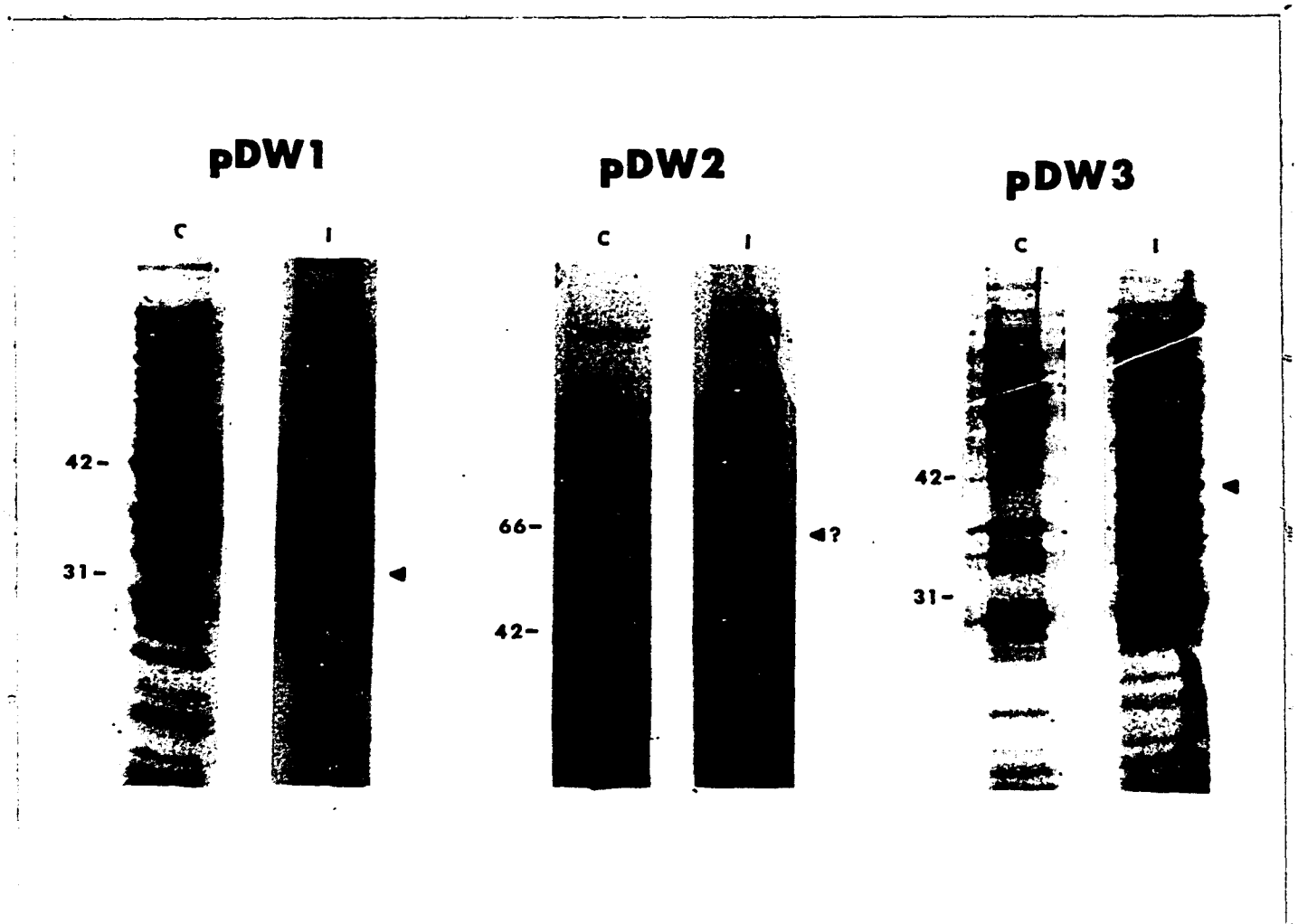
Figure 16. Immunoblot analysis of crude cell extracts using anti-α-agglutinin. A *AGα1::LEU* 2 disruption mutant (Lane A) failed to express α-agglutinin peptides while α-agglutinin peptides could be detected in wild type α cell extract (Lane B). Crude extracts (5 ug) were treated with endo-N-acetyl glucosaminidase H prior to Western blotting. Numbers indicate kD.


A
B
200–
116–
97–
66–
42–

resulted from disruption of a gene necessary for α-agglutinin expression.

More definitive evidence suggesting that the gene codes for α-agglutinin was obtained by demonstrating that the ORF product was immunoreactive when tested with anti-α-agglutinin. This was accomplished by expressing parts of the ORF as a fusion protein in *E. coli*. Numerous DNA fragments which encoded different parts of the ORF were cloned into pRA10, a vector which expresses a CII fusion protein at high levels in *E. coli* (Mallon et al. 1986). Three constructs were made which would express different parts of the ORF: pDW2 contained most of the ORF, pDW1 contained most of the amino half and pDW3 contained the carboxy half of the ORF. The ability of these constructs to express fusion products was determined by the ability of transformed *E. coli* strain MZ1 to express a product of the correct size at the derepressing temperature (42°C). SDS-PAGE of cell lysates showed that only MZ1 transformed with construct pDW1 could express a fusion product at high levels (Fig. 17). MZ1 with pDW3 could express a fusion protein at low levels while no detectable fusion product (expected molecular weight of fusion protein, 65 kD) was seen in MZ1 containing plasmid pDW2. The only induced protein in strain MZ1 containing pDW2 was a heat shock protein of 68 kD. This result was verified by demonstrating that induced pDW2 transformants did not

Figure 17. Expression of Fusion Proteins In *E. coli*. Various segments of the *AGα1* ORF were cloned into pRA10 to create pDW1, pDW2, and pDW3. MZ1 cells containing these plasmids were grown under repressing conditions (C) or derepressing conditions (I). The cells were lysed and subjected to SDS-PAGE followed by Coomassie staining. Arrows in pDW1 and pDW3 indicate fusion protein. Arrow ? in pDW2 indicates induction of a 66 kD heat shock protein but no apparent fusion protein. Numbers indicate kD.


pDW1
C
I
42-
31-
pDW2
C
I
66-
42-
?
pDW3
C
I
42-
31-

express an immunoreactive product of the expected molecular weight. The reason for the different levels of fusion protein expression from pDW1 and pDW3 is unknown. Because MZ1 transformed with pDW1 expressed fusion protein at the highest level, fusion protein made from this transformant was used for all subsequent experiments.

Fusion protein was purified so that antibodies could be raised. First, the fusion protein was partially purified by lysing induced cells in lysozyme with 1% Triton X-100. This step lyses the cells and separates soluble endogenous *E. coli* proteins from insoluble fusion protein. The partially purified material was solubilized in SDS and subjected to preparative SDS-PAGE. Fusion protein was then eluted from gel slices (Fig. 18, lane D). The purified fusion protein was then tested by immunoblotting to determine whether it was immunoreactive with anti-α-agglutinin. Fusion protein was recognized by anti-α-agglutinin (Fig. 18, lane E) and neutralized the antibody mediated inhibition of agglutination effected by this antibody (Fig. 19). The neuralization of antibody mediated inhibition of agglutination by fusion protein was specific for mannan had no neutralizing activity even when used in concentrations up to 1 ug/ml (Fig. 19).

Although these results suggest that the gene codes for α-agglutinin, it was necessary to demonstrate that the same population of antibody recognized both

Figure 18. Expression, purification, and immunoreactivity of fusion protein. The fusion protein expressed in *E. coli* from pDW1 consists of the N-terminal 13 amino acids of lambda *cII*, 12 amino acids specified by the M13 polylinker, amino acids 128 to 356 of *AGα1*, and 39 amino acids encoded by vector DNA. Coomassie stains are as follows: lane A, cell lysate from pDW1 containing cells grown under repressing conditions; lane B, cell lysate from cells containing a plasmid with the *AGα1* insert in the opposite orientation, grown under derepressing conditions; lane C, an extract from pDW1-containing cells grown under derepressing conditions; lane D, 250 ng. of gel-purified fusion protein. Lane E, Western blot of 250 ng of fusion protein probed with anti-α-agglutinin antibody, followed by secondary antibodies. Apparent molecular weights in kD.

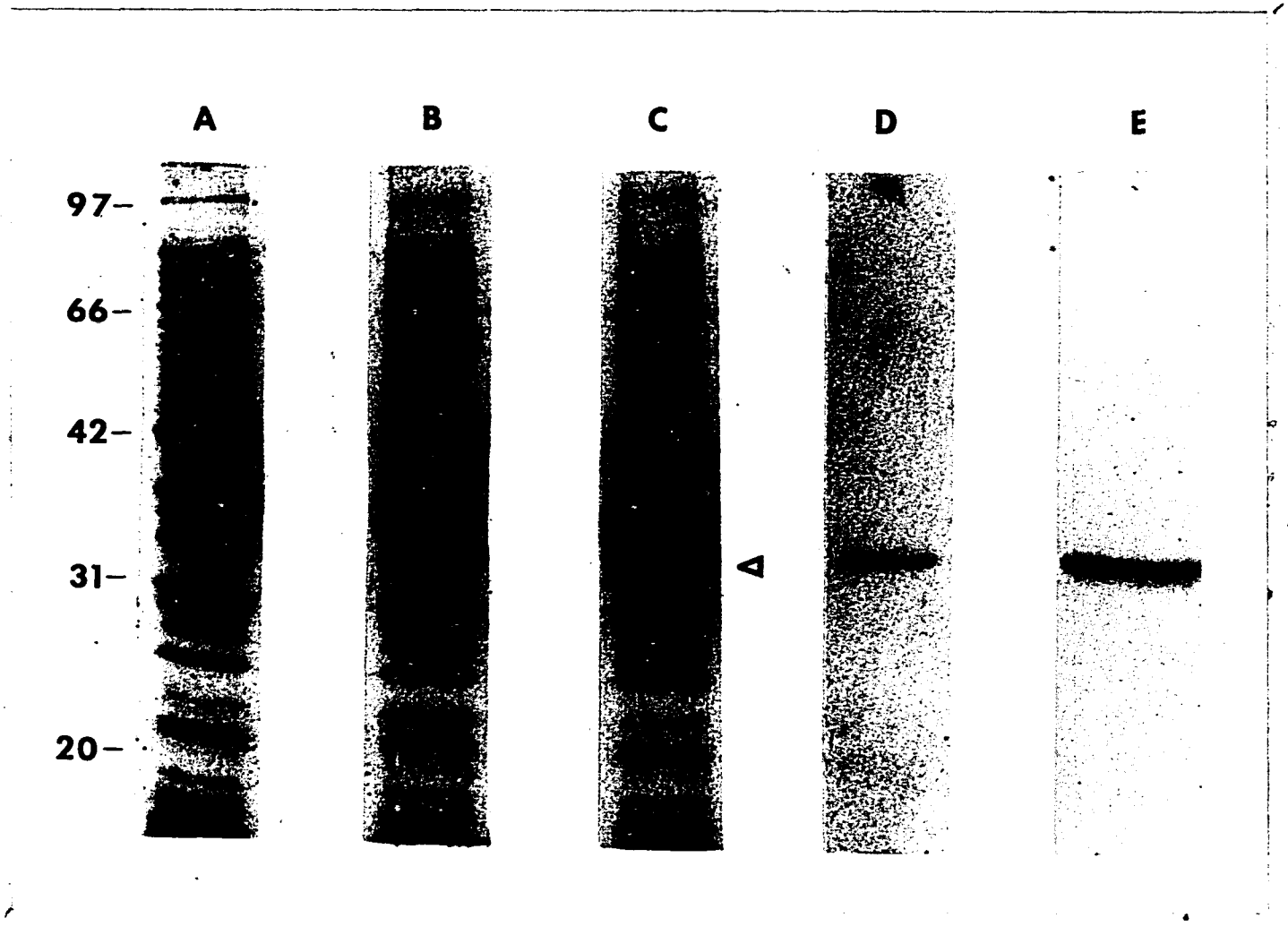
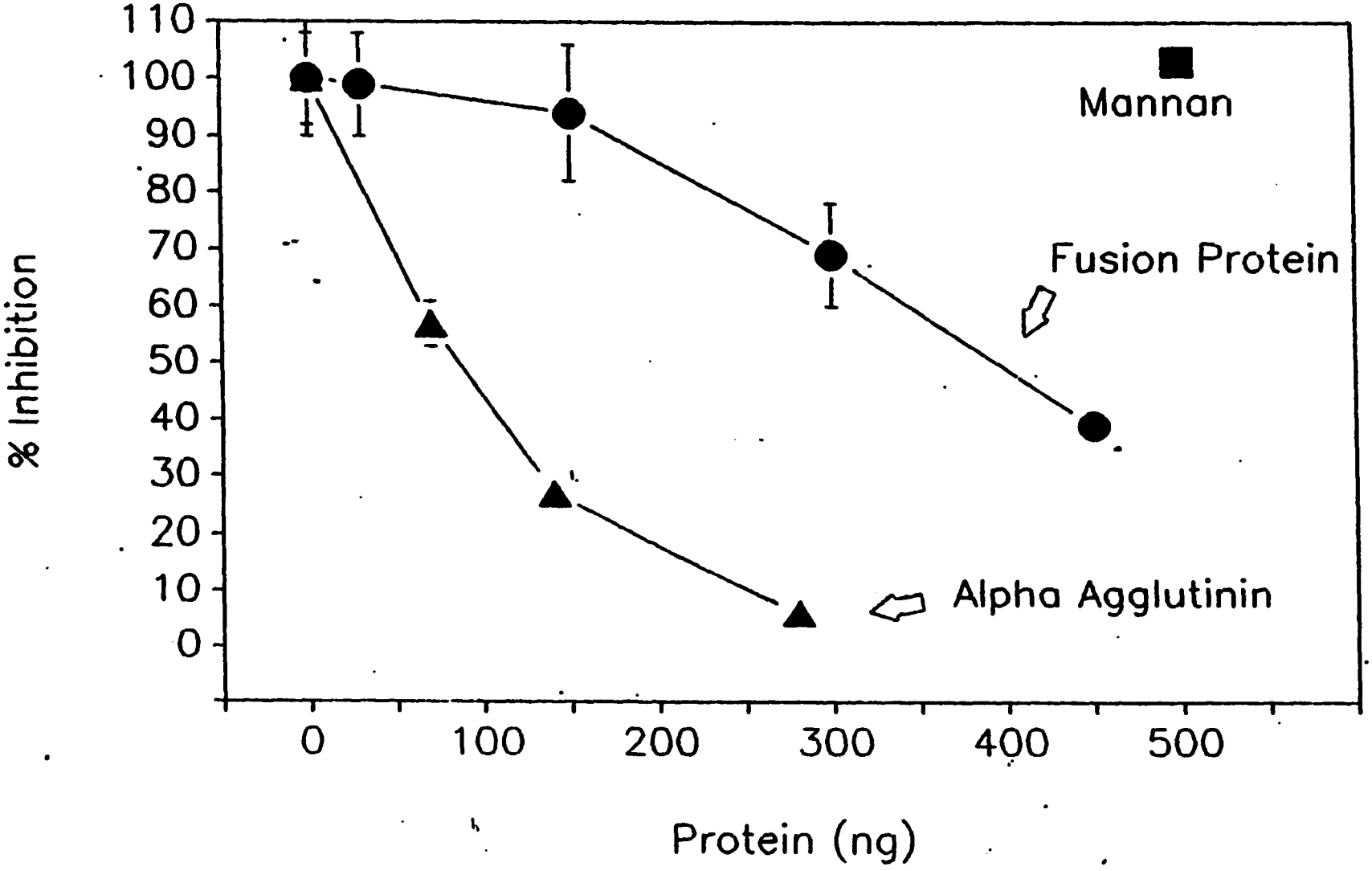

A
B
C
D
E
97–
66–
42–
31–
20–

Figure 19. Fusion Protein Neutralizes the Antibody Mediated Inhibition of Agglutination. Crude yeast mannan (■), increasing amounts of α-agglutinin (▲) or fusion protein (●) were incubated with anti-α-agglutinin followed by incubation with α cells. An equal number of a cells were then added. The agglutinability of the mixture was then assayed.


110
100
90
80
70
60
50
40
30
20
10
0
% Inhibition
0
100
200
300
400
500
Protein (ng)
Mannan
Fusion Protein
Alpha Agglutinin

α-agglutinin and fusion protein. To demonstrate this, antiserum was preadsorbed with purified α-agglutinin and then tested by immunoblotting using fusion protein as antigen. Immunoblots incubated with preadsorbed antiserum show a 70 % reduction in the immunostaining of fusion protein (as compared to a control blot incubated with unadsorbed antibody) (Fig. 20). This result confirms that there is a population of antibody in the antiserum which recognizes both α-agglutinin and fusion protein. This result demonstrates immunological crossreactivity between α-agglutinin and fusion protein.

An antiserum was then raised against fusion protein. The antiserum recognized α-agglutinin peptides in crude cell extracts from wild type α cells but not in extracts from a clone in which the α-agglutinin gene had been disrupted (Figure 21). This result indicates that the antiserum is relatively free of contaminating antibodies since no immunoreactive species (other than α-agglutinin peptides) could be identified in immunoblots.

Collectively, these results indicate that the 2 kb ORF present in the 6.1 kb fragment codes for α-agglutinin.

Figure 20. Immunological crossreactivity between α-agglutinin and fusion protein. Identical amounts (100 ng) of fusion protein were Western blotted and incubated with anti-α-agglutinin (B) or anti-α-agglutinin preadsorbed with purified α-agglutinin (A). This was followed by incubation of blots with secondary antibodies. Comparison of blots (insets) show relative reduction in immunoreactivity of fusion protein when incubated with adsorbed antibody (A) as compared to fusion protein incubated with non-adsorbed antibody (B). Relative difference in immunoreactivity was quantified by densitometric scanning of bands (compare differences in peaks highlighted in black). Fusion protein incubated with adsorbed antibody was shown to be 70% less immunoreactive than that seen for fusion protein incubated with non-adsorbed antibody.

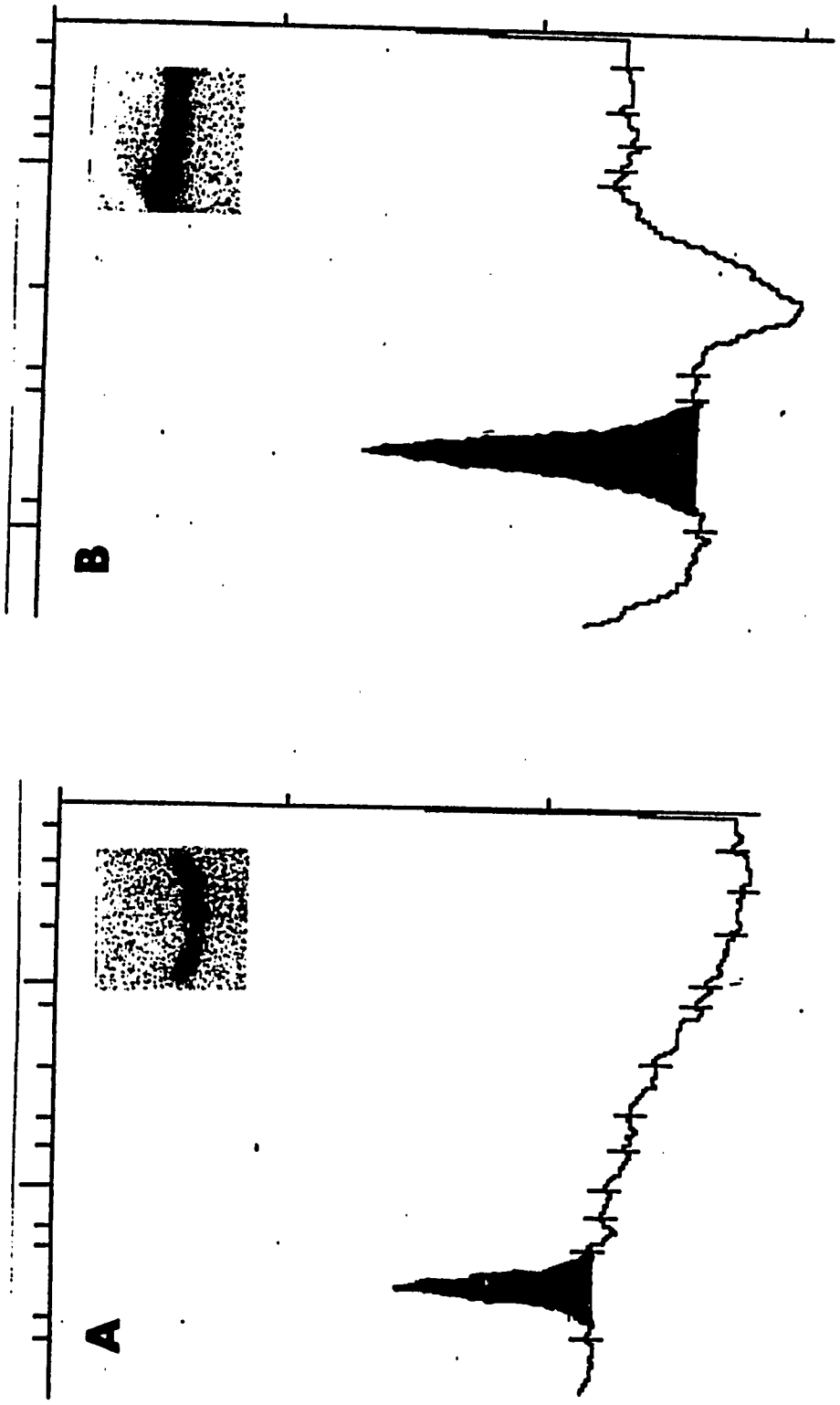
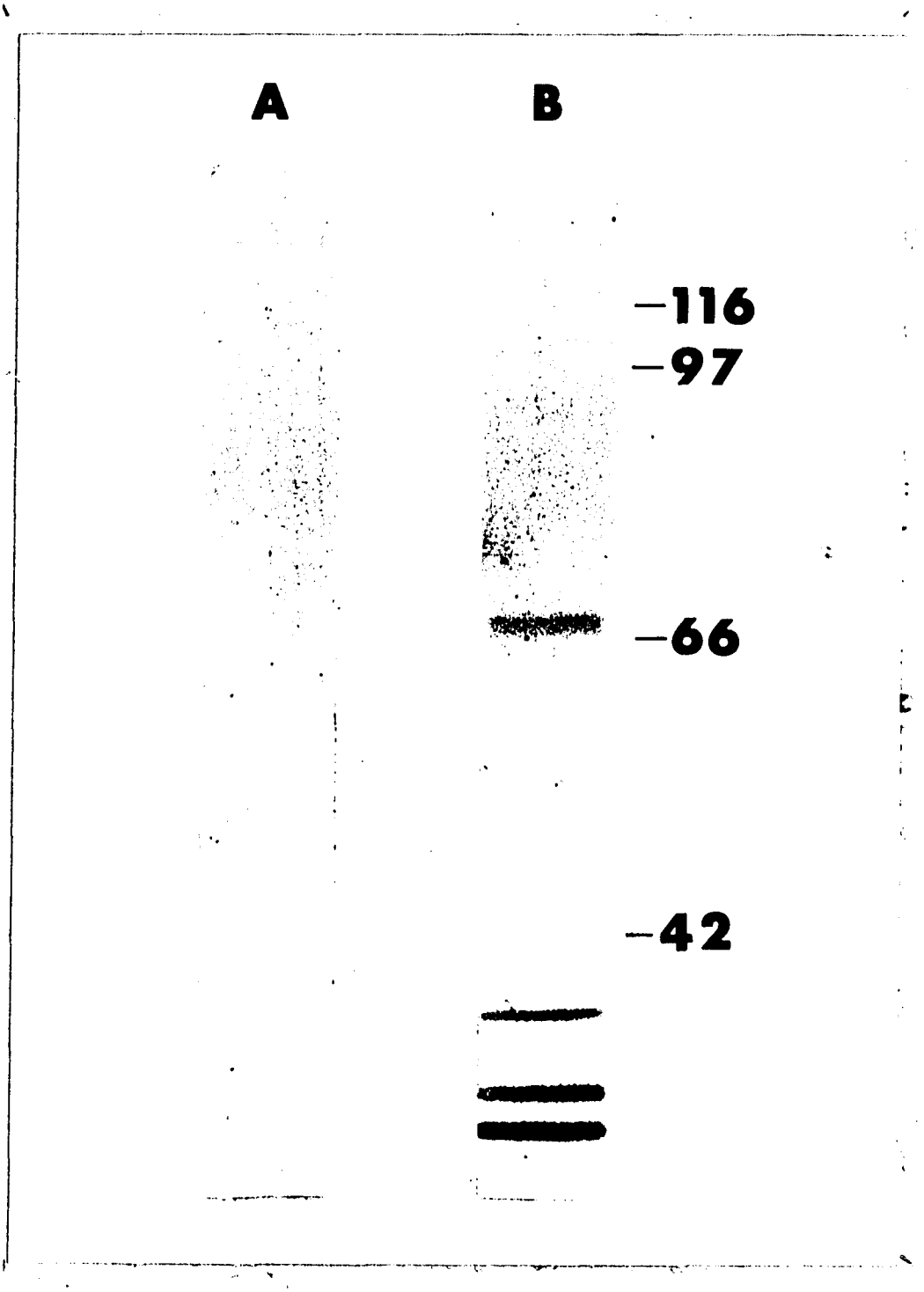

B
A

Figure 21. Immunoblot analysis of crude cell extracts using antibody to fusion protein. An *AGα1::LEU 2* disruption mutant (Lane A) failed to express α-agglutinin peptides while α-agglutinin peptides could be detected in wild type α cell extract from strain W303-1B (Lane B). Crude extracts (5 ug) were treated with endo-N-acetyl glucosaminidase H prior to immunoblotting. Numbers indicate kD.


A
B
116
97
66
42

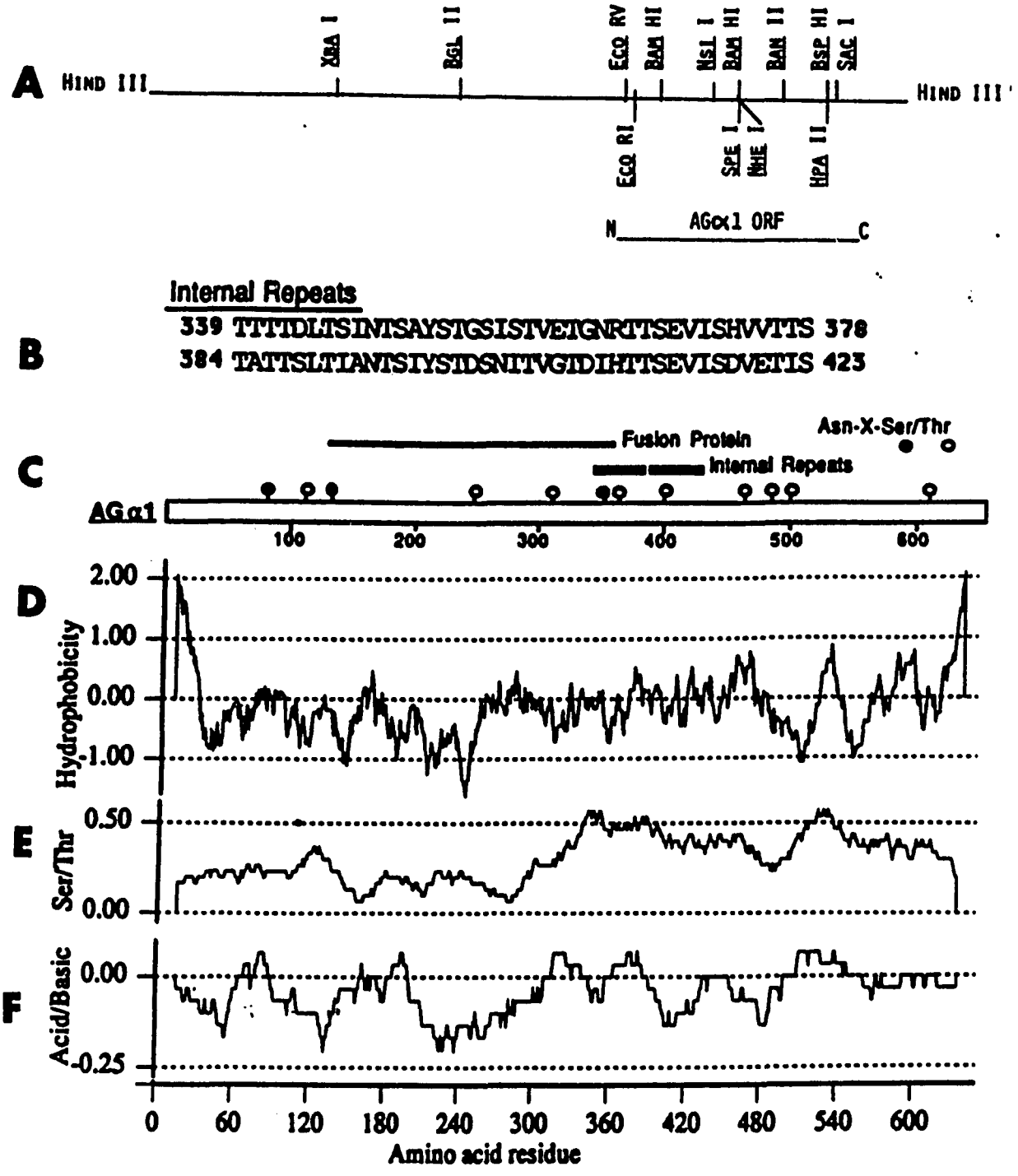
Discussion

1. THE BINDING DOMAIN MAY RESIDE WITHIN THE ACIDIC REGION OF α-AGGLUTININ

The region of the ORF coded by the fusion protein represents the most acidic portion of *AGα1* (Fig. 22). It is speculated (Lipke et al. 1989) that this region of the ORF contains the binding domain of the glycoprotein since ionic interactions between appear to play an important role in the binding of the two agglutinins because: 1) the binding of one agglutinin to the other is pH dependent (maximum binding at pH 5.5) and 2) agglutination is inhibited by ionic (0.05% SDS) but not nonionic detergents (1% Triton-X100) or Urea (up to 3.0 M) (Terrance and Lipke 1981). These observations suggest that agents which perturb ionic interactions disrupt or inhibit binding of α-agglutinin to a-agglutinin. Additionally, binding fragments of α-agglutinin from other related species have been found to be rich in acidic residues (Burke et al. 1980, Pierce and Ballou 1983). The observation that the most acidic part of the ORF is between residues 200 and 300 suggests that the region coded by the fusion protein contains the binding domain of α-agglutinin.

Observations that: 1) fusion protein can neutralize the inhibitory activity of anti-α-agglutinin and 2)

Figure 22. Restriction map and structural features of *AGα1*. (A) Restriction map of 6.1 kb fragment of DNA containing *AGα*1. N= amino terminus and C= carboxy terminus of *AGα1* ORF. (B) Sequences of tandem repeat found approximately 2/3 through the ORF of *AGα1*. (C) Potential N-linked glycosylation sites (O) or (●), position of internal repeats, and sequence contained within fusion protein coded by pDW1. (D) Hydrophobicity as determined by Kyte-Doolittle analysis (Kyte and Doolittle 1982), with a window of 20 amino acids. (E) Frequency of Ser and Thr residues using a value of 1 for Ser and Thr and 0 for all other amino acids, with a window of 30 amino acids. (F) Acidic and basic regions of *AGα1* using a value of 1 for Arg and Lys and His, -1 for Asp and Glu and 0 for all other amino acids, with a window of 30 amino acids.


A
HIND III
XBA I
BGL II
ECO RV
BAM HI
NSI I
BAM HI
BAN II
BSP HI
SAC I
HIND III
ECO RI
SPE I
NHE I
HPA II
N
AGα1 ORF
C
B
Internal Repeats
339 TTTIDLTSINISAYSIGSISTVEIGNRITSEVISHVVITS 378
384 TATTSLTIANISIYSIDSNITVGIDIHITSEVISDVETIS 423
C
Fusion Protein
Asn-X-Ser/Thr
Internal Repeats
AGα1
100
200
300
400
500
600
D
Hydrophobicity
2.00
1.00
0.00
-1.00
E
Ser/Thr
0.50
0.00
F
Acid/Basic
0.00
-0.25
0
60
120
180
240
300
360
420
480
540
600
Amino acid residue

antibody to the fusion protein can inhibit the binding of the two mating types suggests that the fusion protein contains an antigenic sequence or sequences which reside near or within the binding domain of α-agglutinin. These results provide indirect evidence that the binding domain of the glycoprotein resides within the region contained within the fusion protein.

Antibody to the fusion protein recognizes three peptides of 36 to 38 kD in crude extracts of wild type α cells (Fig. 21). It has been shown previously that three deglycosylated fragments of α-agglutinin which are approximately 38 kD have the ability to bind to a cells (Terrance et al. 1987). The observation that the 38 kD peptides are highly immunoreactive suggests that the 38 kD peptides contain at least part of the region contained within the fusion protein. This data suggests that the region contained within the fusion protein may reside near or within the functional domain of the glycoprotein because the 38 kD peptides are functional. In light of these results, it is thought that the 38 kD species are proteolytic fragments of α-agglutinin which contain the amino half of the glycoprotein.

Collectively, data which is presented suggests that the binding domain of α-agglutinin resides within the amino half of the glycoprotein.

2. IMPLICATION OF THE CARBOXY TERMINUS IN CELL WALL ANCHORAGE

It is assumed that α-agglutinin is anchored to the cell wall because 1)*AGα1* does not appear to contain a stretch of hydrophobic amino acids sufficient in length to constitute a transmembrane domain and 2) the hydrophobic stretch of amino acids found in *AGα1* is not followed by basic residues, a feature found in many transmembrane sequences. Additionally, if α-agglutinin were cell membrane associated, it would need to pass through the cell wall to reach the cell surface. Since the cell wall is approximately 100 to 150 nm thick (Ballou 1982), α-agglutinin would need to be in an extended conformation to reach the cell surface from the plasma membrane. It is therefore likely that α-agglutinin is cell wall associated.

Examination of the carboxy terminus reveals a stretch of 15 hydrophobic amino acids (Fig. 22). This stretch is not long enough to constitute a transmembrane domain but is similar in length to hydrophobic stretches seen in proteins which are anchored to cell surfaces through a phosphatidyl inositol glycan tail (PIG-tail) (Ferguson and Williams 1988, Low and Saltiel 1988). In *Saccharomyces cerevisiae*, a 125 kd membrane glycoprotein has been found to be associated with the

lipid bilayer by a similar anchor (Conzelmann et al. 1988). This result suggests that in this organism, anchorage by a mechanism similar to that of a PIG-tail is possible. At present it is difficult to speculate whether mannoproteins are anchored to the cell wall by such a mechanism because: 1) α-agglutinin is the first cell wall protein to be cloned in yeast, and 2) the molecular architecture of the cell wall is largely unknown.

3. SUMMARY

AGα1 has been identified as the gene which codes for α-agglutinin. Identification was made by demonstrating that: 1) the gene product is recognized by antibody made to α-agglutinin 2) fusion protein neutralizes the inhibitory effect of anti-α-agglutinin and 3) disruption of the gene results in the loss of expression of α-agglutinin peptides. In addition, an antibody was produced against fusion protein and found to recognize α-agglutinin peptides by Western blotting.

It is speculated that an acidic region within the amino half of *AGα1* codes for the binding domain of α-agglutinin while the carboxy terminus contains sequences important for cell wall anchorage. Genetic analysis of *AGα1* was then undertaken to determine whether these regions were involved in α-agglutinin function.

Part 3

1.INTRODUCTION AND STRATEGY

α-agglutinin is reported to be transported to the cell surface through the secretory pathway (Tohoyama and Yanagishima 1985). This is supported by the observation that *AGα1* has a putative secretion signal sequence at the amino terminal. I reasoned that products of *AGα1* would be secreted into the culture medium if sequences responsible for cell wall anchorage were deleted. Therefore, if the carboxy end of *AGα1* was responsible for cell wall attachment, then termination of the ORF prior to this sequence should result in an *AGα1* product which is secreted.

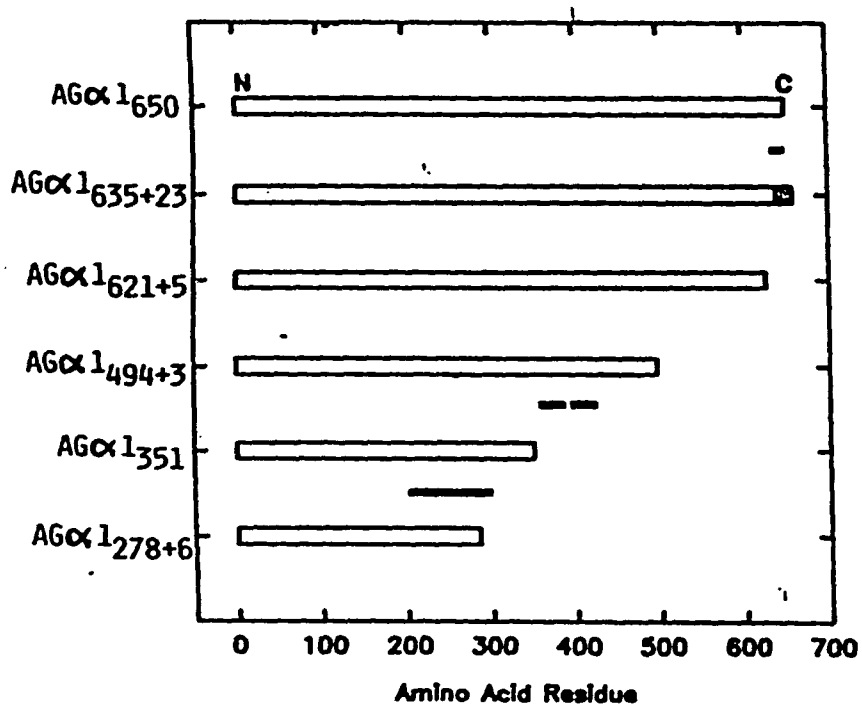
To test this hypothesis, premature terminations of translation in *AGα1* were made by cutting the DNA coding the ORF at specific restriction sites and either filling-in or removing the single stranded DNA overhangs created by the restriction endonuclease with Klenow fragment of DNA polymerase or T4 DNA polymerase. Modifying the restriction sites in this manner resulted in the addition (or deletion) of 4 base-pairs to the coding sequence of the ORF. The addition or subtraction of these base-pairs caused sequences 3' to the restriction site to be read out of frame resulting in premature termination of translation at the next out of

frame termination codon.

Restriction sites selected for modification were chosen to obtain information about the possible function of specific regions within the protein. Five restriction sites were chosen (Fig. 23) for modification which were just upstream of, or within sequences coding 1) the hydrophobic carboxy terminus, 2) the imperfect tandem repeat and 3) the acidic region, located between amino acids 200 and 300 of the ORF.

Once a restriction site was modified, fragments of DNA containing the modified sites were subcloned into YEp351-*AGCX1* (see Material and Methods, Part 3 for diagram of this plasmid). YEp351 is a high copy plasmid which has *LEU2* (a selectable marker allowing transformants of strain LCX21 to grow on medium devoid of leucine). *LEU2* codes for isopropylmalate dehydrogenase, an enzyme used in the biosynthesis of leucine from pyruvate. YEp351 also contains a 2u segment of DNA which allows autonomous replication of plasmid in *Saccharomyces cerevisiae*. Lastly, YEp351 contains an origin of replication (Ori) and an ampicillin resistance marker (Amp^R) which allows replication and selection in *E. coli*.

Figure 23. Truncations made in *AGα1* and summary of the biochemistry of transformants expressing these truncations. Bars represent the lengths of truncated products as compared to wild type *AGα1* ($AG\alpha 1_{650}$). N= amino terminus, C= carboxy terminus. Stippled area at carboxy end of $AG\alpha 1_{635+23}$ indicates residues coded by out of frame sequence. Black bars indicate position and length of: 1) the hydrophobic tail (between $AG\alpha 1_{650}$ and $Ag\alpha 1_{635+23}$), tandem repeat (between $Ag\alpha 1_{494+3}$ and $AG\alpha 1_{351}$ and 3) acidic region (between $AG\alpha 1_{351}$ and $AG\alpha 1_{278+6}$). nd=not determined.



	Agglutination Index (AI)	Immunofluorescence	Activity Secreted (U/ml)
$AG\alpha1_{650}$	0.65-0.75	+++	0.18
$AG\alpha1_{635+23}$	0.17	+/-	3.30
$AG\alpha1_{621+5}$	0.03	-	2.70
$AG\alpha1_{494+3}$	0.09	nd	2.80
$AG\alpha1_{351}$	0.02	-	3.40
$AG\alpha1_{278+6}$	0.03	nd	0.00

2.RESULTS

Once subcloned into YEp351, the modified restriction sites were sequenced by double stranded DNA sequencing. As an example, the results of a typical sequencing reaction appear in Figure 24. These plasmids were designated pAGc.1X1+X2 were X1 is the number of amino acids coded by *AGα1* sequence and X2 is the number of residues coded by out of frame sequence (Fig. 25). Wild type *AGα1* (YEp351+AGα1) is designated pAGα1650. Restriction sites modified by T4 DNA polymerase: *Nsi* I(pAGα1278+6), *Ban* II (pAGα1494+3) and *Sac* I (pAGα1635+23) were trimmed back (the 4 base single stranded overhang eliminated (Fig. 25) and those modified by Klenow fragment of DNA polymerase: *Spe* I (pAGα1351) and *Bsp* HI (pAGα1621+5) were filled-in so that a 4 base pair repeat of the single stranded 4 base overhang was created (Fig. 25).

These constructs were then introduced into Lα21 (a strain of α cell which fails to: 1) agglutinate a cells (Lipke et al. 1989) and 2) express immunoreactive α-agglutinin peptides (as determined by immunofluorescence and Western blotting). Transformants were selected on medium devoid of leucine. Transformants

Figure 24. Sequence of modified *Sac* I site in pAGα$_{1835+23}$. T7 DNA polymerase was used to perform double stranded DNA sequencing using an 18 mer oligonucleotide primer. Sequence of modified site (read from the bottom) confirms trimming back of the single stranded 4 base pair overhang by the exonuclease activity of T4 DNA polymerase.

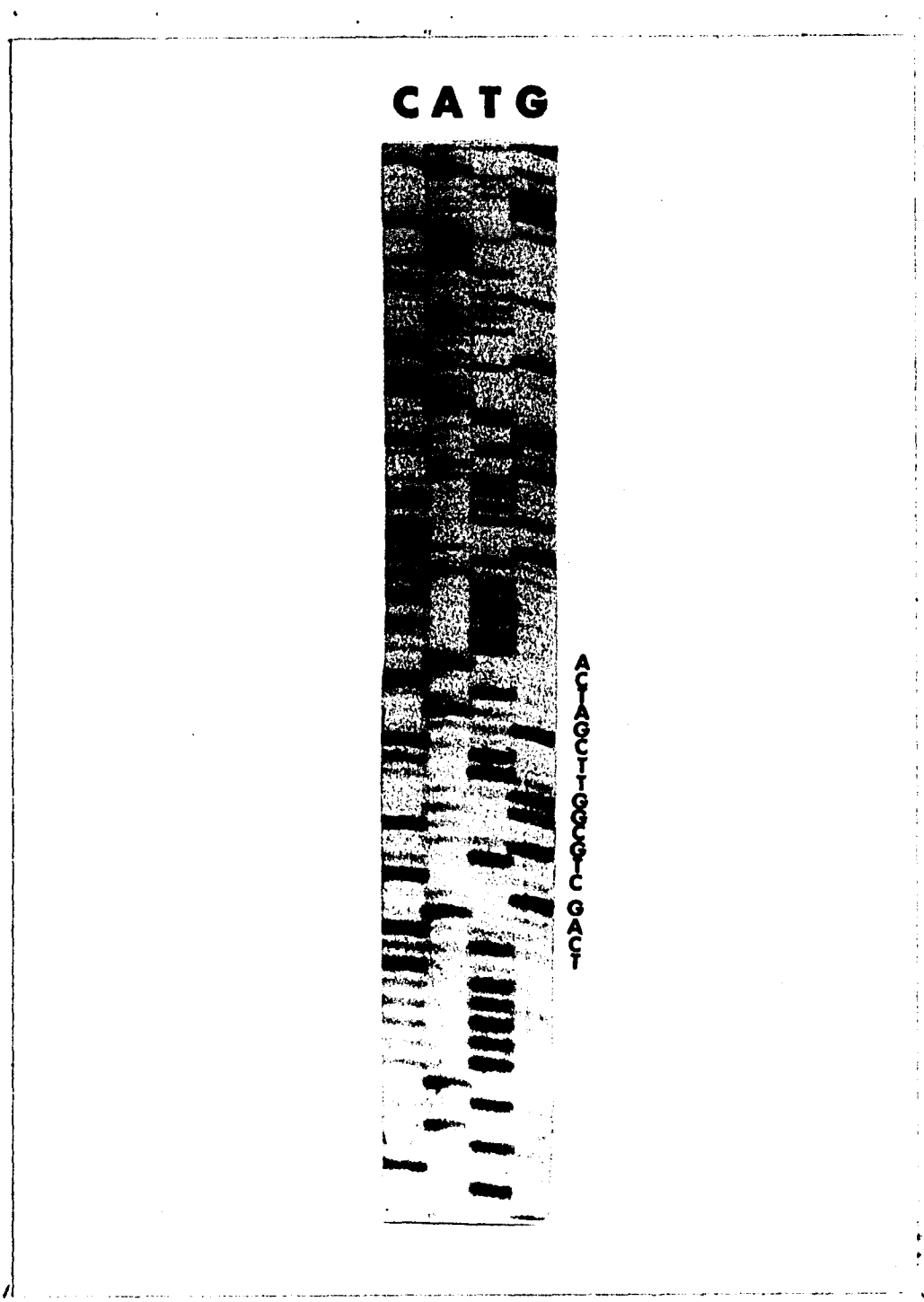

CATG

Figure 25. Nucleotide and amino acid sequences of truncations in *AGα1*. The sequence found in the top half of each box is that found in wild type *AGα1* ($AG\alpha_{1650}$), the bottom sequence is the new nucleotide and amino acid sequence after modification of the restriction site. The double underline in each wild type sequence denotes the sequence of the restriction site used to create the truncation. Number after the last amino acid in the wild type sequence indicates the residue number within the ORF.

	Val Asn Ala Leu Gln Ser Leu Pro Ala Asn_{287}
$AG\alpha1_{650}$	GTT/AAT/GCA/TTA/CAA/TCT/CTA/CCC/GCT/AAT/...
$AG\alpha1_{278+6}$	GTT/AAT/TAC/AAT/CTC/TAC/CCG/CTA/ATG/TAA Val Asn Tyr Asn Leu Tyr Pro Leu Met *
	Asn Thr Ser Ala_{352}
$AG\alpha1_{650}$	AAC/ACT/AGT/GCG/...
$AG\alpha1_{351}$	AAC/ACT/AGC/TAG Asn Thr Ser *
	Glu Glu Pro Thr Phe_{498}
$AG\alpha1_{650}$	GAA/GAG/CCC/ACT/TTT/...
$AG\alpha1_{494+3}$	GAA/GCA/CTT/TTG/TAA Glu Ala Leu Leu *
	Leu Met Ile Ser Thr Tyr Glu Gly_{627}
$AG\alpha1_{650}$	CTC/ATG/ATT/TCA/ACC/TAT/GAA/GGT/...
$AG\alpha1_{621+5}$	CTC/ATG/CAT/GAT/TTC/AAC/CTA/TGA Leu Met His Asp Phe Asn Leu *
	Ala Glu Leu Gly Ser Ile Ile Phe Leu Leu Leu Ser Tyr Leu Leu Phe_{650}
$AG\alpha1_{650}$	GCT/GAG/CTC/GGT/TCG/ATC/ATT/TTT/CTG/CTT/TTG/TCG/TAC/CTG/CTA/TTC/TAA
$AG\alpha1_{623+23}$	GCT/GCG/GTT/CGA/TCA/TTT/TTC/TGC/TTT/TGT/CGT/ACC/TGC/TAT/TCT/AAA/ACG/GGT/ACT/GTA/CAG/TTA/GTA/CAT/TGA Ala Val Val Arg Ser Phe Phe Cys Phe Ser Arg Thr Cys Phe Ser Lys Thr Gly Thr Val Gln Leu Val His *

were then assayed for their ability to 1) agglutinate with $\underline{a}$ cells, and 2) secrete functional α-agglutinin peptide (as determined by the ability of spent culture medium, when preincubated with $\underline{a}$ cells, to inhibit the binding of these cells to α cells). Once α-agglutinin activity was detected in culture media, the secreted product was isolated from culture medium by ion exchange chromatography (DEAE Sephadex). The isolated product was then deglycosylated with endo-N-acetylglucosaminidase H (endo H) and analyzed by Western blotting using antibody to the fusion protein (See Part 2) to determine the molecular weight of the secreted peptide (Figs. 26 and 27).

3. AGGLUTINABILITY OF TRANSFORMANTS AND CHARACTERIZATION OF SECRETED PRODUCTS

3a. pAGα1635+23

The glycoprotein produced from pAGα1635+23, designated AGα1635+23, is 8 amino acids longer than AGα1650 with 23 residues at the carboxyl end coded from sequences which are out of frame (Fig. 25). This out of frame sequence codes for a carboxy terminus which is markedly less hydrophobic (Fig. 29).

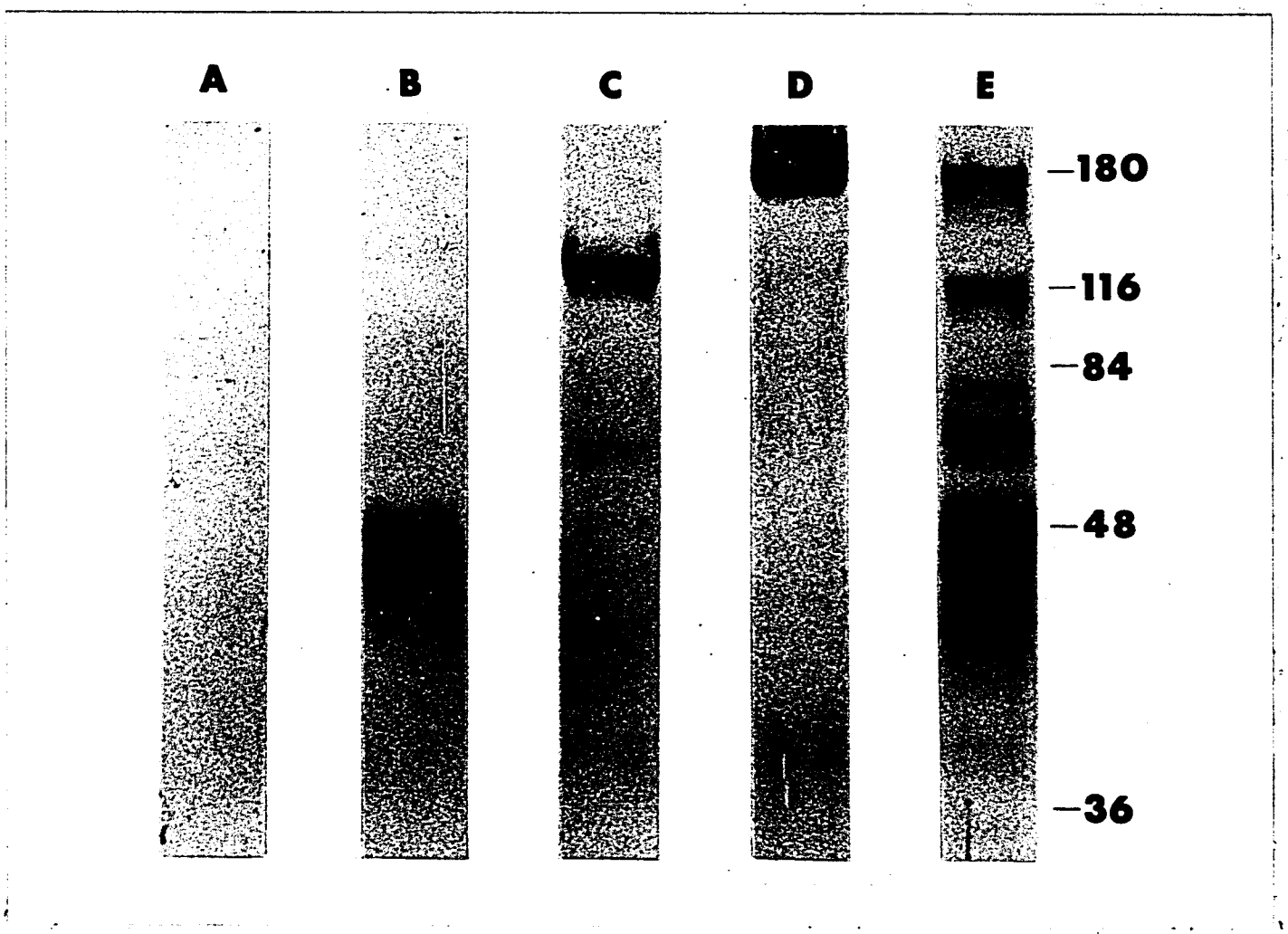
Cells transformed with pAGα1635+23 were

different from cells transformed with pAGα1650 in that they agglutinated poorly (Agglutination Index (AI)=0.17 as compared to an AI of 0.65 for pAGc.1650 transformants) and expressed only 10 % of the cell surface associated α-agglutinin as compared to wild type as determined by immunoquantitation.

However, pAGα1635+23 transformants secreted α-agglutinin activity. Secreted activity was deglycosylated with endo H and analyzed by Western blotting. Secreted deglycosylated peptides ranged in molecular weight from 45 kd to 180 kD (Fig. 26). pAGα1635+23 transformants secreted approximately 3.3 U/ml of activity (Fig. 23). Conversely, transformants carrying wild type *AGα1* (pAGα1650) secreted only 0.18 U/ml or only 5% of the activity secreted by pAGα1635+23 1 unit of activity is defined as the amount of α-agglutinin, when incubated with induced a cells, that will lower the AI of these cells by 0.1 AI unit below the control value. If purified α-agglutinin has an average specific activity of 27,000 U/mg (Terrance et al. 1987), then there is about 150 ug of AGα1635+23 peptide present per liter of spent culture medium. In contrast, there is only about 8 ug of AGα1650 present per liter of spent culture medium from transformants of pAGα1650.

Material isolated from culture medium of cells transformed with AGα1650 showed the same peptides

Figure 26. Identification of secreted peptides by Western blotting. Peptide was isolated from media by ion exchange chromatograhy and 4 units of each peptide were deglycosylated with endo H. Peptides were subjected to Western blotting. Membrane was probed with antibody to fusion protein followed by secondary antibodies. Lane A, sample of a mock peptide isolation using medium in which cells transformed with YEp351 were grown. Lane B, AGα_{1351}; Lane C, AGα_{1484+6}; Lane D, AGα_{1621+5}; and Lane E, AG$\alpha_{1635+23}$ peptides. Numbers indicate kD.


A
B
C
D
E
180
116
84
48
36

(which ranged in molecular weight from 45 to 180 kD) by Western blotting. However, in strains of α cell (W303-1B, X2180-1B) which contain a single copy of *AGα1*, α-agglutinin activity could not be detected in the culture medium. This suggests that the material isolated from the medium of transformants carrying the wild type gene secrete product because of *AGα1* overexpression on a multicopy plasmid (YEp351).

Most of the product made from pAGα1$_{635+23}$ is secreted because: 1)only 10 % of the peptide (as compared to wild type) is stably expressed on the cell surface and 2) pAGα1$_{635+23}$ transformants secrete as much active peptide (3.3 U/ml) as other transformants which do not express any active cell surface *AGα1* product (Fig. 23).

This data suggests that the hydrophobic tail is necessary for efficient anchorage of α-agglutinin to the cell surface and confirms the earlier speculation (see Part 2) that the carboxy terminus is involved in anchorage of α-agglutinin to the cell wall.

3b. pAGα1$_{621+5}$

AGα1$_{621+5}$ has a truncated carboxy terminus and is 29 amino acids shorter than full length

AGα1. Five residues, coded by out of frame sequence, are present and comprise the carboxy terminus (Fig. 25)

pAGα1$_{\Delta 621+5}$ transformants were non-agglutinable (AI=0.03) and did not express cell surface associated α-agglutinin (as determined by immunofluorescence) (Fig. 23). These transformants secreted a functional α-agglutinin. A deglycosylated peptide of approximately 180 kD is detected by Western blotting (Fig. 26). The observation that pAGα1$_{\Delta 621+5}$ transformants are non-agglutinable confirms the supposition that the carboxy terminus of *AGα1* is necessary for anchorage. Approximately 2.7 U/ml of activity is secreted from pAGα1$_{\Delta 621+5}$ transformants (Fig 23).

3c. pAGα1$_{\Delta 494+3}$

AGα1$_{\Delta 494+3}$ is 497 residues in length (153 amino acids shorter than wild type *AGα1*) and has the addition of three amino acids coded by sequence which is out of frame (Fig. 25). Cells transformed with pAGα1$_{\Delta 494+3}$ were also non-agglutinable (AI=0.09) (Fig. 23). Functional α-agglutinin was secreted by this transformant. Once deglycosylated with endo H, the peptide has an apparant molecular weight of 120 kD (Fig.

26). The secreted peptide is still functional (2.8 U/ml secreted, Fig. 23), indicating that the binding domain of α-agglutinin is intact and resides closer to the amino terminus.

3d. pAGα1351

Transformants with pAGα1351 secreted functional α-agglutinin peptide. The deglycosylated peptide has a molecular weight of 45 kD (Fig. 26) which contains approximately half of the ORF but lacks the imperfect tandem repeat (Fig. 23). No residues are added due to out of frame sequence (Fig. 25). Approximately 3.4 U/ml of activity is secreted by pAGα1351 transformants (Fig. 23). A functional AGα1351 peptide indicates that the tandem repeat does not comprise part of the binding domain and demonstrates that the binding domain of the glycoprotein resides within the amino half of the ORF (possibly within the acidic region).

Endo H-treated AGα1351 was incubated with either induced a cells or Lα21 transformed with YEp351. These cells were then incubated in anti-α-agglutinin and fluorescein conjugated IgG. a cells incubated with AGα1351 immunostained confirming that AGα1351 had bound to the cell surface. Conversely, YEp351

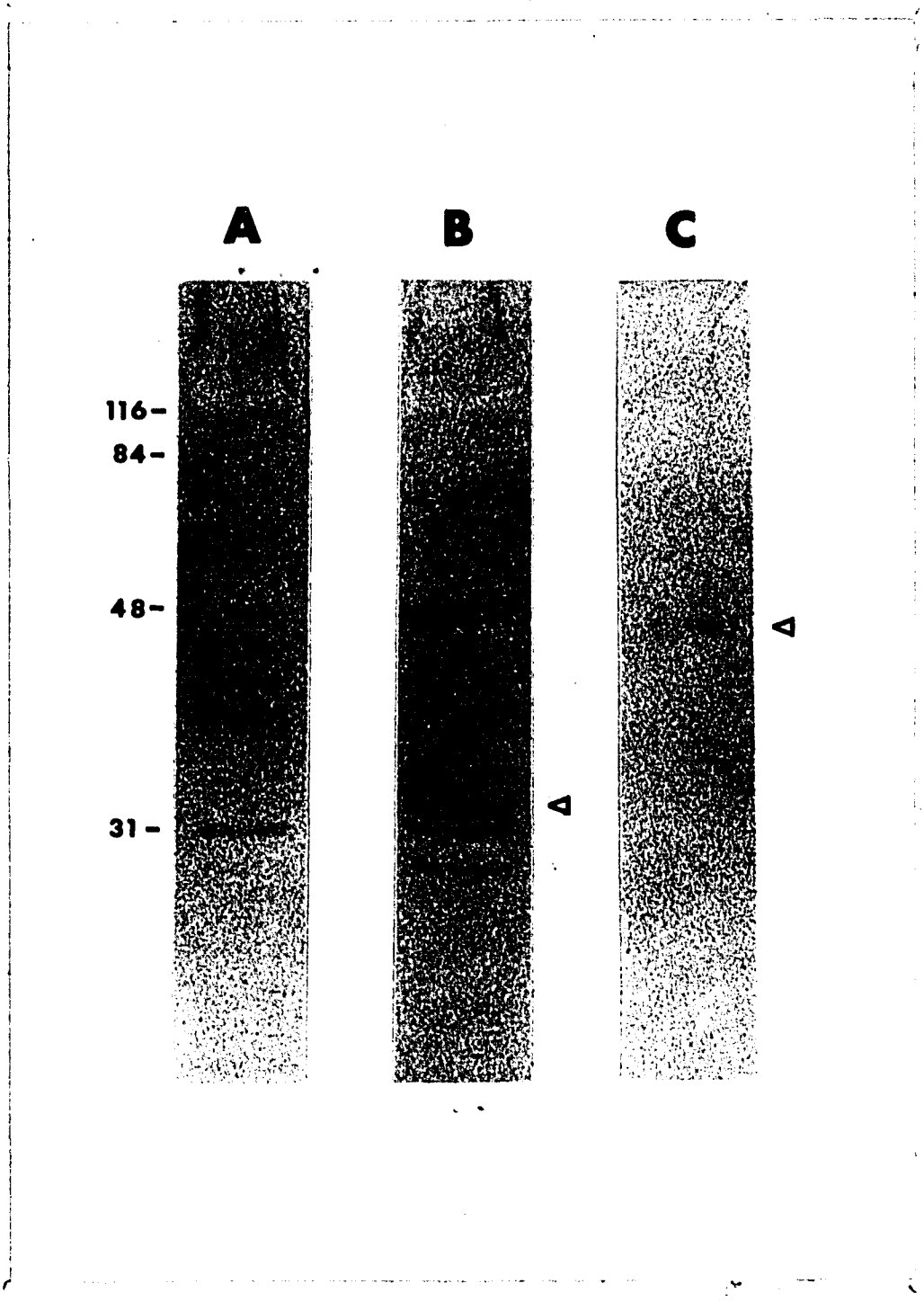
transformants of Lα21 did not immunostain. This data confirms that deglycosylated AGα1351 binds specifically to a cells.

3e. pAGα1278+6

A 32 kD species can be identified in endo H treated spent culture medium of pAGα1278+6 transformants (Fig. 27). By comparing the immunostaining intensity of the AGα1278+6 and AGα1351 peptides by Western blotting (Fig. 27) I estimate that the amount of AGα1278+6 secreted by pAGα1278+6 transformants is 200-fold less than AGα1351 secreted by the pAGα1351 transformants. The low yield of AGα1278+6 peptide may be due to peptide lability. Immunoblots of culture media from these transformants show a broad immunoreactive smear from approximately 25 kD to 20 kD. This suggests that the product is susceptible to proteolysis.

Comparative immunoquantitation of the AGα1351 and AGα1278+6 products was performed by immunoblotting (Fig. 27). Using equivalent amounts of immunoreactive AGα1351 and AGα1278+6 peptides in bioassay, it was found that activity could be detected with the AGα1351 peptide (0.4 units) but not with the AGα1278+6 peptide. Up to five-fold more AGα1278+6 was assayed and still no activity could

Figure 27. Identification of AGα_{1278+6} peptide from concentrated culture medium and its immunoquantiation as compared to the immunoreactivity of AGα_{1351}. 5 ml of spent medium from cultures of pAGα_{1278+6} (Lane B) and YEp351 (Lane A) transformants were concentrated and treated with endo H. Samples were then subjected to Western blotting. The membrane wes incubated with antibody to fusion protein followed by secondary antibodies. Lane B, arrow identifies 32 kD AGα_{1278+6} product in medium of pAGα_{1278+6} transformant. Lane C, arrow identifies the minimum amount of immunoreactive AGα_{1351} needed to be detected by bioassay (0.4 units). From comparison of the intensity of immunostaining of AGα_{1351} (Lane C) and AGα_{1278+6} (Lane B), it is estimated that there is twice the amount of immunoreactive AGα_{1278+6} in Lane B as there is AGα_{1351} in Lane C. Up to 2.5 times the amount of AGα_{1278+6} has been bioassayed without detecting activity.


A
B
C
116-
84-
48-
31-

be demonstrated. This data suggests that the AGα1278+6 peptide is at least five-fold less active than the AGα1351 peptide. Since no activity has yet been detected with the AGα1278+6 peptide, it is possible that this peptide is completely inactive. The inability to demonstrate that AGα1278+6 binds to a-agglutinin on a cells indicates that the binding domain has been at least partially perturbed in this peptide.

AGα1278+6 could not be isolated by ion-exchange chromatography. Although the peptide binds to DEAE sephadex, only a small percentage of the peptide elutes with NaCl concentrations up to 1M. This suggests that may be the peptide is adhering to the column through non-specific interactions (such as hydrophobic coalescing). If so, then the results of experiments demonstrating lack of binding activity with AGα1278+6 should be taken cautiously. It is possible that the peptide may be unable to interact with a-agglutinin not because it is inactive but because it non-specifically binds to plastic or cell surface before it can interact with ligand. However, the observation that deglycosylated AGα1351 binds specifically to a cells suggests that *AGα1* products do not bind in a non-specific fashion.

The *Nsi* I site modified to make pAGα1278+6 resides near the end of the acidic region (Figs. 22 and 23). The acidic region is located

between amino acids 200 and 300 of the ORF (Figs. 22 and 23). Within this region the protein has 16 acidic and 3 basic residues. Although most of the acidic region is contained within AGα1$_{278+6}$ (pAGα1$_{278+6}$ eliminates only 3 acidic and 1 basic residue), it is possible that the secondary structure of the acidic region is sufficiently perturbed (by the elimination of downstream sequences) so as to make AGα1$_{278+6}$ non-functional (see Discussion, Part 3). Regardless of the mechanism by which function is lost, it is apparent that the acidic region comprises an integral part of the binding domain of α-agglutinin. This conclusion is consistent with the observation that the binding fragments of α-agglutinin from other species of yeast (*Hansenula wingei* and *Saccharomyces kluyveri*) are acidic in composition (Burke et. al. 1980, Pierce and Ballou, 1983).

4. PEPTIDE SYNTHESIS, PROCESSING AND SECRETION

It was important to determine the relative rate of synthesis and secretion of the truncated peptides. Therefore, cellular fractions of transformants were prepared from Lα21 cells transformed with either pAGα1$_{351}$ or YEp351 (a control plasmid) and analyzed by Western Blotting to determine the relative amount of

peptide present in culture medium, the periplasmic fraction and cell extract (cytoplasm). Western blots demonstrate that almost all of the AGα_{1351} peptide was secreted and that only a very small percentage of this peptide may be present intracellularly in the form of slightly lower molecular weight species (Fig. 28). These species could represent underglycosylated forms of AGα_{1351}. No AGα_{1351} peptide was detected in the periplasmic space, indicating efficient export of peptide from the plasma membrane to the culture medium.

No detectable amount of AGα_{1278+6} peptide was found intracellularly, indicating that low levels of peptide secretion was not attributable to inefficient processing or export of this peptide through the secretory pathway. Transformants containing the other constructs showed the same result: all of the truncated products were efficiently secreted.

5. GLYCOSYLATION OF α-AGGLUTININ: PATTERNS OF GLYCOSYLATION

In all cases, the molecular weight of the secreted peptide was substantially larger than the weight predicted from the peptide sequence even after treatment with endo H. However, the disparity between the expected and observed molecular weight (by SDS-PAGE) lessened as

Figure 28. Cellular distribution of AGα1 peptide. pYEp351 and pAGα1$_{351}$ transformants were grown and fractionated (see Material and Methods). Culture medium (250 ul) and periplasmic fraction and cell extract made from cells present in 250 ul of culture medium were analyzed by Western blotting. Material from transformants carrying YEp351 (Y) and pAGα1$_{351}$ (S) were compared. AGα1$_{351}$ peptides are present in culture medium (arrows) at high levels and possibly in cell extract at barely detectable levels in the form of slightly lower molecular weight species (arrows ?).

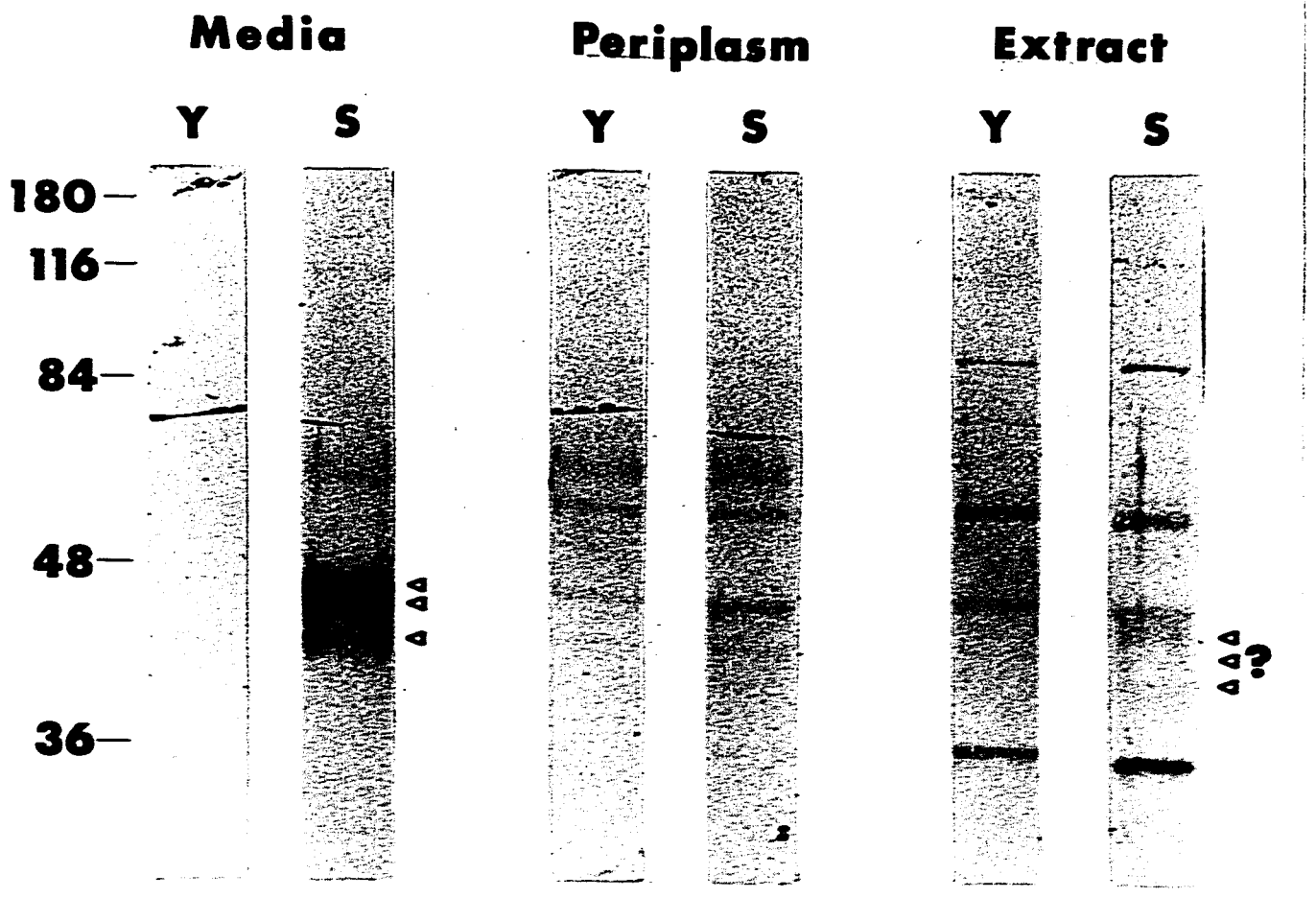
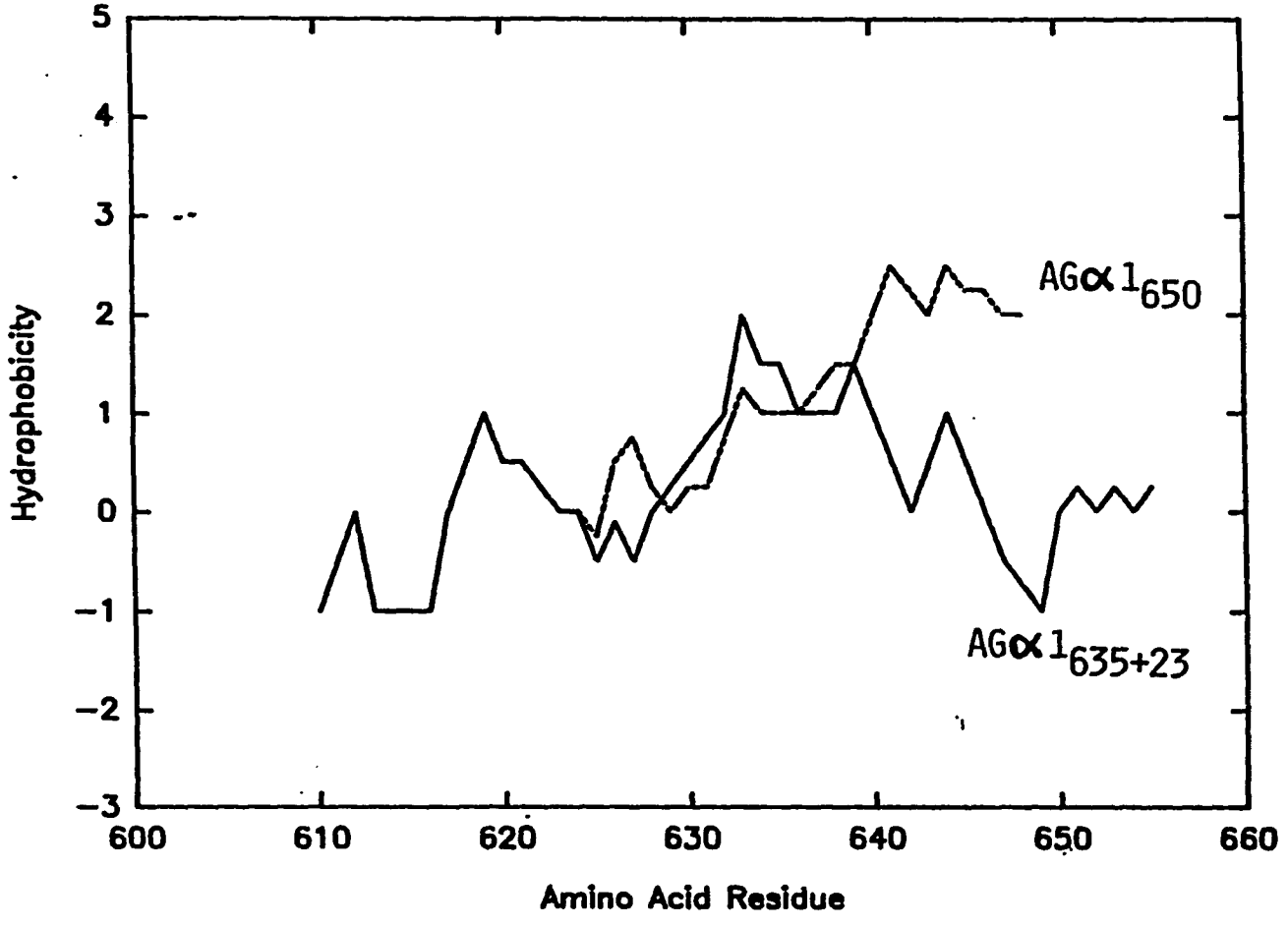

Media
Periplasm
Extract
Y
S
Y
S
Y
S
180
116
84
48
36
?

Figure 29. Comparison of hydrophobicity of AGα$_{1650}$ and AGα$_{1635+23}$ carboxy termini. Hydrophobicity was determined as described (Kyte and Doolittle 1982) with a window of 8. Plot starts at amino acid 610 in *AGα1*. Starting at residue 638, AGα$_{1635+23}$ (solid line) becomes markedly less hydrophobic than wild type AGα$_{1650}$ (stippled line).


5
4
3
2
1
0
−1
−2
−3
Hydrophobicity
600
610
620
630
640
650
660
Amino Acid Residue
AGα1650
AGα1635+23

longer stretches of the Ser/Thr rich region were eliminated from the secreted product (Table 5). This suggests that numerous Ser/Thr residues within this region are sites of attachment for O-linked sugars. Although comparative trends in the degree of glycosylation of the peptides can be made from comparative examination of their molecular weights by SDS-PAGE, the absolute amount of glycosylation cannot be determined.

N-linked carbohydrate adds substantial mass to α-agglutinin and inhibits the ability of the peptide portion of the molecule to be recognized by antibody (Fig. 7). To efficiently detect small amounts of secreted product by Western blotting, all the secreted products needed to be first treated with endo H. This observation indicates that regardless of their size, some N-linked carbohydrate is associated with the truncated peptides. This suggests that N-linked glycosylation sites throughout the ORF are utilized (See Fig. 22 for sites).

Collectively, these observations confirm that α-agglutinin is heavily glycosylated and that glycosylation is due to the presence of significant amounts of O and N linked sugar.

Table 5

Comparison of Predicted and Observed Molecular Weights of Secreted Products*

Product	Expected	Observed	(Observed)-(Expected)
AGα1$_{650}$	70,000	180,000	110,000
AGα1$_{635+23}$	70,000	180,000	110,000
AGα1$_{621+5}$	67,000	180,000	113,000
AGα1$_{494+3}$	53,000	120,000	67,000
AGα1$_{351}$	37,000	45,000	8,000
AGα1$_{278+6}$	29,500	32,000	2,500

* All values determined by SDS-PAGE and expressed in Daltons.
All products deglycosylated with endo H prior to SDS-PAGE.
All products produced in transformants of Lα21.

Discussion

The data presented confirms that the carboxy terminus of *AGα1* is involved in cell wall attachment and that the acidic region in the amino half of the protein is neccessary for function of the binding domain of α-agglutinin.

1. α-AGGLUTININ: PIG-TAIL ASSOCIATED?

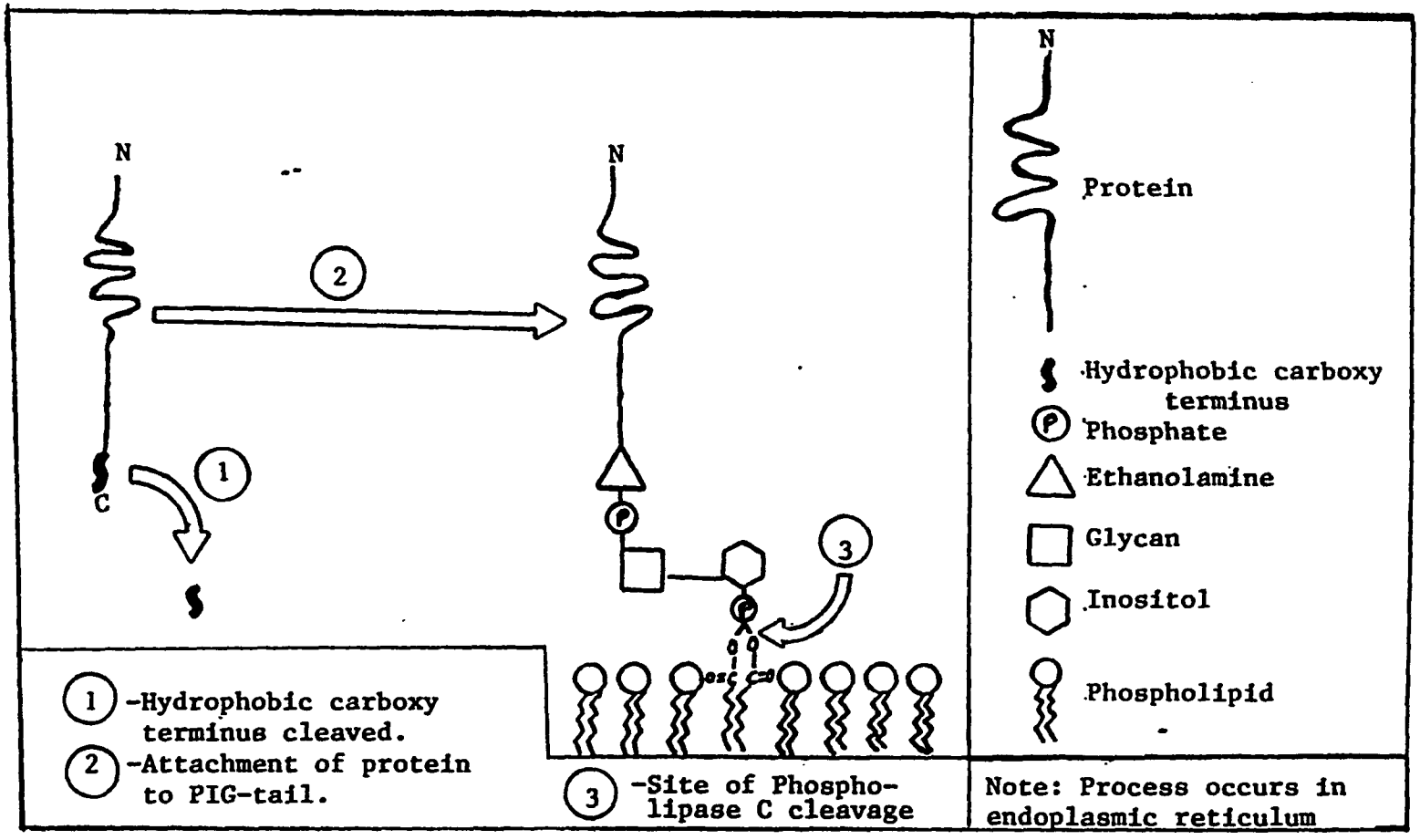
Results demonstrate that removal of the hydrophobic carboxy terminus eliminates cell surface association of α-agglutinin. This data confirms the role of the hydrophobic carboxy terminus in anchoring α-agglutinin to the cell surface. It has been speculated that α-agglutinin is anchored to the cell wall by a mechanism similar to that seen for proteins bound to cell membranes by a phosphatidyl inositol glycan anchor (PIG-tail)(Lipke et al. 1989).

PIG-tails have been shown to mediate the anchorage of numerous cell surface cell glycoproteins to the plasma membrane in a wide variety of eucaryotic cell types (Ferguson and Williams 1988). A schematic representation of the process by which a protein is anchored to a PIG-tail is depicted in Figure 30. An important feature of PIG-tail associated proteins is that a hydrophobic

stretch of amino acids (15 to 25 amino acids in length) is located at the carboxy end of the primary translation product of a PIG-tail associated protein and is cleaved prior to attachment of the PIG-tail (Fig. 30). The hydrophobic sequence serves as a signal to a protein (or protein complex) in the endoplasmic reticulum to have the PIG-tail added to the carboxy end of the protein. After cleavage of the carboxy terminus, the protein is then transferred to a PIG-tail which is probably preassembled (Fig. 30). The PIG-tail associated protein is then transported through the secretory pathway to the plasma membrane. Removal of the hydrophobic carboxy termini from various PIG-tail associated proteins by site directed mutagenesis results in their secretion (Ferguson and Williams 1988, Su and Bothwell 1989). This result confirms the importance of the hydrophobic carboxy terminus in signalling PIG-tail attachment.

Although a direct comparison between *AGα1* and PIG-tail associated proteins cannot be made (because *AGα1* is believed to be cell wall associated and PIG-tail associated proteins are membrane associated), a number of observations point to a relationship which may exist between *AGα1* and PIG-tail associated proteins. First, *AGα1* has a hydrophobic sequence similar to that seen in PIG-tail associated proteins (Lipke et al. 1989). Furthermore, removal of the hydrophobic carboxy terminus from *AGα1* results in

Figure 30. Mechanism of protein attachment to a PIG-tail.


N
N
C
1
2
3
1 -Hydrophobic carboxy terminus cleaved.
2 -Attachment of protein to PIG-tail.
3 -Site of Phospho-lipase C cleavage
N
Protein
Hydrophobic carboxy terminus
Phosphate
Ethanolamine
Glycan
Inositol
Phospholipid
Note: Process occurs in endoplasmic reticulum

its secretion. This result is similar to what is observed when the hydrophobic carboxy terminus is eliminated from PIG-tail associated proteins and suggests a role for the hydrophobic carboxy terminus in the cell wall anchorage of *AGα1*. Second, there is a positively charged residue (Lys) 22 amino acids upstream from the carboxy end of *AGα1*. A positively charged residue appears at approximately the same location in many PIG-tail associated proteins (Ferguson and Williams 1988). Third, the sequence Leu-Leu-Phe or some closely related sequence appears in the hydrophobic tail of many PIG tail associated proteins (Williams and Ferguson 1988). Both related and identical matches to this sequence are found in the carboxy terminus of *AGα1*: Leu-Leu-Ser-Tyr-Leu-Leu-Phe$_{550}$. It is evident that structural and functional similarities do exist between the carboxy termini of PIG-tail associated proteins and *AGα1*.

I think that *AGα1* is bound by a PIG-tail until it reaches the plasma membrane at which time it is cleaved (possibly by a phospholipase, see Fig. 30). By some unknown mechanism, α-agglutinin is then integrated into the cell wall. The apparent relationship between the loss of the hydrophobic tail in *AGα1* and peptide secretion suggests that PIG-tail association and cell wall integration are not independent events. This infers that PIG-tail association may be the signal which directs

the deposition of α-agglutinin into the cell wall.

2. DEFINING THE BINDING DOMAIN OF α-AGGLUTININ

The observation that the AGα$_{1351}$ peptide is active but the AGα$_{1278+6}$ peptide is not indicates that sequences near or within the acidic region are important for binding to a-agglutinin. This confirms earlier speculation that this region contains the binding domain (see Part 2). The acidic region also contains 4 out of the 7 cysteine residues found in the glycoprotein. It is possible that the secondary structure of the peptide (due to the presence of disulfide bridges between cysteine residues) may also play an important role in defining the binding domain of α-agglutinin (Heller and Lipke, unpublished observation). If this is the case, then the binding domain may not consist of a linear stretch of residues but residues separated by some distance but brought into close proximity by peptide folding and the formation of disulfide linkages. The AGα$_{1278+6}$ peptide contains most of the acidic region (Fig. 23), but due to termination at the *Nsi I* site, lacks one of these cysteines. Therefore, the peptide may be inactive not because of the deletion of part of the acidic region *per se*, but because the region cannot maintain a proper secondary structure (due

to loss of a disulfide bond). Treatment of α cells with 50 mM DTT for 30 minutes at room temperature does not diminish their ability to agglutinate a cells (Terrance and Lipke 1981). This result suggests that disulfide linkages are not necessary for the glycoprotein to maintain a functional conformation. However, because the mature secreted form of α-agglutinin is so highly glycosylated, there is no assurance that all (or any) of the disulfide linkages in α-agglutinin are accessible to DTT. In addition, the large amount of carbohyrate associated with α-agglutinin may be sufficient to keep the molecule in a functional conformation even in the absence of disulfide bonds.

The loss of a disulfide linkage in the AGα1_{278+6} peptide may also place the peptide in an alternate conformation which makes the product more susceptible to proteolysis. This would explain why so little AGα1_{278+6} peptide is identified in culture medium.

3. SUMMARY

By truncating the *AGα1* ORF at selected locations it is shown that: 1) elimination of the hydrophobic carboxy terminus results in the secretion of *AGα1* products 2) elimination of a tandem repeat sequence within *AGα1* does not diminish the products ability to bind to a cells, suggesting that this region does not comprise part of the binding domain and 3) elimination of residues within the acidic region abolishes the ability of the glycoprotein to bind to a cells. This suggests that the acidic region may contain the binding domain of α-agglutinin.

CONCLUSION

In previous studies α-agglutinin has been biochemically purified and its function and expression partially characterized. Biochemical purification of α-agglutinin revealed that it is a glycoprotein which is heavily glycosylated (50 to 75 % carbohydrate by weight), is monovalent in its binding ability and has two binding states: a tight and weak binding. Endo H treatment of biochemically purified α-agglutinin generates peptides of: 38, 72, 105, 145 and 160 kD. All of these species bind to a cells. Treatment of purified α-agglutinin with sodium periodate does not diminish its binding activity, suggesting that it is the peptide portion of the molecule which confers the ability to bind to a cells. Treatment of α cells with the peptide pheromone a-factor (a-factor is secreted by a cells) increases slightly the cellular agglutinability of α cells. It was assumed that α-agglutinin is bound to the cell surface by attachment to the cell wall. Additionally, a gene (*AGα1*) was cloned which could complement mutants in α-agglutinin expression.

The work presented here further clarifies the structure, function and expression of α-agglutinin. I have shown that the *AGα1* codes for α-agglutinin. This gene is α cell-specific and shown to be required for agglutinability of *MATα* cells. Notable features

of *AGα1* include that the ORF 1)codes for a 70 kD protein 2)contains an acidic region within the amino half of the glycoprotein 3)contains a 15 amino acid hydrophobic carboxy terminus 4)has a putative signal secretion sequence 5)has numerous potential N-linked glycosylation sites throughout the coding sequence and 6)is comprised of 40% Ser/Thr within the carboxy half of the ORF.

Phenotypes of *AGα1* truncation mutants further elucidate the domain structure of α-agglutinin. Specifically, I have shown that 1)the carboxy hydrophobic terminus of α-agglutinin is necessary for cell wall anchorage and 2) the amino half of the glycoprotein (possibly the acidic region) contains the binding domain of α-agglutinin. Molecular weight analysis shows that α-agglutinin is highly glycosylated with both O and N-linked carbohydrate. Most of the O-linked sugar is associated with the carboxy half of the molecule and that N-linked carbohydrate is found throughout α-agglutinin. Lastly, I show that α-agglutinin is efficiently transported through the secretory pathway, since only small amounts of α-agglutinin precursors can be found intracellularly.

I have shown that the increase in the agglutinability of α cells in response to a-factor is due to an increase in the expression of cell surface α-agglutinin. There are 5 x 10^4 molecules of

α-agglutinin constitutively expressed per α cell. Induction with a-factor causes a modest increase in α-agglutinin expression to 6.5 x 10^4 molecules per cell. The spatial distribution of α-agglutinin on the cell surface in uninduced cells is polar while induced cells express α-agglutinin more evenly. Buds and daughter cells do not express cell surface α-agglutinin. Lastly, α-agglutinin is expressed in the shmoo tip region of induced α cells, the location were a and α cells fuse to form a diploid zygote.

Bibliography

Achstetter, T. 1989. Regulation of α-factor production in *Saccharomyces cerevisiae*:a-factor pheromone-induced expression of the *MFα1* and *STE13* genes. Mol. Cell. Biol. 9:4507-4514.

Anderegg, R.J., Betz, R., Carr, S.A., Crabb, J.W., and Duntze, W. 1988. Structure of *Saccharomyces cerevisiae* mating hormone a-factor: identification of S-farnesyl cysteine as a structural component. J. Biol. Chem. 263:18236-18240.

Ballou, C.E. 1970. A study of the immunochemistry of three yeast mannans. J. Biol. Chem. 245:1197-1203.

Ballou, C.E. 1982. Yeast cell wall and cell surface. pp. 335-360. In J.N. Strathern, E.W. Jones, and J.R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Bender, A., and Sprague, G.F. 1987. MATα1 protein, a yeast transcription activator, binds synergistically with a second protein to a set of cell-type-specific-genes. Cell 50:681-691.

Betz, R. J., Duntze, W., and Manney, T.R. 1978. Mating factor-mediated sexual agglutination in *Saccharomyces cerevisiae*. FEMS Lett. Microbiol. 4:107-110.

Betz, R. J., Crabb, J.W., Meyer, H.E., Wittig, R., and Duntze, W. 1987. Amino acid sequences of a-factor mating peptides from *Saccharomyces cerevisiae*. J. Biol. Chem. 262:546-548.

Brake, A.J., Brenner, C., Najarian, R., Laybourn, P., and Merryweather, J. 1985. Structure of genes encoding precursors of the yeast peptide mating pheromone a-factor. pp.103-108. In: Mary-Jane Gething (ed.), Current communications in molecular biology: protein transport and secretion. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Burke, D., Mendonca-Previato, L., and Ballou, C.E. 1980. Cell-cell recognition in yeast: Purification of *Hansenula wingei* 21-cell sexual agglutination factor and comparison of the factors from three genera. Proc. Natl. Acad. Sci. (USA), 77:318-322.

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

Carter, B.L.A., and Jagadish, M.N. 1978. Control of cell division in the yeast *Saccharomyces cerevisiae* cultured at different growth rates. Exp. Cell Res. 112:373-383.

Chvatchko, Y., Howald, I., and Riezman, H. 1986. Two yeast mutants defective in endocytosis are defective in pheromone response. Cell 46:355-364.

Conzelmann, A., Riezman, H., Desponds, C., and Bron, C. 1988. A major 125-kD membrane glycoprotein of *Saccharomyces cerevisiae* is attached to the lipid bilayer through an inositol-containing phospholipid. EMBO J. 7:2233-2240.

Conzelmann, A., Fankhauser, C., and Desponds, C. 1990. Myoinositol gets incorporated into numerous membrane glycoproteins of *Saccharomyces cerevisiae*; incorporation is dependent on phosphomannomutase (sec 53). EMBO J. 9:653-661.

Cross, F.R. 1988. *DAF1*, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Saccharomyces cerevisiae*. Mol. Cell Biol. 8:4675-4684.

Cross, F.R., Hartwell, L.H., Jackson, C., and Konopka, J.B. 1988. Conjugation in *Saccharomyces cerevisiae*. Ann. Rev. Cell Biol. 4:429-457.

Cross, F., Roberts, J., and Weintraub, H. 1989. Simple and complex cell cycles. Ann. Rev. Cell Biol. 5:341-395.

Dietzel, C., and Kurjan, J. 1987. The yeast *SCG1* gene: a Gα-like protein implicated in the a and α -factor response pathway. Cell 50:1001-1010.

Dmochowska, A., Dignard, D., Henning, D., Thomas, D. Y., and Bussey, H. 1987. Yeast *KEX1* gene encodes a putative protease with a carboxypeptidase B-like function involved in killer toxin and α-factor precursor processing. Cell 50:573-584.

Doi, S., and Yoshimura, M. 1977. Temerature-sensitive loss of sexual agglutinability in *Saccharomyces cerevisiae*. Arch. Microbiol. 114:287-288.

Doi, S., and Yoshimura, M. 1978. Temperature-dependent conversion of sexual agglutinability in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 162:251-257.

Doi, S., Suzuki, Y., and Yoshimura, M. 1979. Induction of sexual cell agglutinability of a mating type cells by

α-factor in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. 91:849-853.

Doi, S., and Yoshimura, M. 1985. Alpha mating type-specific expression of mutations leading to constitutive agglutinability in *Saccharomyces cerevisiae*. J. Bacteriol. 161:596-601.

Dolan, J.W., Kirkman, C., and Fields, S. 1989. The yeast STE12 protein binds to the DNA sequence mediating pheromone induction. Proc. Natl. Acad. Sci. (USA), 86:5703-5707.

Duntze, W., MacKay, V., and Manney, T. R. 1970. *Saccharomyces cerevisiae*: a diffusible sex factor. Science, 168:1472-1473.

Elion, E.A., Grisafi, P.L., Fink, G.R. 1990. FUS3 encodes a *cdc2+/CDC28*-related kinase required for the transition from mitosis into conjugation. Cell, 60:649-664.

Fahey, J. L., and Lawrence, M. E. 1963. Quantitative determination of 6.6 S γ-Globulins, β_{2A}-globulins and γ, -macroglobulins in human serum. J. Immunol. 91:597-603.

Falcone, G., and Nickerson, W.J. 1956. Cell-wall mannanprotein of bakers yeast. Science 124:272-273.

Fehrenbacher, G., Perry, K., and Thorner, J. 1978. Cell-cell recognition in *Saccharomyces cerevisiae*: regulation of mating-specific adhesion. J. Bacteriol. 134:893-901.

Ferguson, M.J., and Williams, A.F. 1988. Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. Ann. Rev. Biochem. 57:285-320.

Fields, S., and Herskowitz, I. 1985. The yeast *STE12* product is required for expression of two sets of cell-type-specific genes. Cell 42:923-930.

Fields, S., Chaleff, D.T, and Sprague, G.F. 1988. Yeast *STE7*, *STE11*, and *STE12* genes are required for expression of cell-type-specific genes. Mol. Cell Biol. 8:551-556.

Fuller, R.S., Brake, A.J., Julius, D.J. and Thorner, J. 1985. The *KEX2* gene product required for processing of yeast prepro-α-factor is a calpain-like endopeptidase specific for cleaving at pairs of basic residues. pp. 97-102. In Mary-Jane Gething (ed.), Current communications in molecular biology: protein transport and secretion. Cold Spring Harbor Laboratory, Cold Spring

Harbor, New York.

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

Hagiya, M., Yoshida, K., and Yanagishima, N. 1977. The release of sex-specific substances responsible for sexual agglutination from haploid cells of *Saccharomyces cerevisiae*. Exp. Cell Res. 104: 263-272.

Harashima, S., Miller, A.M., Tanaka, K., Kusumoto, K.-I., Tanaka, K.-I., Mukai, Y., Nasmyth, K. and Oshima, Y. 1989. Mating-type control in *Saccharomyces cerevisiae*: isolation and characterization of mutants defective in repression by a1-α2. Mol. Cell. Biol. 9:4523-4530.

Hartwell, L.H., and Unger, M.W., 1977. Unequal division in *Saccharomyces cerevisiae* and its implications for the control of cell division. J. Cell Biol. 75:422-435.

Herskowitz, I and Oshima, Y. 1981. Control of cell type in *Saccharomyces cerevisiae*: mating type and mating-type interconversion. pp. 181-209. In J.N. Strathern, E. W. Jones, and J.R. Broach (ed.s), The molecular biology of the yeast *Saccharomyces*: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Herskowitz, I. 1989. A regulatory heirarchy for cell specialization in yeast. Nature, 342:749-757.

Jarvis, E.E., Hagen, D.C., and Sprague, G.F. 1988. Identification of a DNA segment that is necessary and sufficient for α-specific gene control in *Saccharomyces cerevisiae*: implications for regulation of c.-specific and a-specific genes. Mol. Cell. Biol. 8:309-320.

Jenness, D.D., and Spatrick, P. 1986. Down regulation of the α-factor pheromone receptor in *S. cerevisiae*. Cell 46:345-353.

Johnson, A.D., and Herskowitz, I. 1985. A repressor (*MAT ALPHA-2* Product) and its operator control expression of a set of cell type specific genes in yeast. Cell 42:237-247.

Johnston, D.D.,Ehrhardt, C.W., Lorincz, A., Carter, B.L.A. 1979. Regulation of cell size in the yeast *Saccharomyces cerevisiae*. J. Bacteriol., 137:1-5.

Johnston, G.C., Singer, R.A., Sharrow, S.O., and Slater, M.L. 1980. Cell division in the yeast *Saccharomyces cerevisiae* growing at different rates. J. Gen. Microbiol. 118:479-484.

Johnston, J.R. 1988. Yeast genetics, molecular aspects. In; I. Campbell and J. H. Duffus (ed.), Yeast: a practical approach. IRL Press, Oxford, Washington D.C.

Julius, D., Brake, A. Blair, L., Kunisawa, R., and Thorner, J. 1984. Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-α-factor. Cell 37:1075-1089.

Konigsberg, W.H., and Henderson, L. 1983. Removal of sodium dodecyl sulfate from proteins by ion-pair extraction. Meth. Enzymol. 91:254-259.

Kostriken, R., Strathern, J.N., Klar, A., Hicks, J.B., and Heffron F., 1983. A site-specific endonuclease essential for mating-type switching in *Saccharomyces cerevisiae*. Cell 35:167-174.

Kronstad, J. W., Holly, J.A., and MacKay, V.L. 1987. A yeast operator overlaps an upstream activation site. Cell 50:369-377.

Kurjan, J. and Herskowitz, I. 1982. Structure of a yeast pheromone gene (*MFα*): a putative alpha-factor precursor contains four tandem copies of mature alpha factor. Cell 30:933-943.

Kyte, J., and Doolittle, R.F. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157:105-132.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 277:680-685.

Lasky, R.D., and Ballou, C. E. 1988. Cell-cell recognition in yeast: isolation of intact α-agglutinin from *Saccharomyces kluyveri*. Proc. Natl. Acad. Sci. (USA) 85: 349-353.

Lipke, P.N., Taylor, A., and Ballou, C.E. 1976. Morphogenic effects of α-factor on *Saccharomyces cerevisiae* a cells. J. Bacteriol. 127:610-618.

Lipke, P.N., Terrance, K., and Yu-Sheng Wu. 1987. Interaction of α-agglutinin with *Saccharomyces cerevisiae* a cells. J. Bacteriol. 169:483-488.

Lipke, P.N., Wojciechowicz, D., and Kurjan, J. 1989. *AGα1* is the structural gene for the *Saccharomyces cerevisiae* α-agglutinin, a cell surface glycoprotein involved in cell-cell interactions during mating. Mol. Cell Biol. 9:3155-3165.

Lorincz, A.T., and Reed, S.I. 1984. Primary structure homology between the product of yeast cell division control gene *CDC28* and vertebrate oncogenes. Nature (London) 307:183-185.

Low, M.G., and Saltiel, A.R. 1988. Structural and functional roles of glycosyl-phosphatidylinositol in membranes. Science 239:268-275.

Mallon, R.G., Sisk, W., and Defendi, V. 1986. Expression of the open reading frame of *Papillomavirus* BPV1 and HPV6b in *Escherichia coli*. Gene 42:241-251.

Manney, T.R., and Meade J.H. 1977. Cell-cell interactions during mating in *Saccharomyces cerevisiae*. p. 281-321. In. J. L. Reissig (ed.) Microbial Interactions, Chapman and Hall Publishers, London.

Manney, T.R., Jackson, P., and Meade, J. 1983. Two temperature-sensitive mutants of *Saccharomyces cerevisiae* with altered expression of mating-type functions. J. Cell Biol., 96:1592-1600.

Matile, Ph., Moor, H., and Robinow, C.F. 1969. Yeast cytology, p. 220-297. In: Rose and Harrison (ed). The yeasts, vol 1. Academic Press, London.

Matsudaira, P. 1987. Sequence from picomole quantities of protein from polyvinylidene difluoride membranes. Millipore Corp., Bedford, Ma.

Mendonca-Previato, L., Burke, D., and Ballou, C. E. 1982. Sexual agglutination factors from the yeast *Pichia amethionina*. J. Cell. Biochem. 19:171-178.

Mierendorf, R.C., and Pfeffer, D. 1987. Direct sequencing of denatured plasmid DNA. Meth. Enzymol. 152:556-562.

Miyajima, I., Nakafuku, M., Nakayama, N., Brenner, C., Miyajima, A., Kaibuchi, K., Arai, K., Kaziro, Y., and Matsumoto, K. 1987. *GPA1*, a haploid-specific essential gene, encodes a yeast homolog of mammalian G protein which may be involved in mating factor signal transduction. Cell 50:1011-1019.

Mitchell, A.P., and Herskowitz, I. 1986. Activation of meiosis and sporulation by repression of the *RME1*

product in yeast. Nature (London) 319:738-742.

Moore, S. A. 1983. Comparison of Dose-Response Curves for α Factor-induced cell division arrest, agglutination, and projection formation of yeast cells. J. Biol. Chem. 258:13849-13856.

Mort, A.J., and Lamport, D.T.A. 1977. Anhydrous hydrogen fluoride deglycosylates glycoproteins. Anal. Biochem. 82:289-309.

Murray, A.W., and Kirschner, M.W. 1989. Cyclin synthesis drives the early embryonic cell cycle. Nature (London) 339:275-280.

Murray, A.W., Solomon, M.J., and Kirschner, M.W. 1989. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature (London) 339:280-286.

Nakagawa, Y., and Yanagishima, N. 1982. Changes in production of the mating-type-specific glycoproteins, agglutination substances in association with mating type interconversion in Homothallic strains of the yeast, *Saccharomyces cerevisiae*. Mol. Gen. Genet. 207-210.

Nasmyth, K. 1985. A repetetive DNA sequence that confers cell-cycle START (*CDC28*)-dependent transcription of the *HO* gene in yeast. Cell 42:225-235.

Nasmyth, K., Seddon, A., and Ammerer, G. 1987. Cell cycle regulation of *SWI5* is required for mother-cell-specific transcription in yeast. Cell 49:549-558.

Nash, R., Tokiwa, G., Anand, S., Erickson, K., and Futcher, A.B. 1988. The *WHI1+* gene of *Saccharomyces cerevisiae* teters cell division to cell size and is a cyclin homolog. EMBO J., 7:4335-4346.

Orlean, P., Ammer, H., Watzele, M., and Tanner, W. 1986. Synthesis of an O-glycosylated cell surface protein induced in yeast by c. factor. Proc. Natl. Acad. Sci. (USA) 83:6263-6266.

Pastor, F.I.J., Valentin, E., Herrero, E., and Sentandreu, R. 1984. Structure of the *Saccharomyces cerevisiae* cell wall: mannoproteins released by zymolase and their contribution to wall architecture. Biochemica et. Biophysica Acta 802:292-300.

Pierce, M., and Ballou, C.E. 1983. Cell-cell recognition in yeast: characterization of the sexual agglutination factors from *Saccharomyces kluyveri*. J. Biol. Chem.

258:3576-3582.

Pringle, J.R., and Hartwell, L.H. 1981. The *Saccharomyces cerevisiae* cell cycle, pp. 97-142. In J. N. Strathern, E. W. Jones and J.R Broach (ed.), The molecular biology of the yeast *Saccharomyces*: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Roberts, R.L., Bowers,B., Slater, M.L., and Cabib, E. 1983. Chitin synthesis and localization in cell division cycle mutants of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 3:922-930.

Roy, A., Marykwas, D.L., Fen Lu, C., Lipke, P., and Kurjan, J. 1990. *AGa1* encodes the *Saccharomyces cerevisiae* cell surface attachment domain of a-agglutinin, a cell surface glycoprotein involved in cell-cell interactions during mating. (Submitted for publication).

Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. (USA) 74:5463-5467.

Su, B., and Bothwell, A. 1989. Biosynthesis of a phosphatidylinositol-glycan-linked membrane protein:signals for posttranslational processing of the Ly-6E antigen. Mol. Cell. Biol. 9:3369-3376.

Strazdis, J.R., and MacKay, V.L. 1983. Induction of yeast mating pheromone a-factor by α cells. Nature (London), 305:545-547.

Suzuki, K., and Yanagishima, N. 1985. An α-mating-type-specific mutation causing specific defect in sexual agglutinability in the yeast *Saccharomyces cerevisiae*. Curr. Genet. 9:185-189.

Tabor, S., and Richardson, C.C. 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. (USA) 84:4767-4771.

Tan, S., Ammerer, G., and Richmond, T.J., 1988. Interactions of purified transcription factors: binding of yeast *MAT*α*1* and PRTF to cell type-specific, upstream activating sequences. EMBO J. 7:4255-4264.

Taylor ,N. W., and Orton, W. L. 1971. Cooperation among the active binding sites in the sex-specific agglutinin from the yeast *Hansenula wingei*. Biochem. 10:2043-2049.

Taylor, N. W., Orton, W. L., and Babcock, G. E. 1968.

Sexual agglutination in yeast. vi. role of disulfide bonds in 5-agglutinin. Arch. Biochem. and Biophys. 123:265-270.

Teague, M. A., Chaleff, D.T., and Errede, B. 1986. Nucleotide sequence of the yeast regulatory gene *STE7* predicts a protein homologous to protein kinases. Proc. Natl. Acad. Sci. (USA) 83:7371-7375.

Terrance, K., and Lipke, P.N. 1981. Sexual agglutination in *Saccharomyces cerevisiae*. J. Bacteriol. 148:889-896.

Terrance, K., and Lipke, P.N. 1987. Pheromone induction of agglutination in *Saccharomyces cerevisiae* a cells. J. Bacteriol. 169:4811-4815.

Terrance, K., Heller, P., Yu-Sheng Wu, and Lipke, P.N. 1987. Identification of glycoprotein components of α -agglutinin, a cell adhesion protein from *Saccharomyces cerevisiae*. J. Bacteriol. 169: 475-482.

Tkacz, J.S.,Cybulska, E.B., and Lampen, J.O. 1971. Specific staining of wall mannan in yeast cells with fluorescein-conjugated Concanavalin A. J. Bacteriol. 105:1-5.

Tkacz, J.S., and Lampen, J.O. 1972. Wall replication in *Saccharomyces* species: Use of fluorescein-conjugated Concanavalin A to reveal the site of mannan insertion. J. Gen. Microbiol. 72:243-247.

Tkacz, J.S., and MacKay, V.L. 1979. Sexual conjugation in yeast:cell surface changes in response to the action of mating hormones. J. Cell Biol. 80:326-333.

Towbin, H., Stachelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. (USA) 76:4350-4354.

Tohoyama, H., Hagiya, M., Yoshida, K., and Yanagishima, N. 1979. Regulation of the production of the agglutination substances responsible for sexual agglutination in *Saccharomyces cerevisiae*: changes associated with conjugation and temperature shift. Molec. Gen. Genet. 174:269-280.

Tohoyama, H., and Yanagishima, N. 1985. The sexual agglutinin substance is secreted through the yeast secretory pathway in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 201: 446-449.

Trimble, R.B., and Maley, F. 1984. Optimizing hydrolysis

of N-linked high-mannose oligosaccharides by endo-B-N-acetylglucosaminidase H. Analyt. Biochem. 141:515-522.

Trueheart, J., Boeke, J.D., and Fink, G.R. 1987. Two genes required for cell fusion during yeast conjugation: evidence for a pheromone-induced surface protein. Mol. Cell. Biol. 7:2316-2328.

Watzele, M., Klis, F., and Tanner, W. 1988. Purification and characterization of the inducible a agglutinin of *Saccharomyces cerevisiae*. EMBO J. 7:1483-1488.

Weinstock, K., and Ballou, C.E. 1986. Cell-cell recognition in yeast: molecular nature of the sexual agglutinin from *Saccharomyces kluyveri* 17-cells. J. Biol. Chem. 261:16174-16179.

Wojciechowicz, D., and Lipke, P N. 1989. α-agglutinion expression in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. 161:46-51.

Whiteway, M., Hougan, L., Dignard, D., Thomas, D.Y., Bell, L., Saari, G.C., Grant, F. J., O'Hara, P., and MacKay, V.L. 1989. The *STE4* and *STE18* genes of yeast encode potential *β* and *γ* subunits of the mating factor receptor-coupled G protein. Cell 56:467-477.

Yamaguchi, M., Yoshida, K., and Yanagishima, N. 1982. Isolation and partial characterization of the cytoplasmic α agglutination substance in the yeast *Saccharomyces cerevisiae*. FEBS Let. 139:125-129.

Yamaguchi, M., Yoshida, K., and Yanagishima, N. 1984. Isolation, and biochemical and biological characterization of an a-mating-type-specific glycoprotein responsible for sexual agglutination from the cytoplasm of a-cells, in the yeast *Saccharomyces cerevisiae*. Arch. Microbiol. 140:113-119.

Yanagishima, N., Yoshida, K., Hamada, K., Hagiya, M., Kawanabe, Y., Sakurai, A., and Tamura, S. 1976. Regulation of sexual agglutinability in *Saccharomyces cerevisiae* of a and α types by sex-specific factors produced by their respective opposite mating types. Plant and Cell Physiol. 17:439-450.

Yanagishima, N., and Nakagawa, Y. 1980. Mutants inducable for sexual agglutinability in *Saccharomyces cerevisiae*. Molec. Gen. Genet. 178:241-251.

Yen, P. H., and Ballou, C. E. 1974. Partial characterization of the sexual agglutination factor from *Hansenula wingei* Y-2340 type 5 cells. Biochem.

13:2428-2437.

Yoshida, K., Hagiya, M., and Yanagishima, N. 1976. Isolation and purification of the sexual agglutination substance of mating type a cells in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. 71:1085-1094.