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A pathway for cell wall anchorage of alpha-agglutinin from *Saccharomyces cerevisiae*

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A PATHWAY FOR CELL WALL ANCHORAGE OF ALPHA-AGGLUTININ FROM *SACCHAROMYCES CEREVISIAE*

by

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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.

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Abstract

A PATHWAY FOR CELL WALL ANCHORAGE OF ALPHA-AGGLUTININ FROM *SACCHAROMYCES CEREVISIAE*

by

Chafen Lu

Advisor: Professor Peter N. Lipke

Saccharomyces cerevisiae α-agglutinin is a cell wall-anchored adhesion glycoprotein. It can be released from the cell wall by ß-glucanase treatment. The predicted α-agglutinin polypeptide contains a potential signal sequence for glycosyl phosphatidylinositol (GPI) anchor addition. It was shown in this study that the α-agglutinin was synthesized with a GPI anchor. Two GPI-anchored intermediate forms were identified: the 140 kD ER form and the >300 kD plasma membrane form. GPI linkage to these forms was demonstrated by susceptibility to PI-PLC cleavage and metabolic labeling with [^{3}H]myo-inositol and [^{3}H]palmitic acid. A soluble form of >300 kD that lacked the GPI anchor had properties of a periplasmic intermediate between the plasma membrane form and the cell wall-anchored form. Additional forms of 80 kD, 150 kD and 250-300 kD were detected in temperature-sensitive secretory mutants. The 80 kD form is likely to represent the unmodified α-agglutinin peptide; the 150 kD and 250-300 kD forms were membrane-bound and are likely to be intermediates between the 140 kD ER form and the >300 kD plasma membrane form. N- and O-glycosylation, and probably other modifications resulted in successive increases in size during transport

to the cell surface.

The mature cell wall α-agglutinin, but not the intermediate forms, was immunoreactive with antibodies against ß-1,6-glucan. The cell wall α-agglutinin from *kre* mutants, which have reduced size of the cell wall ß-1,6-glucan, exhibited lower molecular size and less immunoreactivity with the anti-ß-1,6-glucan. These observations demonstrate that the cell wall α-agglutinin is covalently associated with the cell wall ß-1,6-glucan. A C-terminal 29 amino acid truncated form of α-agglutinin, which was secreted into the medium due to a defect in GPI anchor addition, did not contain ß-1,6-glucan, suggesting a role for the GPI anchor in cross-linking of α-agglutinin and ß-1,6-glucan.

The results constitute the first experimental support for a novel cell wall anchorage mechanism: the GPI anchor acts to localize α-agglutinin to the plasma membrane and the cell wall anchorage involves release from the GPI anchor to produce a periplasmic intermediate, followed by cross-linkage to the wall ß-glucan.

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Section I

Introduction

The cell wall of *S. cerevisiae*

The *S. cerevisiae* cell wall is a rigid outer layer surrounding the plasma membrane. It maintains the shape of the cell, mechanically protects the cell from the environment, and acts as a sieve to limit the passage of large molecules through the cell wall.

Composition of the cell wall

A schematic diagram of the *S. cerevisiae* cell wall is shown (Fig. 1). The cell wall contains two major components: the fibrous glucan and mannoproteins. These two components are present in approximately equal amounts and account for more than 90% of the cell wall material. A small amount of chitin is also present in the cell wall (Cabib et al. 1988,1991; Ballou 1982,1988; Fleet 1991).

Glucan. The glucan component of the cell wall is composed of two classes. The major class is ß-1,3-glucan that consists mainly of linear ß-1,3-linked glucan with some branches through ß-1,6-linkage. This class of glucan has an average of 1500 glucose residues per molecule (Manners et al. 1973a). The minor ß-1,6-glucan contains predominantly ß-1,6-linkages with some ß-1,3-linked branch points. This glucan has a polymerization of approximately 140 glucose residues, and accounts for about 7% of the cell wall dry weight (Manners et al. 1973b). The wall glucan forms a fibrillar network around the cell, and provides a strong framework for the cell wall (Necas 1971; Cabib et al. 1991; Fleet 1991).

Mannoproteins. Some wall mannoproteins are distributed through the glucan network, and others are located over the glucan layer and form a proteinaceous outer

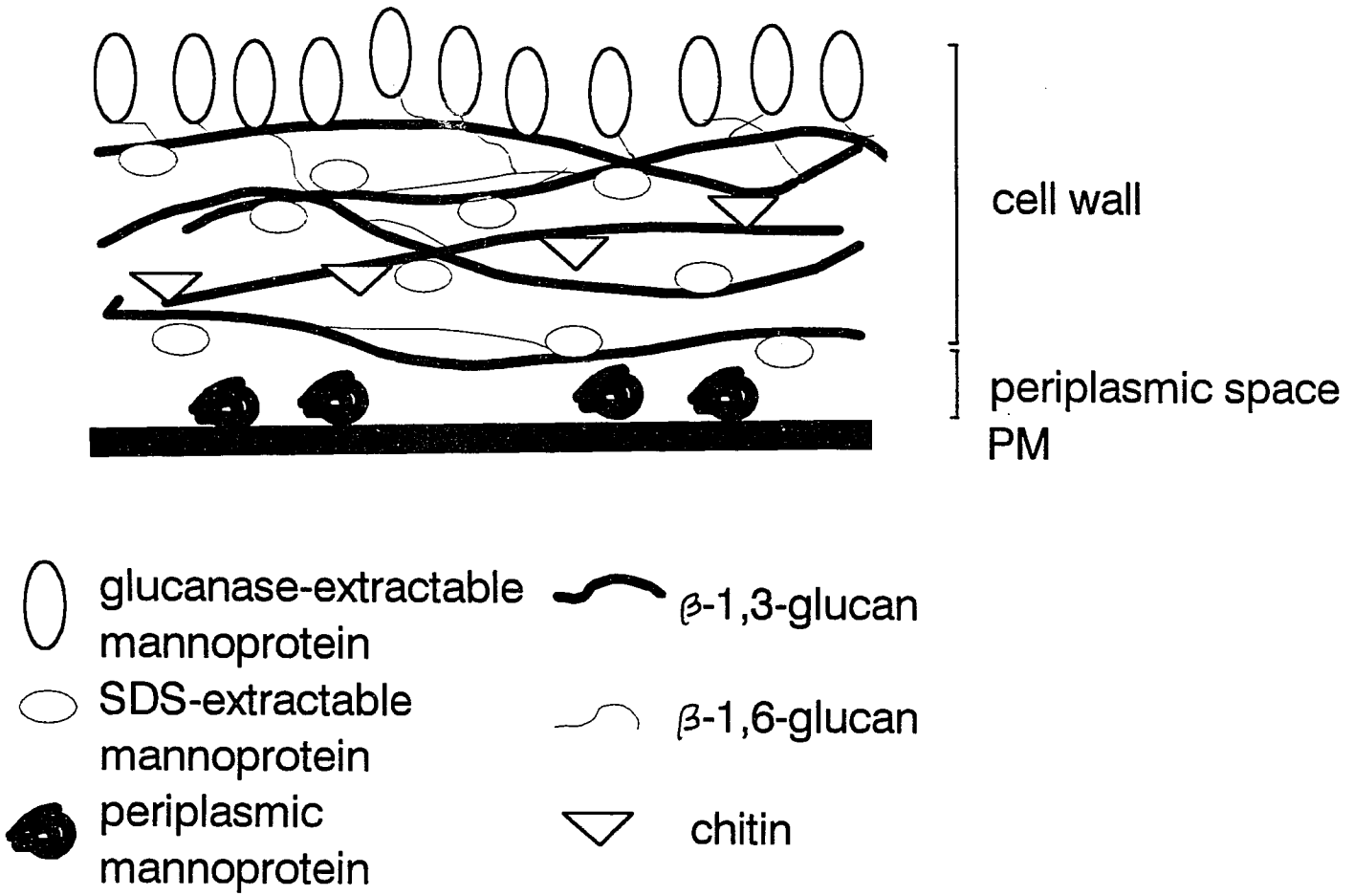


Figure 1. Schematic Diagram of S. cerevisiae Cell Wall

layer which determines the wall permeability (Zlotnik et al. 1984; Ballou 1982; De Nobel et al. 1990). Based on solubility, the wall mannoproteins can be divided into four types. The first type mannoproteins are soluble. Some of them are precursors of the wall-bound mannoproteins transiently present in the periplasmic space (Pastor et al. 1982,1984). Others including periplasmic enzymes, such as invertase and acid phosphatase, are retained within the cell wall by physical entrapment (Esmon et al. 1987; De Nobel et al. 1989). The second type of wall mannoproteins can be extracted by SDS treatment, and appear to be associated with the cell wall by non-covalent interactions (Zlotnik et al. 1984; Valentin et al. 1984). The third type, usually having very high molecular mass, can not be extracted by SDS, but can be released from the cell wall by treatment with ß-glucanases. It has been proposed that this type of wall mannoproteins are covalently associated with the ß-glucan (Shibata et al. 1983; Valentin et al. 1984; Ballou 1982; De Nobel et al. 1989). The fourth type of wall mannoproteins are disulfide-bonded to other wall mannoproteins. They can be released from the cell wall by treatment with reducing reagents (Orlean et al. 1986).

Chitin. Chitin is a linear polysaccharide composed of ß-1,4-linked N-acetylglucosamine residues. The majority of the chitin is located in the primary septum that separates the mother and daughter cells. A small percentage (around 10%) is distributed in the lateral cell wall (Cabib et al. 1988, 1991; Fleet 1991; Molano et al. 1980). The chitin together with most of the glucan constitute the insoluble components of the cell wall (Fleet 1991).

Biosynthesis of cell wall components

Glucan. The ß-1,3-glucan synthetase is plasma membrane-bound, and is activated

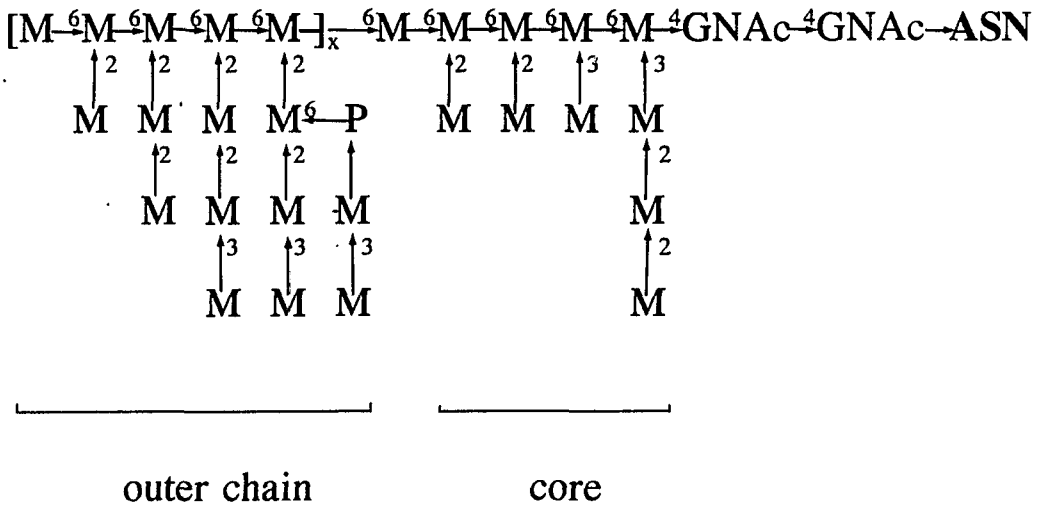
by GTP (Shematek et al. 1980; Shematek and Cabib 1980). The ß-1,3-glucan is synthesized from UDP-glucose at the cytoplasmic face of the plasma membrane, and probably extruded through the membrane to the extracellular face as it is synthesized (Cabib et al. 1988). The biosynthesis of the cell wall ß-1,6-glucan is not well understood yet, despite the isolation of a number of genes required for the wall ß-1,6-glucan synthesis. It has been proposed that ß-1,6-glucan core is assembled intracellularly from UDP-glucose, and the side chains are added to the core through ß-1,3-linked branchpoints on the cell surface. The products of *KRE5, KRE6, KRE9* and *KRE11* genes appear to be required for the core assembly; the *KRE1* gene product, which is likely to be a cell wall protein, is involved in the side chain addition (Boone et al. 1990; Brown et al. 1993; Brown and Bussey 1993; Roemer and Bussey 1991; Roemer et al. 1993).

Chitin. The synthesis of chitin is catalyzed by chitin synthetases which are bound to the plasma membrane. Three chitin synthetases (Chs1, Chs2 and Chs3) have been identified, and they catalyze the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to a growing chitin chain (Cabib et al. 1988,1991). Chs2 is essential for septum formation, whereas Chs1 has a repair function (Silverman et al. 1988; Cabib et al. 1989). Chs3 appears to be responsible for the synthesis of cell wall chitin distributed in the lateral wall (Shaw et al. 1991). Because of the localization of chitin synthetases in the plasma membrane, it seems probable that the polysaccharide is synthesized at the cytoplasmic side and simultaneously extruded through the membrane to the external face (Cabib et al. 1991).

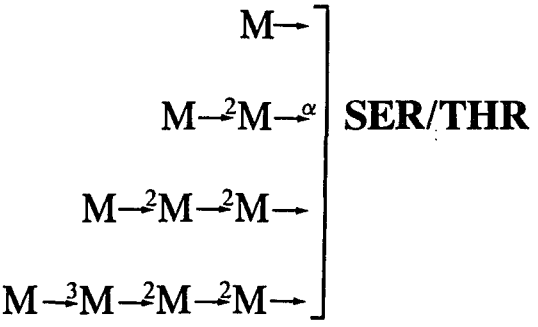
Mannoproteins. The protein moieties of wall mannoproteins are synthesized in the ER and transported to the cell surface through the secretory pathway (Esmon et al.

Figure 2. General structures of N-linked and O-linked oligosaccharide chains of *S. cerevisiae* glycoproteins. A. The N-linked chain consists of a core unit (8-15 mannose residues) and a highly branched outer chain (50-100 mannose residues). Some of the side chains in the outer chain contain phosphate. **B.** The O-linked chain contains up to 4 mannose residues.

A


[M→6M→6M→6M→6M→]x→6M→6M→6M→6M→6M→4GNAc→4GNAc→ASN
outer chain
core

B


M→
M→2M→α
M→2M→2M→
M→3M→2M→2M→
SER/THR

1981; Schekman and Novick 1982; Novick and Schekman 1983; Schekman 1985; Sanz et al. 1987). As the proteins are synthesized and translocated into the lumen of the ER, the preassembled core oligosaccharides ($GlcNAc_2Man_9Glc_3$) are transferred from the dolichol phosphate carrier lipid to the asparagine residues of the proteins. Three glucose residues and one mannose are subsequently removed from the N-linked core oligosaccharides, and the glycoproteins are transported from the ER to the Golgi, where the modified core oligosaccharides are elongated in a stepwise manner by a highly branched outer chain consisting of 50-100 mannose residues (Fig. 2). Although the N-linked core oligosaccharides of some glycoproteins are not extended, the cell wall mannoproteins contain N-linked outer chains, resulting in high sugar to protein ratios (Ballou 1982; Tanner and Lehle 1987; Kukuruzinska et al. 1987; Cabib et al. 1988). Unlike O-glycosylation in mammalian cells, O-glycosylation in yeast initiates in the ER. The first mannose residue is transferred from Dol-P-Man to serine or threonine residues in the ER. The extension of O-linked chains takes place in the Golgi apparatus (Tanner and Lehle 1987; Kukuruzinska et al. 1987).

Although many features about the composition of the cell wall and the biosynthesis of individual wall component are known, little is known about how the cell wall components are assembled into a supramacromolecular structure of the cell wall. Presumably the assembly occurs through extensive cross-linking among the wall components by various enzymatic, chemical and physical forces. There is evidence for covalent linkages between ß-1,3-glucan and ß-1,6-glucan (Fleet 1991). The ß-glucan is likely to be associated with chitin (Sietsma and Wessels 1981). Roncero et al (1988) have observed that the insolubility of ß-glucan is greatly reduced in a mutant that has

a defect in the synthesis of lateral cell wall chitin. This finding implies a role for chitin in stabilizing the wall ß-glucan. Covalent associations of wall mannoproteins with ß-glucans have been suggested, based on the observation that some ß-glucanase extracted wall mannoproteins contain ß-linked glucose residues (Kitamura 1982; Shibata et al. 1983; Elorza et al. 1989). Recently, Van Rinsum et al (1991) and Montijn et al (1994) have characterized the glucose-containing carbohydrate chain derived from *S. cerevisiae mnn9* cell wall glucomannoproteins. It contains N-acetylglucosamine, mannose and glucose in a molar ratio of 1:17:18. This type of carbohydrate chain cannot be removed from the proteins by treatments that remove N-linked or O-linked carbohydrate chains. The nature of the attachment of the glucose-containing side chain to the wall mannoproteins remains unknown.

The *S. cerevisiae* agglutinins

The *S. cerevisiae* **a**-agglutinin and α-agglutinin are cell adhesion proteins expressed by haploid **a** and α cells, respectively. The **a**-agglutinin on **a** cells binds to the α-agglutinin on the surface of α cells, causing cellular agglutination. The agglutination facilitates the fusion of two haploid cells to form a diploid zygote during mating process (Cross et al. 1988; Terrance et al. 1987; Yanagishima and Fujimura 1981; Yanagishima 1984). Other species of budding yeast express cell surface agglutinins biochemically similar to the *S. cerevisiae* **a**- or α-agglutinin, but the agglutinin interactions are highly species specific (reviewed by Lipke and Kurjan 1992).

Biochemical characterization

Both **a**- and α-agglutinin are glycoproteins. The α-agglutinin is present in a single polypeptide. The α-agglutinin extracted from the cell wall by ß-glucanase digestion has molecular weight of 250-400 kD. Endoglycosidase F (Endo F) treatment, which removes N-linked carbohydrate, yields five fragments from 51 kD to 72 kD. All of these fragments have biological activity (binding to the **a**-agglutinin), and share an identical N-terminal sequence, indicating that these fragments are proteolytic products from a single protein (Hauser and Tanner 1989). A soluble 160 kD form of α-agglutinin has been isolated from broken cells (Terrance et al. 1987). This form may represent an intracellular intermediate of the cell wall α-agglutinin. Active α-agglutinin fragments can also be released from the cells by trypsin treatment (Sijmons et al. 1987). The N-linked carbohydrate has been reported to account for 30-50% of the molecular mass of α-agglutinin, and is not required for the activity (Terrance et al. 1987; Lipke et al. 1987; Hauser and Tanner 1989).

a-agglutinin analogs from other yeasts consist of a core subunit, which mediates cell surface attachment, and one or more binding subunits, which bind to the complementary agglutinin on cells of opposite mating type. The core and the binding subunits are bound together by disulfide linkage (Burke et al. 1980; Pierce and Ballou 1983; Lipke and Kurjan 1992). A 22 kD glycoprotein is released from *S. cerevisiae* **a** cells by treatment with reducing agents (Orlean et al. 1986; Watzele et al. 1988). The purified 22 kD protein inhibits the agglutinability of α cells, indicating that it is the binding subunit of the **a**-agglutinin. The 22 kD binding subunit contains solely O-linked carbohydrates, which are not essential for interaction with α-agglutinin (Cappellaro et al. 1991). Larger forms representing **a**-agglutinin complex have also been isolated using

different isolation approaches (Yamaguchi et al. 1984; Shimoda and Yanaghishima 1975; Hagiya et al. 1977). Genetic analyses have confirmed that the **a**-agglutinin is composed of a core subunit and a binding subunit (Roy et al. 1991).

Pheromone induction of agglutinin expression

The α-agglutinin is constitutively expressed, and the levels vary from strain to strain. The **a**-agglutinin is constitutively expressed in some strains, while in other strains, it is not detectable before pheromone induction. Increased expression of both agglutinins is seen after the cells are exposed to peptide pheromone secreted by opposite mating type cells. A 2-10-fold increase in the level of α-agglutinin expression has been observed after incubation of α cells with **a**-factor (Wojciechowicz and Lipke 1989; Hauser and Tanner 1989; Terrance and Lipke 1981). Treatment of **a** cells with α-factor increases the amount of the 22 kD binding subunit of **a**-agglutinin by 30-fold (Orlean et al. 1986). The constitutively expressed and pheromone-induced agglutinins are biochemically indistinguishable (Terrance and Lipke 1981). The pheromone induction of agglutinin expression occurs at mRNA level. The transcripts of agglutinin genes are rapidly induced upon exposure of cells to pheromone (Lipke et al. 1989; Hauser and Tanner 1989; Roy et al. 1991; Cappellaro et al. 1991).

Structural genes

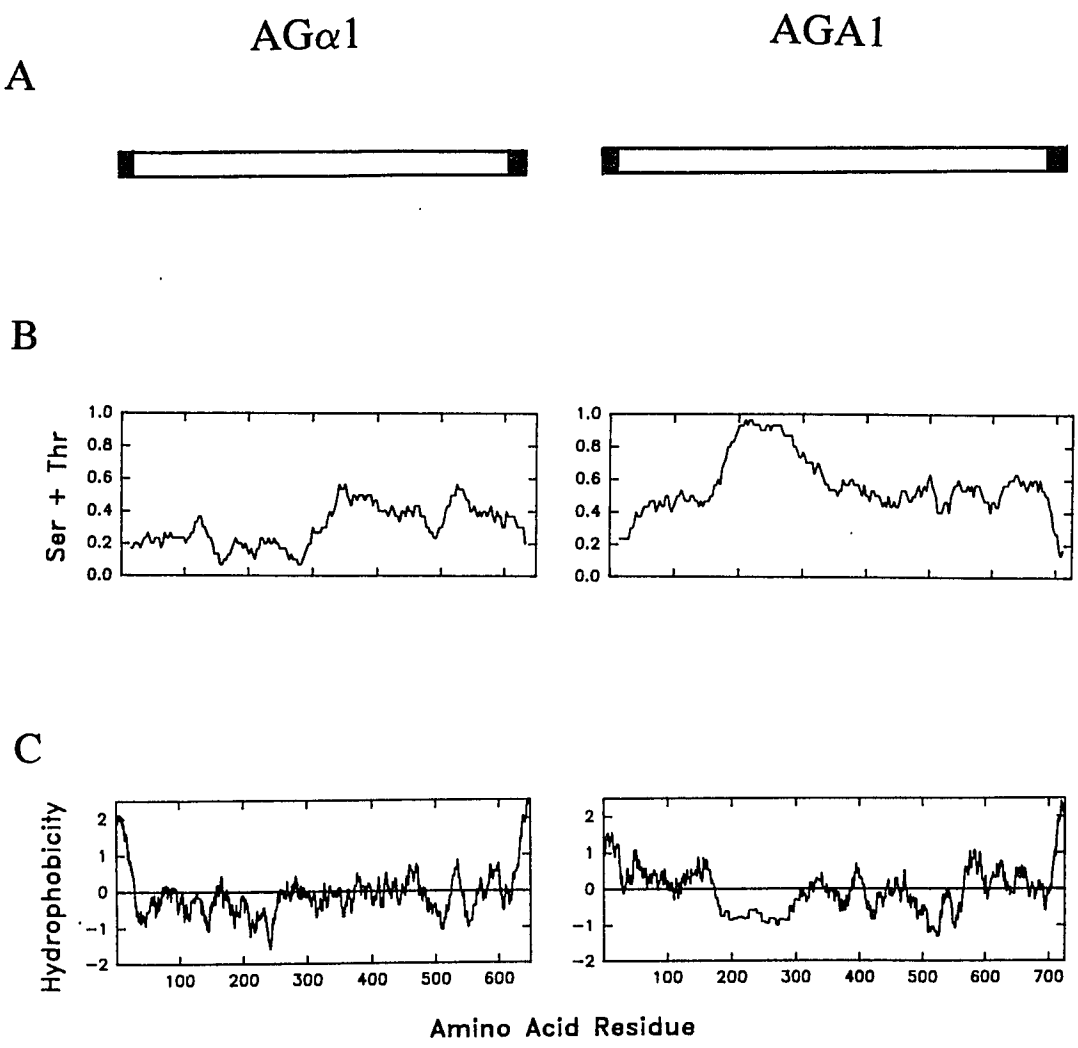
The genes for both agglutinins have been cloned and sequenced (Lipke et al. 1989; Hauser and Tanner 1989; Roy et al. 1991; Cappellaro et al. 1991). There is no sequence homology between the **a**- and α-agglutinin genes. The α-agglutinin gene, *AGα1*, encodes a protein of 650 amino acids with predicted molecular weight of 70.5

kD. The open reading frame of *AGα1* contains 12 potential N-glycosylation sites (Asn-X-Ser/Thr) and 29% serine and threonine residues, which are potential O-glycosylation sites. The Ser and Thr residues are clustered in the C-terminal half (Fig. 3). The binding domain of α-agglutinin has been mapped to the N-terminal half. A segment of α-agglutinin within the binding domain shows sequence similarity to variable-type domains of immunoglobulin (Ig) superfamily of adhesion molecules (Wojciechowicz et al. 1993).

The **a**-agglutinin core subunit is encoded by *AGA1* gene (Roy et al. 1991). The *AGA1* open reading frame could code for a protein of 725 amino acids with 50% Ser and Thr residues (Fig. 3). There are no potential N-glycosylation sites in the *AGA1* open reading frame. These features are similar to the purified core subunits of **a**-agglutinin analogs in other budding yeast (Burke et al. 1980). The **a**-agglutinin binding subunit is encoded by the *AGA2* gene (Cappellaro et al. 1991). This gene codes for a protein of 87 amino acids, 27% of which are Ser and Thr. The structure of *AGA2* is consistent with its encoding a highly O-glycosylated protein.

All three agglutinin genes (*AGα1, AGA1* and *AGA2*) contain an N-terminal hydrophobic secretion signal sequence. Consistent with this structural feature, both **a**- and α-agglutinins are transported to the cell surface through the vesicular secretory pathway (Tohoyama and Yanagishima 1985,1987; Cappellaro et al. 1991). The *AGα1* and *AGA1* also contain a second hydrophobic sequence located at the C-terminus (Fig. 3). The expression of AGα1 and AGA2 is mating-type specific (Lipke et al. 1989; Cappellaro et al. 1991). However, the **a**-agglutinin core subunit gene (*AGA1*) is transcribed in both **a** and α cells (Roy et al. 1991).

Figure 3. Structural features of α-agglutinin gene (*AGα1*) and a-agglutinin core subunit gene (*AGA1*). **A**. Open reading frames of *AGα1* and *AGA1*. Hydrophobic regions are shown by solid rectangles. **B**. Frequency of Ser and Thr residues. **C**. Hydrophobicity plot.


AGα1
AGA1
A
B
C
Ser + Thr
Hydrophobicity
Amino Acid Residue

Cell surface attachment

The predicted C-terminal hydrophobic sequence of α-agglutinin resembles signal sequences for glycosyl phosphatidylinositol (GPI) anchor addition (see below). This finding would imply that α-agglutinin were attached to the plasma membrane by a GPI membrane anchor. However, several lines of evidence suggest that the mature α-agglutinin is anchored in the cell wall rather than plasma membrane. First, α-agglutinin can be released from cells by treatment with ß-glucanases (Hauser and Tanner 1989; Lasky and Ballou 1988; Schreuder et al. 1993), this would not occur if the protein were membrane-bound. Second, the C-terminal half of the α-agglutinin peptide (about 300 amino acids) is not long enough, even in highly extended confirmation, to allow membrane attachment and simultaneous exposure of the N-terminal half binding domain at the surface of the cell wall, which is 100 to 200 nm thick (Jentoft 1990; Ballou 1982). Third, electron-microscopic observation of spheroplasting process reveals that spheroplasts pop out from small openings in the wall, as though there were no adhesion at all between the cell wall and the plasma membrane (Ballou 1988). This observation implies that the bulk wall mannoproteins are not integrated into the plasma membrane. Schreuder et al (1993) have reported that the C-terminal half of α-agglutinin is sufficient to localize a soluble enzyme of plant origin to the cell wall of *S. cerevisiae*. Wojciechowicz et al (1993) have shown that truncation of the C-terminal hydrophobic sequence from *AGα1* eliminates cell surface attachment and allows active α-agglutinin secretion into the medium. Similar results are obtained when the C-terminal hydrophobic sequence is replaced by a more hydrophilic sequence. Therefore, the C-terminal hydrophobic sequence is required for cell surface anchorage of α-agglutinin.

The cell surface attachment of **a**-agglutinin is mediated by the core subunit. *AGA1*- defective mutants secrete the binding subunit of **a**-agglutinin into the medium. Truncations of the C-terminal hydrophobic sequence from *AGA1* result in the loss of cell surface **a**-agglutinin (Roy et al. 1991). It appears that a similar mechanism is involved in the cell surface anchorage of both **a**- and α-agglutinins.

GPI protein membrane anchors

Many proteins in mammalian cells, as well as in lower eukaryotes such as yeast and protozoa, are anchored in the plasma membrane by covalently attached glycolipids containing inositol called glycosyl phosphatidylinositols or GPI anchors. A wide variety of proteins have GPI anchors, including parasite coat proteins, cell surface hydrolytic enzymes, lymphocyte antigens, and cell adhesion molecules (reviewed by Ferguson and Williams 1988; Low and Saltiel 1988; Doering et al 1990; Cross 1990; Englund 1993).

Signal sequences for GPI anchor addition

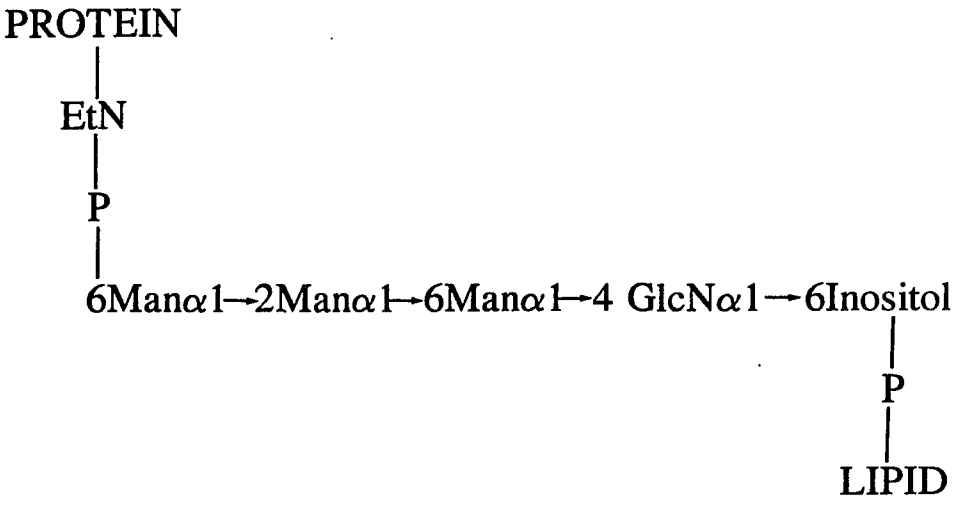
The predicted peptide sequences for GPI-anchored proteins contain a cleavable N-terminal hydrophobic signal sequence, which directs the polypeptide to the ER, and a C-terminal sequence, which consists of 17-30 predominantly hydrophobic amino acids. The C-terminal hydrophobic sequences are not present in the mature GPI-anchored proteins. Truncations of the C-terminal hydrophobic sequences from the genes eliminate GPI-anchoring of proteins (Su and Bothwell 1989; Caras and Weddell 1988; Berger et al. 1988; Nuoffer er al. 1991). These observations indicate that the C-terminal hydrophobic sequences function as signals for GPI anchor addition. However, the C-terminal sequences for various GPI-anchored proteins share no sequence homology. The

similarity among these sequences is the presence of a hydrophobic region at the C-terminus, which is not followed by a cluster of basic amino acid residues characteristic of typical transmembrane domains. Mutational studies have shown that it is the hydrophobicity rather than the sequence itself that is important for GPI anchor addition (Kodukula et al. 1992,1993; Lowe 1992; Waneck et al. 1988). The GPI anchors are preformed and transferred to the protein after the C-terminal hydrophobic sequence is cleaved from the primary translation product (Mayor et al. 1990,1991; Vidugiriene and Menon 1993). The addition of GPI anchor occurs quickly; in the case of trypanosome variant surface glycoprotein (VSG), replacement of the C-terminal peptide with the GPI anchor takes place within 1 min of peptide synthesis (Bangs et al. 1985; Ferguson et al. 1986; Conzelmann et al. 1987).

Structures of GPI anchors

GPI anchors from yeast to mammal share a conserved core structure (Fig. 4). The core structure consists of the tetrasaccharide Manα1-2Manα1-6Manα1-4GlcN linked glycosidically to the inositol ring of phosphatidylinositol. The terminal mannose is linked via a phophodiester bond to ethanolamine, which is in turn joined by an amide bond to the α-carboxyl group of the C-terminal amino acid of the protein. Various modifications to the core structure have been detected. Mammalian GPI anchors contain up to three phosphoethanolamine residues. The ethanolamine phosphate can be added to any of the mannose residues in the core structure (Kamitani et al. 1992). In trypanosome VSG GPI anchor, the core glycan is modified by a side chain consisting of galactosyl residues (Ferguson et al. 1985). Some GPI anchors, such as human erythrocyte acetylcholinesterase, contain an extra fatty acid chain esterified to the inositol ring

Figure 4. Core structure of GPI anchors. EtN, ethanolamine; P, phosphate. The ethanolamine is linked in an amide bond to the α-carboxyl group of the C-terminal amino acid residue of the protein.


PROTEIN
EtN
P
6Manα1→2Manα1→6Manα1→4 GlcNα1→6Inositol
P
LIPID

(Roberts et al. 1988). The acylation of inositol confers the GPI anchor resistance to the cleavage by phophatidylinositol-specific phospholipase C (PI-PLC) (Roberts et al. 1988; Walter et al. 1990; Wong and Low 1992). The lipids of GPI anchors from different organisms also exhibit a wide variation. Most anchors contain diacylglycerol. Some anchors in *Dictyostelium discoideum* or *Saccharomyces cerevisiae* contain ceramide (Stadler et al. 1989; Conzelmann et al. 1992).

Functional roles of GPI anchors

GPI anchors effectively attach proteins to the membranes. Apart from providing a means for membrane attachment, GPI anchoring possesses some unique features. One of the obvious features presented by GPI anchoring is the regulated protein release from the membrane. Specific phospholipases could mediate rapid release of enzymes, receptors or adhesion molecules from the cell surface. The *Trypanasoma brucei* VSG is rapidly cleaved to a soluble form by an endogenous GPI-specific PLC (Fox et al. 1986; Bulow and Overath 1986). GPI-PLC activity has been isolated from mammalian liver (Fox et al. 1987). Davitz et al (1987) have detected a GPI-specific PLD in human plasma. Some GPI-anchored proteins such as alkaline phosphatase and 5'nucleotidase are also detected in soluble forms, which may result from GPI anchor cleavage (Romero et al. 1988; Sorimachi and Yasumura 1988; Spychala et al. 1988; Stochaj et al. 1989).

Another obvious consequence of GPI anchoring is the increased lateral mobility in cell membranes. Proteins with GPI anchors diffuse in the lipid bilayer at a rate closer to that of the lipids than to that of intrinsic membrane proteins (Cross 1990). Some GPI-anchored proteins may require increased lateral mobility for function. GPI anchoring can affect the sorting of proteins in the secretory pathway in polarized epithelial cells. It has

been reported that the GPI anchor acts as an apical targeting signal in Madin-Darby canine kidney (MDCK) cells (Lisanti et al. 1988,1989; Brown et al. 1989). However, a recent study by Zurzolo et al (1993) shows that in Fisher rat thyroid (FRT) epithelial cells, the GPI anchor behaves rather as a basolateral targeting signal. This finding raises new questions regarding the mechanism for sorting of GPI-anchored proteins in polarized epithelial cells.

Yeast GPI-anchored proteins

The first GPI-anchored protein identified in *S. cerevisiae* is a 125 kD glycoprotein called Gas1 (or Ggp1) (Conzelmann et al. 1988; Vai et al. 1990). This protein can be metabolically labeled with both [^{3}H]myo-inositol and [^{3}H]palmitic acid, and after PI-PLC treatment, the palmitate label is lost whereas the myo-inositol label still remains associated with the protein. Sequence analysis of the gene (*GAS1*) reveals that the predicted Gas1 peptide contains a cleavable N-terminal secretion signal, and a C-terminal hydrophobic domain characteristic of signal sequences for GPI anchor addition (Nuoffer et al. 1991; Vai et al. 1991). The C-terminal hydrophobic sequence is essential for GPI anchor addition, as truncations of this sequence or point mutations that affect the hydrophobicity of this sequence abolish membrane attachment and allow secretion of Gas1p into the medium (Nuoffer et al. 1991). In a secretory mutant (*sec18*) which blocks protein vesicular transport from the ER to the Golgi at restrictive temperature, the Gas1p is detected as a 105 kD precursor which already contains a GPI anchor (Fankhauser and and Conzelmann 1991). Therefore, like in mammalian cells, the addition of GPI anchors occurs in the ER in yeast. The mature Gas1p is attached to the external face of the plasma membrane (Nuoffer et al. 1991). Analyses of mutants reveal

that Gas1p is involved in morphogenesis and cell separation (Popolo et al. 1993).

Gas1 is one of the major GPI-linked proteins in *S. cerevisiae*. A cAMP-binding glycoprotein with molecular weight of 54 kD is attached to the plasma membrane by a GPI anchor (Muller and Bandlow 1991; Muller et al. 1992). Numerous other membrane glycoproteins of unknown function can be labelled with myo-inositol (Conzelmann et al. 1990). Structural analyses reveal that the core region of the yeast GPI anchors is identical to the conserved core structure found in protozoan and mammalian GPI anchors (Fankhauser et al. 1993). This finding implies that a similar GPI biosynthetic pathway exists in all eukaryotes.

Aim of this thesis research

The presence of a predicted C-terminal hydrophobic sequence that resembles the signal sequences for GPI anchor addition suggests that α-agglutinin is likely to be linked to a GPI membrane anchor. However, as mentioned above, it is unlikely that the mature α-agglutinin is attached to the plasma membrane by a GPI anchor, this implies that the α-agglutinin would have to be released from the membrane before becoming anchored in the cell wall. The aim of the research described in this thesis was to determine whether α-agglutinin indeed contains a GPI anchor, and if so, what role of the GPI anchor plays in the cell wall anchorage of α-agglutinin. In section II, the identification and characterization of GPI-anchored and soluble intermediates of the mature cell wall α-agglutinin are described. In section III, the mechanism for cell wall anchorage of α-agglutinin is explored. The results demonstrate that the cell wall α-agglutinin is cross-linked to the wall ß-1,6-glucan, and that the cross-linking of the two components appears dependent on addition of a GPI anchor to the α-agglutinin.

Section II

Intermediates in Secretion and Cell Wall Anchorage of α-Agglutinin from *S. cerevisiae*

INTRODUCTION

α-agglutinin is a cell surface adhesion glycoprotein expressed by haploid α cells from *S. cerevisiae*. It binds to **a**-agglutinin on **a** cell surface during mating (Cross et al. 1988). The predicted α-agglutinin peptide contains two hydrophobic sequences: one is the N-terminal secretion signal sequence and the other is located at the C-terminus (Lipke et al. 1989; Hauser and Tanner 1989). The C-terminal hydrophobic sequence of α-agglutinin is characteristic of signals for GPI anchor addition (Ferguson and Willians 1988; Cross 1990; Englund 1993). Truncation of the C-terminal hydrophobic sequence from *AGα1* eliminates cell surface anchorage and allows secretion of active α-agglutinin into the medium (Wojiechowicz et al. 1993). These findings strongly imply that the α-agglutinin contains a GPI anchor. However, unlike GPI-anchored proteins, which are attached to the plasma membrane (Ferguson and Willians 1988; Cross 1990), the mature α-agglutinin appears to be covalently anchored in the cell wall (Lasky and Ballou 1988; Weinstock and Ballou 1986; Hauser and Tanner 1989; Schreuder et al. 1993).

To test whether α-agglutinin is synthesized with a GPI anchor, intermediate forms of the cell wall-associated α-agglutinin were identified, and GPI-linkage to the intermediate forms was determined. Two GPI-anchored and one soluble forms of α-agglutinin were identified in wild-type cells. Additional forms were detected in temperature-sensitive secretory mutants. The temporal and spatial relationships among various forms of α-agglutinin were also studied. A pathway for α-agglutinin processing is proposed at the end of this section.

MATERIALS AND METHODS

Yeast strains and growth conditions. The following standard *S. cerevisiae* strains were used: X2180-1A, *MAT***a** *SUC2 mal mel gal2 cup1*; X2180-1B, *MATα SUC2 mal mel gal2 cup1*; W303-1A, *MAT***a** *ade2 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15*; and W303-1B, *MATα ade2 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15*. The temperature-sensitive secretory mutants NY432 (*MATα ura3-52 sec18-1*), NY761 (*MATα his4-619 sec7-1*), NY4 (*MATα his4-619 sec1-1*), and their isogenic wild type strain NY191 (*MATα his4-619*) were provided by Dr. Peter Novick. The protease-deficient strain BJ3501 (*MATα pep4 his3 prb1-1.6R his3-200 ura3-52 can1 gal2*) was obtained from the Yeast Genetics Stock Center. BYF106-4D (*MATα can1-100 ade2*^{oc} *his3-11 leu2-3 trp1-1 ura3-1 kex2-2 his3-A*) was provided by Dr. Robert S. Fuller and PRY95 (*MATα alg4-4(sec53) ura3-52*) was provided by Dr. Peter Orlean. Yeast cells were grown in a minimal medium (yeast nitrogen base plus 2% glucose) supplemented with amino acids to exponential phase.

Plasmid construction and transformation of yeast cells with plasmid DNA. A 4.5 kb HindIII-XbaI fragment containing the full length α-agglutinin gene *AGα1* was isolated from plasmid pH27 (Lipke et al. 1989), and ligated into the HindIII-XbaI sites of a yeast-*E. coli* shuttle plasmid YEp352 (Hill et al. 1986). The resultant plasmid was designated as pAGα1'. The pAGα1' was used to transform W303-1B and NY432 (*sec18*) strains. Standard DNA manipulation and yeast transformation procedures were followed.

Metabolic labeling of cells. For labeling with [^{3}H]myo-inositol (American Radiolabeled Chemicals, 20 mCi/mmol), pAGα1'-transformed W303-1B cells were grown in inositol-free minimal medium at 30°C. Exponentially growing cells were centrifuged and resuspended in the same medium to a density of 3 x 10^7 cells per ml. [^{3}H]myo-inositol was added to 10 uCi per ml cell suspension. 10 min after the addition of the label, synthetic **a**-factor (provided by Dr. Fred Naider) was added to 50 ng per ml. The labeling was continued for 2 hr at the same temperature. Labeling with [^{3}H]palmitic acid was done as [^{3}H]myo-inositol labeling, except that the cells were grown in minimal medium containing inositol, and [^{3}H]palmitic acid (Du Pond, 52.4 mCi/mmol) was added to 33 uCi per ml cell suspension.

For labeling with [^{35}S]methionine, cells were grown at 30°C in low sulfate minimal medium containing 100 uM $(NH_4)_2SO_4$ to exponential phase (Rothblatt and Schekman 1989). Cells were then collected and resuspended to 7 x 10^7 cells per ml in labeling medium containing 10 uM $(NH_4)_2SO_4$, and incubated for 30 min at 30°C before addition of **a**-factor. 10 min after addition of **a**-factor, [^{35}S]methionine (TRAN^{35}S-LABEL^{TM}, ICN Biochemicals) was added to 15-20 uCi per ml cell suspension, and the labeling was continued for 20-30 min. For pulse-chase labeling, cells were labeled with [^{35}S]methionine for 2 min, after which a chase was initiated by adding unlabeled sulfate to 10 mM, and methionine and cysteine to 1 mM. Labelings were terminated by adding NaN_3 to 10 mM and chilling the culture on ice. The labeled cells were collected, washed twice with 30 mM Tris-HCl (pH 7.4), 1 mM phenyl methylsulfonylfluoride (PMSF) and 1mM EDTA, and stored at -70°C.

When temperature-sensitive secretory mutants were used for labeling at non-

permissive temperature (37°C), cells were grown at permissive temperature (24°C) to exponential phase. The cell suspension was incubated at 37°C for 30 min before addition of the label.

Cell lysis and isolation of cell walls. Two procedures were used in different experiments. **Procedure A**: 1 x 10^9 cells were resuspended in 1 ml lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1mM PMSF, 30 ug/ml each of leupeptin, pepstatin, and antipain) using 15 ml polypropylene tube (Fisher Scientific). The cell suspension was mixed with an equal volume of cold glass beads (0.5 mm, Sigma), and broken by vortexing at maximal speed (Fisher Vortex Genie 2 ^{TM}) four times for 1 min each, with 1 min cooling intervals on ice. At least 90 % of the cells were broken as determined by phase-contrast microscopy. After glass beads were removed, cell walls were collected by spinning at 1000 x g for 5 min at 4°C, and extracted twice with 2% SDS in lysis buffer by heating for 5 min at 100°C each time. The wall pellet was then washed three times with cold 1 N NaCl containing 1 mM PMSF, and three times with 1 mM PMSF in distilled water. **Procedure B**: 1 x 10^9 cells were resuspended in 350 ul SDS lysis buffer (lysis buffer containing 2% SDS) and broken by vortexing with glass beads for 4 x 1 min, followed by heating for 5 min at 100°C. The insoluble material (cell walls) was separated from the SDS extract by spinning for 5 min at 13,600 x g. The wall pellet was extracted once more with SDS lysis buffer by heating for 5 min at 100°C, and washed as in procedure A.

Separation of membrane proteins from soluble proteins. Procedure A: Triton X-114 (TX-114) phase separation. Triton X-114 (Sigma) was pre-condensed as described (Bordier 1981), and added to the low speed supernatant (1000 x g) from cell lysis

procedure A to a final concentration of 2% to solubilize membrane proteins. After incubation on ice for 1 hr, the insoluble material was removed by spinning at 13,600 x g for 5 min at 4°C. The supernatant was warmed to 35°C, and the lower detergent phase and upper aqueous phase were separated by centrifuging at 13,600 x g for 20 seconds at room temperature. The detergent phase (membrane protein fraction) was re-extracted three times by addition of a 10-fold volume of lysis buffer to remove remaining water-soluble proteins; the aqueous phase was extracted once more with TX-114 to remove remaining detergent-soluble proteins. **Procedure B**: high speed centrifugation. The low speed supernatant was span at 100,000 x g for 1 hr at 4°C. After centrifugation, the supernatant (soluble protein fraction) was removed, and the pellet (membrane fraction) was dissolved in SDS lysis buffer. For Na_2CO_3 treatment, Na_2CO_3 (pH 11) was added to the low speed supernatant to a final concentration of 0.1 M. The mixture was incubated for 30 min at 0°C, followed by high speed centrifugation to separate membranes from soluble proteins.

Laminarinase treatment of isolated cell walls. Cell walls from 1 x 10^9 cells were treated with 1.5 U laminarinase (Sigma) in 300 ul 50 mM sodium acetate (pH 5.5) containing 3 mM PMSF, 2 mM EDTA, 90 ug/ml each of leupeptin, pepstatin, and antipain at 35°C for 4 hr. After treatment, the insoluble material was separated from the laminarinase extract by spinning at 13,600 x g for 5 min.

Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment. Procedure A: 70 ul re-extracted detergent phase sample was diluted to 250 ul with 100 mM Tris-HCl, pH 7.4, 50 mM NaCl, 250 mM α-methylmannoside, 1 mM EDTA, and protease inhibitors as in lysis buffer. PI-PLC from *Bacillus thuringiensis* was provided by Dr.

Martin Low (purity, >90% by SDS-PAGE; activity, 390 umol/min/ml using [^{3}H]phosphatidylinositol as substrate at pH 7). 3 ul of the enzyme was added to the diluted detergent sample, and the mixture was incubated for 1 hr at 35°C. After incubation, 0.5 ml buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and protease inhibitors as in lysis buffer) containing 2% TX-114 was added and the phases were separated. Each phase was then re-extracted as described above. The detergent phase was diluted in 5-fold volume of lysis buffer. Proteins in each phase was precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 10%. Bovine serum album (BSA) (final concentration of 90 ug/ml) was added to the aqueous phase as carrier protein before TCA precipitation. The precipitated proteins were washed three times in cold acetone, air-dried, and dissolved in Laemmli sample buffer for SDS-gel electrophoresis and immunoblotting. **Procedure B**: The low speed supernatant (1000 x g) prepared from 5 x 10^8 pAGα1'-transformed W303-1B cells was centrifuged at 13,600 x g for 15 min at 4°C, and the membrane pellet was resuspended in 400 ul lysis buffer and split into two portions. One portion was treated with PI-PLC (3 ul) for 90 min at 35°C. The other portion of the membrane suspension received mock treatment without the enzyme. Following the incubation, membranes were resedimented by spinning at 100,000 x g for 1 hr at 4°C, and dissolved in SDS lysis buffer.

Endo H and α-mannosidase treatment. Membrane proteins from 5 x 10^8 pAGα1'-transformed W303-1B cells were prepared in the TX-114 detergent phase, and treated with PI-PLC as described above. The PI-PLC released membrane proteins in the aqueous phase were treated with 0.005 U endo-ß-N-acetylglucosaminidase H (Endo H) (Boehringer Mannheim) in 240 ul buffer (50 mM NaAc, pH 5.5 containing protease

inhibitors as in the lysis buffer) for 2 hr at 37°C. For α-mannosidase treatment, two third of the Endo H treated sample was treated with 1 U α-mannosidase from Jack Beans (Sigma) in 300 ul buffer at 25°C. After 2 hr, half of the sample was collected and the enzyme reaction was stopped by addition of TCA to 10%. The remaining half of the sample was incubated at 25°C for 14 more hours. Proteins in the Endo H treated or Endo H and α-mannosidase double treated samples were precipitated with TCA as described above, and analyzed by immunoblotting.

Proteinase K treatment of intact cells. Exponentially growing W303-1B cells were labeled with [^{35}S]methionine as described above. The labeled cells were resuspended in 100 mM DTT containing 30 mM Tris-HCl, pH 7.4, 25 mM EDTA, 20 mM NaN_3, and 50 ug/ml cycloheximide, and incubated for 30 min at room temperature (20°C) with gentle agitation. The cells were then washed with buffer (30 mM Tris-HCl, pH 7.4, 15 mM NaN_3, and 50 ul/ml cycloheximide), and resuspended to a density of 6 x 10^7 cells per ml in buffer containing 600 mM sorbitol. The cell suspension was split, and proteinase K was added to different concentrations as indicated. The samples were incubated for 40 min at 35°C with gentle agitation. The reactions were terminated by addition of PMSF to 2 mM. The treated cells were washed with buffer containing 600 mM sorbitol, and lysed using procedure A.

Binding of α-agglutinin to intact cells. Cell membranes prepared from [^{35}S]methionine-labeled W303-1B[pAGα1'] cells were treated with PI-PLC, and resedimented as described above. The supernatant was adjusted to pH 5.5 by addition of NaAc (pH 5.5) to 100 mM. Cycloheximide (20 ug/ml final concentration) and BSA (500 ug/ml final concentration) were also added to the supernatant prior to the

incubation with **a** or α cells.

1 x 10^8 unlabeled **a** cells (X2180-1A) or α cells (X2180-1B) were preincubated with 2 mg/ml BSA in 50 mM NaAc, pH 5.5, 25 ug/ml cycloheximide, and 1 mM PMSF for 30 min at 30°C. The labeled supernatant was split and incubated with the **a** and α cells for 90 min at 30°C with agitation. The cells were harvested, and the bound labeled proteins were released from the cells by heating at 95°C for 5 min in 50 mM Tris-HCl, pH 7.4, containing 1% SDS, 2 mM PMSF. The released labeled proteins were analyzed by immunoprecipitation.

Immunoprecipitation and fluorography. The antiserum was raised in rabbit against purified N-terminal half of α-agglutinin, and was pre-adsorbed twice with heat-killed **a** cells as described (Wojciechowicz and Lipke 1989). The samples (SDS-extracts of total cellular proteins, laminarinase-extracts of cell wall proteins, membrane protein fractions or soluble protein fractions) prepared from 7.5 x 10^7 pAGα1'-transformed cells or 5 x 10^8 cells without pAGα1' were adjusted to 1% Triton X-100 (or 1% TX-114 when TX-114 detergent phase samples were used), 0.2% SDS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1mM PMSF, 30 ug/ml each of leupeptin, pepstatin, and antipain in a final volume of 700 ul, to which 4 ul of anti-α-agglutinin-antiserum was added. The mixture was incubated overnight at 4°C. For antibody competition experiments, the same amount of antiserum was pre-incubated overnight with purified unlabeled α-agglutinin (N-terminal half of α-agglutinin, 120 units). Immune complexes were precipitated by addition of 100 ul protein A-Sepharose beads (15% suspension in lysis buffer), followed by incubation for 2 hr at 4°C with agitation. The beads were pelleted by spinning at 13,600 x g for 20 seconds, and washed three

times with 1 ml wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid) and one time with wash buffer without detergents. The α-agglutinin was eluted from the beads in 50 ul Laemmli sample buffer by heating at 100°C for 4 min, and separated by 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were fixed, soaked in 1 M sodium salicylate, pH 6 (Chamberlain 1979), dried, and exposed to Kodak X-Omat XAR film at -70°C for fluorography.

Immunoblotting. After SDS-PAGE, proteins were transferred from gels to nitrocellulose membranes (0.2 um pore size, Sigma) in 25 mM Tris, 192 mM glycine and 20% methanol, pH 8.2. The transfer was carried out at 40 volts overnight using a Trans-Blot cell (Bio-Rad). Blots were blocked with 3% gelatin at 30°C for 2 hr, followed by incubation in anti-α-agglutinin for 2 hr at room temperature. The blots were then washed and incubated in goat anti-rabbit IgG (Sigma) for 1 hr followed by incubation in rabbit peroxidase-anti-peroxidase (Sigma) for 45 min. The antibodies were diluted 1:1000 in PBS containing 0.2% Tween-20. The α-agglutinin was visualized by incubating the blots in peroxide and 4-chloro-1-naphthol solution.

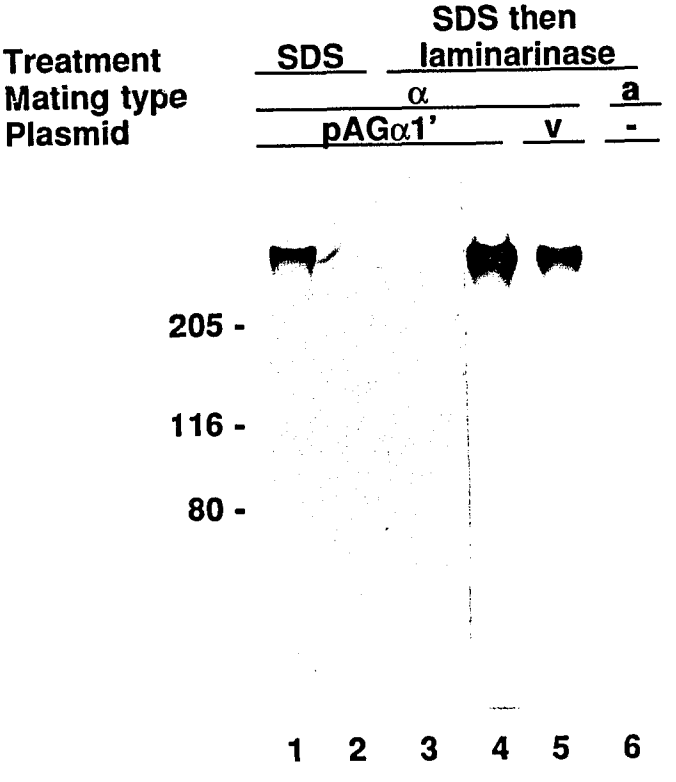
RESULTS

Covalently cell wall-bound form of α-agglutinin

Mature cell wall α-agglutinin is extractable from intact cells by ß-glucanase treatment (Lasky and Ballou 1988; Hauser and Tanner 1989), implying a covalent linkage of α-agglutinin to the cell wall ß-glucan. However, since the ß-glucanases used by previous investigators contained proteases, the liberation of α-agglutinin could be through the action of proteases associated with the glucanases (Zlotnik et al. 1984; Weinstock and Ballou 1986). Schreuder et al. (1993) have reported extraction of α-agglutinin from cell walls with laminarinase, which contains predominantly ß-1,3-glucanase activity and a small amount of ß-1,6-glucanase (7%) and mannanase (6%). Protease activity towards casein is not detectable in the laminarinase preparation (Van Rinsum et al. 1991). Laminarinase was used in this research to extract cell wall α-agglutinin.

To extract mature cell wall α-agglutinin, [^{35}S]methionine labeled cells were mechanically lysed, and cell walls were collected and extracted with hot SDS to remove contaminating membrane proteins and non-covalently associated cell wall proteins. The SDS-extracted cell wall material was then treated with laminarinase in the presence of protease inhibitors. α-agglutinin was immunoprecipitated from the SDS extracts or laminarinase extract of the cell walls. A >300 kD band of α-agglutinin was extracted by a single SDS treatment of the cell wall (Fig. 5, lane 1), indicating that it was not covalently linked to the cell wall. Laminarinase treatment released additional >300 kD α-agglutinin (lanes 4 and 5). The laminarinase-released α-agglutinin was not extractable

Figure 5. Cell wall form of α-agglutinin. Yeast strains W303-1A (**a**) and W303-1B (α) transformed with pAGα1' or vector alone (v) were labeled with [^{35}S]methionine and broken in lysis buffer with glass beads (procedure A). Cell walls were collected, extracted twice with hot SDS, and subsequently treated with laminarinase. α-agglutinin was immunoprecipitated from the SDS extracts or laminarinase extracts of cell walls with anti-α-agglutinin antiserum, and analyzed by SDS-PAGE and fluorography. Lane 1, first SDS extract; Lane 2, second SDS-extract; lanes 3-6, laminarinase extracts: lane 3, as lane 4 except that the antiserum was preincubated with purified unlabeled α-agglutinin. Molecular size standards on the left are indicated in kilodaltons.


Treatment
SDS
SDS then laminarinase
Mating type
α
a
Plasmid
pAGα1'
v
-
205 -
116 -
80 -
1 2 3 4 5 6

by SDS, as it was not present in the second SDS extract of the cell wall (lane 2). The cell wall form of α-agglutinin was present at higher level in α cells expressing the high-copy plasmid pAGα1' (lane 4 vs. 5), and was not detected in **a** cells which do not express α-agglutinin (lane 6) or when the antiserum was preincubated with purified unlabeled α-agglutinin (lane 3).

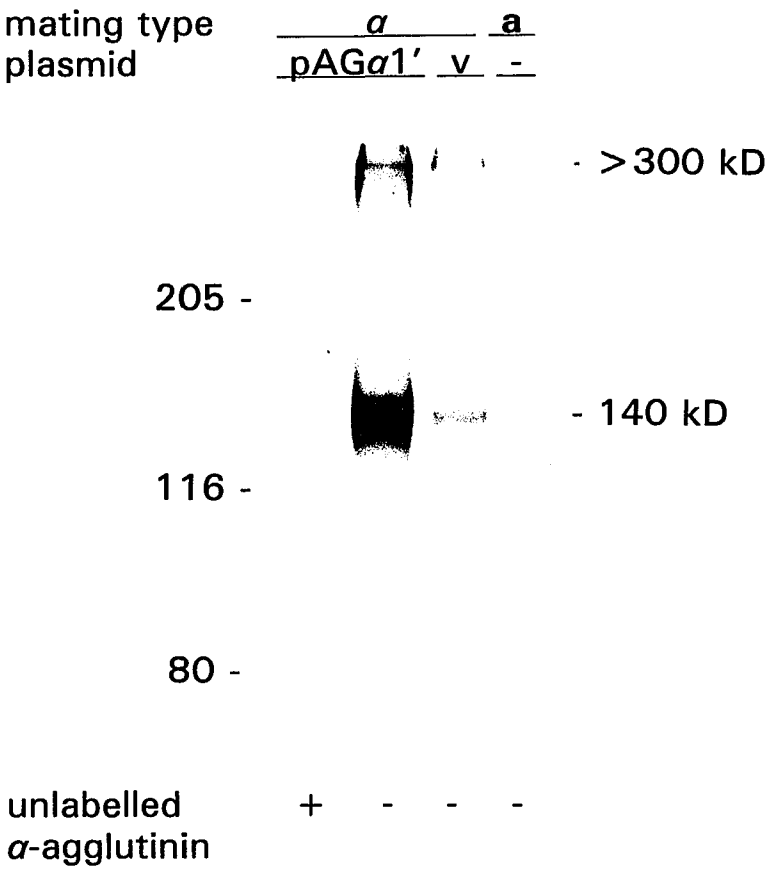
Several control experiments showed that the laminarinase-extractable α-agglutinin had been covalently bound to the cell wall rather than physically trapped within the wall. Multiple extractions with hot SDS or with DTT and EDTA released less than 30% of the wall-associated α-agglutinin. The remaining α-agglutinin could be released only by digestion with laminarinase. No laminarinase-mediated proteolysis of [^{14}C]BSA nor of a soluble form of α-agglutinin was detectable under the experimental conditions (data not shown). GPI-cleaving activity was not detectable in the laminarinase preparation, as laminarinase treatment did not release GPI-anchored forms of α-agglutinin from membranes (data not shown).

Membrane-bound forms of α-agglutinin

To identify intermediates of the covalently cell wall-bound α-agglutinin, the cells were lysed in SDS. Anti-α-agglutinin antiserum precipitated two proteins with molecular sizes of apparent 140 kD and >300 kD from the SDS extracts of α cells, but not from **a** cells (Fig. 6). These proteins were present at higher levels in α cells expressing pAGα1'. Preincubation of the antiserum with purified unlabeled α-agglutinin inhibited the precipitation of these proteins. These results indicate that these two proteins are two forms of α-agglutinin.

To determine whether the SDS-extractable 140 kD and >300 kD forms of α-

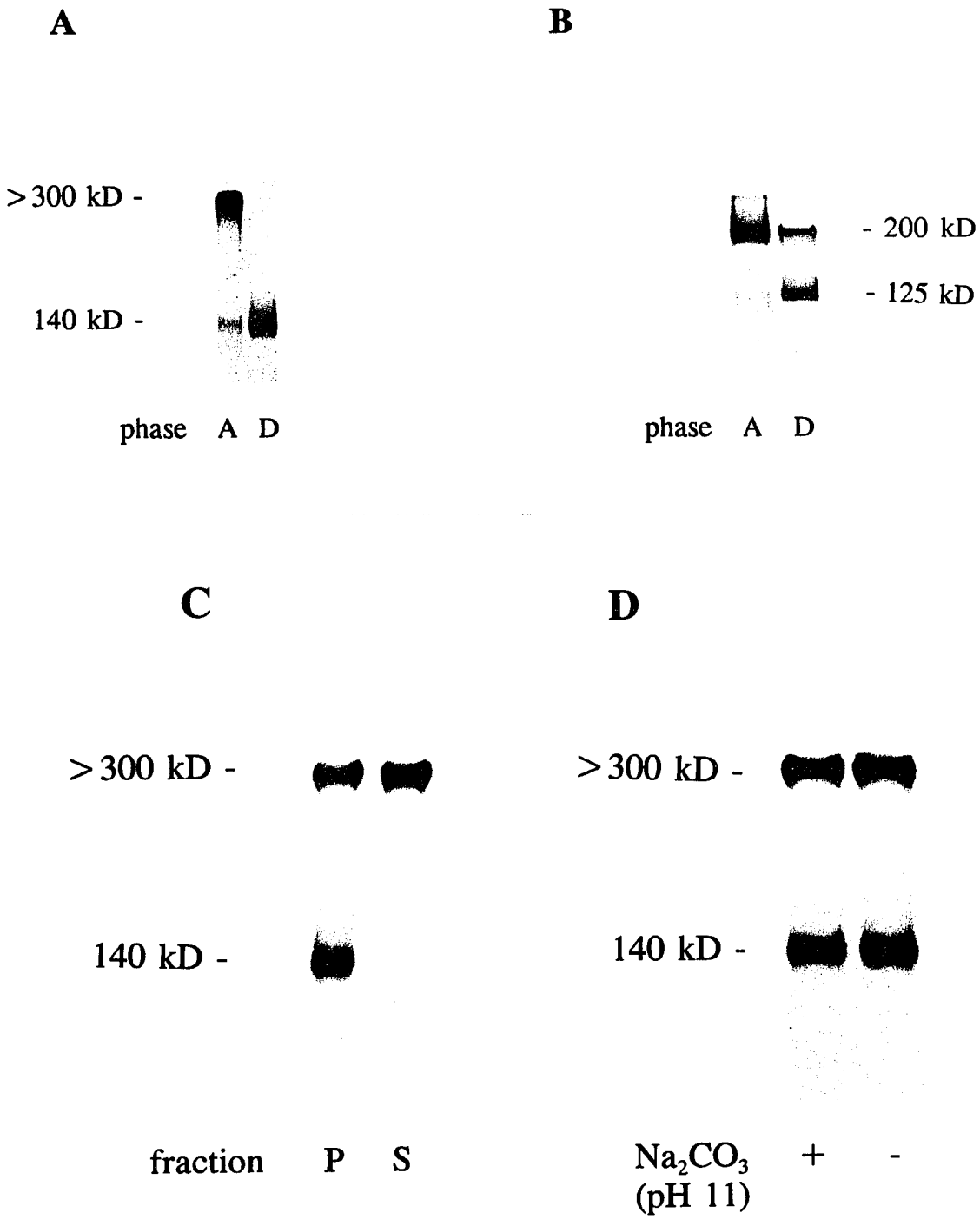
Figure 6. SDS-extracted forms of α-agglutinin. W303-1A (**a**) and W303-1B (α) cells harboring pAGα1' or vector alone (v) were labeled with [^{35}S]methionine and lysed in SDS lysis buffer (procedure B). Cell walls were removed by spinning at 13,600 x g for 5 min. α-agglutinin was immunoprecipitated from the SDS extracts of the cells, separated by SDS-PAGE, and visualized by fluorography. Molecular size standards on the left are indicated in kilodaltons.


mating type
plasmid
α
pAGα1'
v
a
-
>300 kD
205 -
140 kD
116 -
80 -
unlabelled
α-agglutinin
+ - - -

agglutinin were membrane-bound, cell lysate was extracted with Triton X-114 (TX-114) to solubilize membrane proteins, followed by phase separation in TX-114 (Bordier 1981; Conzelmann et al. 1988). The 140 kD form of α-agglutinin partitioned into the detergent phase of TX-114, as expected for membrane-bound proteins (Fig. 7A). The >300 kD α-agglutinin was recovered from the aqueous phase of TX-114. However, when cell labeling was carried out in the presence of tunicamycin, which inhibits protein N-glycosylation, a portion of the de-N-glycosylated >300 kD α-agglutinin (200 kD) partitioned into the detergent phase of TX-114 (Fig. 7B), suggesting that some fraction of the >300 kD α-agglutinin is membrane-bound, and that the high amount of carbohydrate confers resistance to partitioning into the detergent phase of TX-114.

To further investigate whether the >300 kD α-agglutinin was membrane-bound, membranes and soluble proteins were separated by ultracentrifugation (100,000 x g, 1 hr). The 140 kD form sedimented with the membranes, whereas the >300 kD α-agglutinin was found both in the membrane fraction and in the soluble protein fraction (Fig. 7C). About 40-60% of the total >300 kD α-agglutinin was distributed to the soluble fraction in separate experiments using several wild-type strains, and the level did not vary in the presence or absence of the high-copy pAGα1' (data not shown). The soluble >300 kD protein exhibited no obvious mobility difference from the sedimentable >300 kD protein either on 6% identical polyacrylamide gels (Fig. 7C) or on 4-15% gradient SDS-polyacrylamide gels (data not shown). About 50% of the total >300 kD α-agglutinin was soluble in two mutants with reduced protease activity: a *pep4/prb1* double mutant and a *kex2* mutant. The *pep4/prb1* mutant lacks vacuolar proteinase A and proteinase B, which cause major proteolysis problems during cell lysis

Figure 7. Analysis for membrane association of SDS-extractable forms of α-agglutinin. A and **B**. Partitioning of α-agglutinin in Triton X-114. pAGα1'-transformed W303-1B cells were labeled with [^{35}S]methionine in the absence (**A**) or presence of tunicamycin (15 ug/ml) (**B**). The cells were then broken in lysis buffer with glass beads, and cell walls were removed by low speed centrifugation (1000 x g, 5 min). The low speed supernatant was extracted with TX-114 followed by phase separation. α-agglutinin was immunoprecipitated from the aqueous phase (A) and the detergent phase (D) of TX-114, and analyzed by SDS-PAGE and fluorography. Tunicamycin was added 30 min prior to the labeling. **C.** Separation of membrane-bound and soluble α-agglutinin by ultracentrifugation. The low speed supernatant from cells labeled in the absence of tunicamycin was centrifuged at 100,000 x g for 1 hr at 4°C. The membrane pellet was dissolved in SDS, and α-agglutinin was immunoprecipitated from the supernatant (S) and the SDS extract of the membranes (P). **D.** Na_2CO_3 treatment of the membranes. The low speed supernatant was treated with (+) or without (-) 0.1 M Na_2CO_3, pH 11 for 30 min on ice before cell membranes were pelleted by ultracentrifugation. α-agglutinin was immunoprecipitated from the treated and untreated membranes, and analyzed by SDS-PAGE and fluorography.


A
>300 kD -
140 kD -
phase A D
B
- 200 kD
- 125 kD
phase A D
C
>300 kD -
140 kD -
fraction P S
D
>300 kD -
140 kD -
Na2CO3 + -
(pH 11)

(Jones 1991). The *kex2* mutant is deficient in a Golgi-associated protease that cleaves some protein precursors (Wilcox and Fuller 1991). Because the ratio of the soluble to the membrane-bound >300 kD α-agglutinin in these protease-deficient mutants is comparable to that in wild type strains, these proteases are not involved in the production of the soluble >300 kD form from the membrane-bound >300 kD form or the cell wall-bound form of α-agglutinin either *in vivo* or during cell lysis.

The membrane-bound 140 kD and >300 kD forms of α-agglutinin were not removed from the membranes by treatment with 0.1 M Na_2CO_3, pH 11, which strips peripheral membrane proteins and breaks membrane vesicles open (Fig. 7D) (Fujiki et al. 1982). This result indicates that these two forms of α-agglutinin are tightly membrane-bound.

GPI-linkage to the membrane-bound forms of α-agglutinin

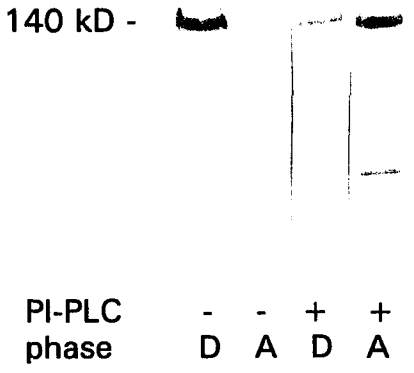
To determine whether membrane-association of the 140 kD and >300 kD forms of α-agglutinin was mediated by a GPI anchor, the susceptibility of these proteins to cleavage by PI-PLC, which cleaves the GPI anchor between inositol phosphate and diacylglycerol, was first tested. Membrane proteins contained in the TX-114 detergent phase were treated with PI-PLC from *Bacillus thuringiensis*, and the phases were separated after the enzyme treatment. PI-PLC released the 140 kD form of α-agglutinin from the detergent phase into the aqueous phase of TX-114, whereas in PI-PLC mock treatment, this protein remained associated with the detergent (Fig. 8A). The 140 kD form was quantitatively released into the aqueous phase with increasing amount of PI-PLC (data not shown).

An alternative approach was used to determine the sensitivity of the membrane-

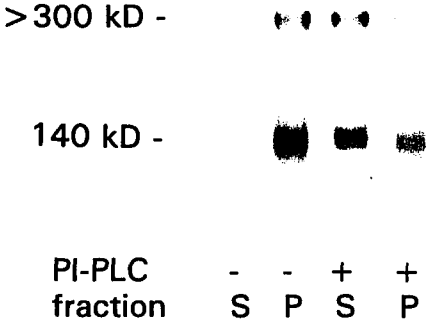
Figure 8. Cleavage of membrane-bound forms of α-agglutinin by PI-PLC.

A. Membrane proteins from pAGα1'-transformed NY432 cells (grown at 24°C) were prepared in the detergent phase of TX-114, and treated with PI-PLC from *B. thuringiensis* (+) or mock treated with the enzyme (-). After treatment, phases were separated, and proteins in the aqueous (A) and detergent (D) phase of TX-114 were precipitated by TCA and analyzed by immunoblotting using anti-α-agglutinin. **B.** The membrane pellet prepared from [^{35}S]methionine-labeled W303-1B[pAGα1'] cells was resuspended in lysis buffer, treated with (+) or without (-) PI-PLC. After treatment, the membranes were resedimented by ultracentrifugation, and α-agglutinin was immunoprecipitated from the supernatant (S) and the SDS extract of membranes (P), and analyzed by SDS-PAGE and fluorography.

A



B

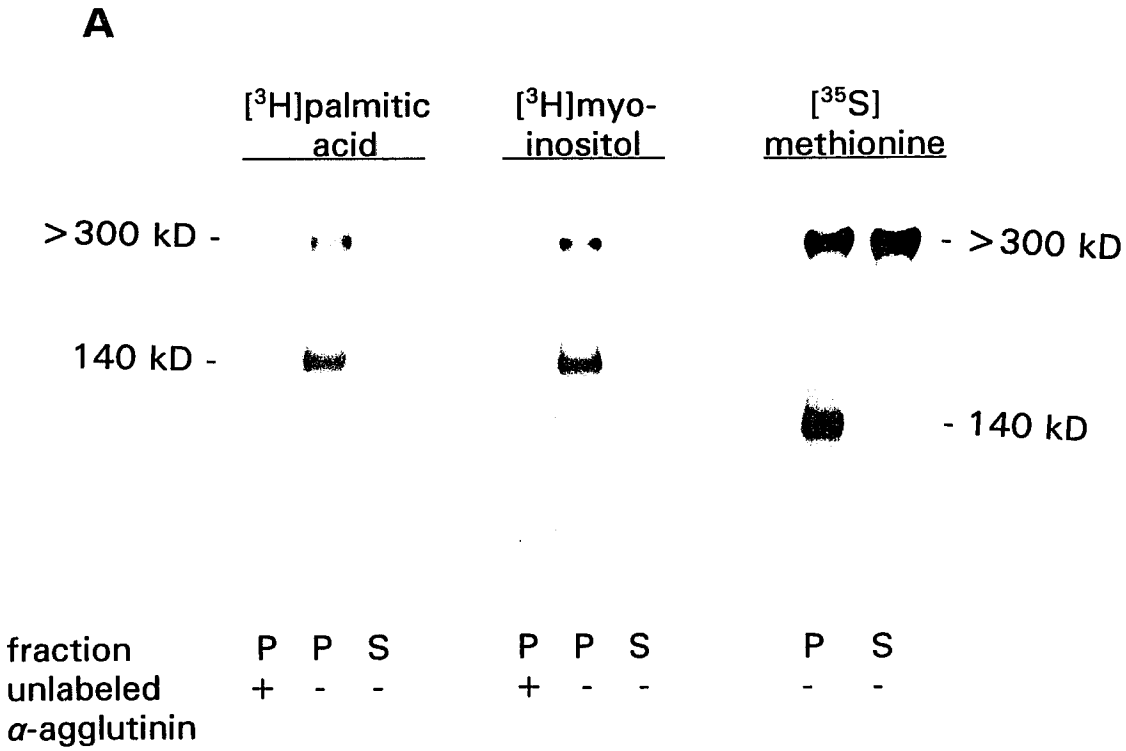


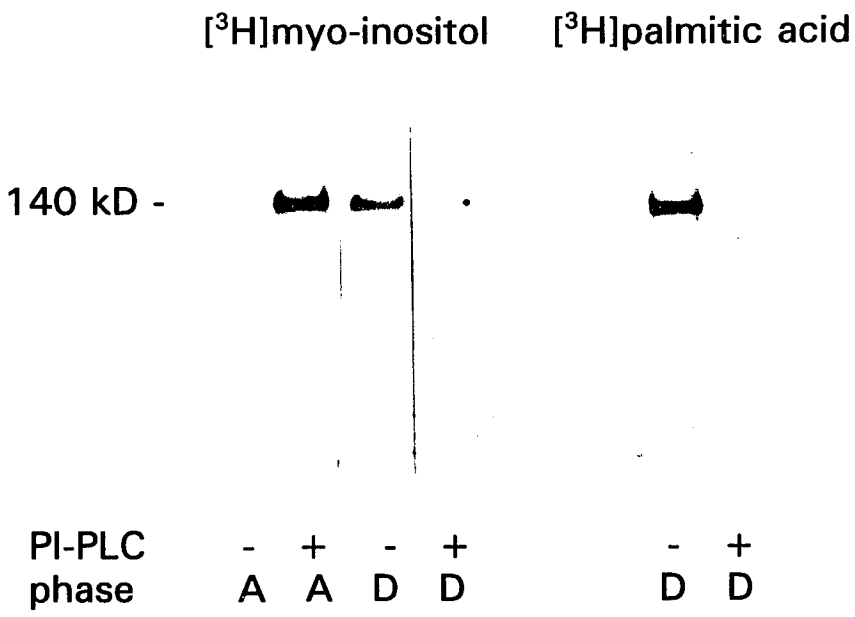
bound >300 kD form of α-agglutinin to PI-PLC cleavage. Cell membranes were pelleted and resuspended, and then treated with PI-PLC. After the treatment, membranes were resedimented by ultracentrifugation. The majority of the 140 kD and the >300 kD proteins were released from the membranes to the soluble fraction by PI-PLC treatment (Fig. 8B). No apparent molecular weight reduction of the PI-PLC released forms was observed (Fig. 8A and 8B).

To determine whether the 140 kD and >300 kD membrane-bound forms of α-agglutinin contain inositol and fatty acid, which are components of the GPI anchor (Fig. 4), cells were metabolically labeled with [^{3}H]myo-inositol or [^{3}H]palmitic acid, and cell membrane and soluble protein fractions were separated by ultracentrifugation. Both [^{3}H]myo-inositol and [^{3}H]palmitic acid labeled 140 kD and >300 kD forms of α-agglutinin were immunoprecipitated from the membrane fractions (Fig. 9A). Preincubation of the antiserum with purified unlabeled α-agglutinin inhibited the precipitation of labeled α-agglutinin from the membrane fractions in both labelings. These results indicate that the membrane-bound 140 kD and >300 kD forms of α-agglutinin are covalently attached by both inositol and palmitic acid. Consistent with the presence of a GPI anchor, the PI-PLC released 140 kD form was still associated with the [^{3}H]myo-inositol label (Fig. 9B), whereas the [^{3}H]palmitic acid label was greatly decreased by PI-PLC treatment. These results demonstrate a covalent attachment of a GPI anchor to the 140 kD and >300 kD membrane-bound forms of α-agglutinin.

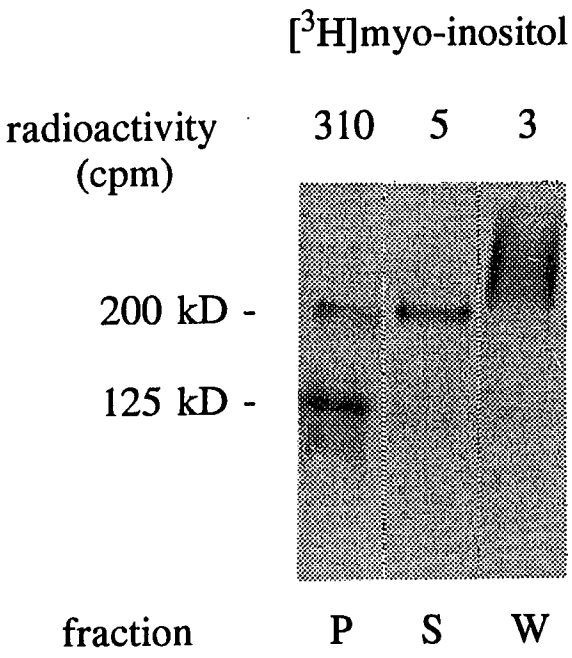
In contrast to the membrane-bound forms, no labeled α-agglutinin was immunoprecipitated from the soluble fraction of either the [^{3}H]myo-inositol or [^{3}H]palmitic acid labelings (Fig. 9A). This fraction contained about 60% of the total

Figure 9. Incorporation of [^{3}H]myo-inositol and [^{3}H]palmitic acid into the membrane-bound forms of α-agglutinin. A. W303-1B[pAGα1'] cells were metabolically labeled with [^{3}H]myo-inositol, [^{3}H]palmitic acid, or[^{35}S]methionine, and lysed in lysis buffer. After cell walls were removed, the lysate was fractionated into the supernatant (S) and membrane pellet (P) by ultracentrifugation. α-agglutinin was immunoprecipitated, and analyzed by SDS-PAGE and fluorography. **B.** Membrane proteins from [^{3}H]myo-inositol or [^{3}H]palmitic acid labeled cells were prepared in TX-114 detergent phase, and treated with (+) or without (-) PI-PLC. The aqueous (A) and detergent (D) phases were separated after the treatment, and α-agglutinin was immunoprecipitated, electrophoresed, and fluorographed. **C.** [^{3}H]myo-inositol-labeled W303-1B[pAGα1'] cells were lysed and fractionated into cell walls (W), membranes (P) and soluble fraction (S). Cell walls were treated with laminarinase. α-agglutinin was immunoprecipitated from each fraction, treated with Endo H, and analyzed by immunoblotting to determine the protein level of various forms of α-agglutinin. The radioactivity contained in the samples loaded in each lane is indicated on the top of the lane. The 140 kD form and the >300 kD forms were reduced to 125 kD and 200 kD, respectively, after Endo H treatment (also see Fig. 15). When the α-agglutinin immunoprecipitate from the laminarinase extract of cell walls was processed for fluorography, no labeled α-agglutinin was seen after exposure for 2 months.


A
[3H]palmitic acid
[3H]myo-inositol
[35S] methionine
>300 kD -
- >300 kD
140 kD -
- 140 kD
fraction P P S P P S P S
unlabeled + - - + - - - -
α-agglutinin


B
[3H]myo-inositol
[3H]palmitic acid
140 kD -
PI-PLC - + - + - +
phase A A D D D D

C


[3H]myo-inositol
radioactivity (cpm)
310
5
3
200 kD -
125 kD -
fraction
P
S
W

>300 kD α-agglutinin as determined by [^{35}S]methionine labeling (Fig. 9A) or by immunoblottig (Fig. 9C). Similarly, the laminarinase extracted cell wall-bound form of α-agglutinin was not labeled by either the [^{3}H]myo-inositol or [^{3}H]palmitic acid (data not shown). It appears, therefore, that the soluble >300 kD form and the cell wall form of α-agglutinin do not have an intact GPI anchor.

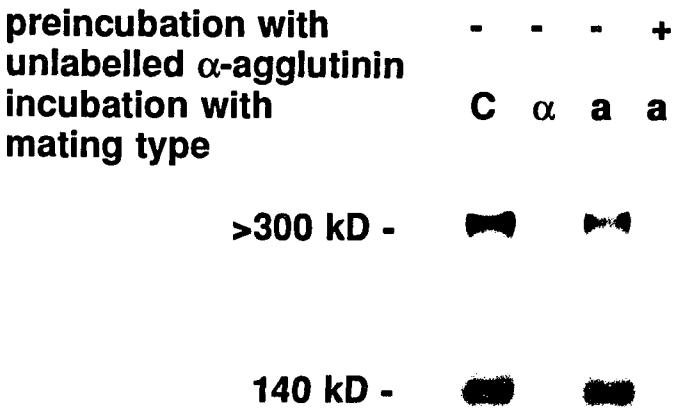
Biological activity of the membrane-bound forms of α-agglutinin

Secreted proteins are folded and assembled in the ER shortly after synthesis and translocation (Pelham 1989). If the GPI-anchored 140 kD and >300 kD proteins are intermediates in the maturation of α-agglutinin, they should be properly folded and might exhibit biological activity. α-agglutinin activity is monitored by binding to **a** cells which express the **a**-agglutinin (Terrance and Lipke 1981; Terrance et al. 1987). To test for activity, [^{35}S]methionine-labeled GPI-anchored forms of α-agglutinin were released from the membranes by PI-PLC and incubated with **a** or α cells and then eluted from the cells with SDS. The labeled 140 kD and >300 kD proteins were able to bind to **a** cells but not to α cells (Fig. 10). The binding of both proteins to **a** cells was competed by unlabeled α-agglutinin. This selective binding indicates that both forms of α-agglutinin are biologically active. The activity of the 140 kD form of α-agglutinin is consistent with the previous finding that complete N-linked oligosaccharide chains are not required for activity of α-agglutinin (Terrance et al. 1987).

Cellular location of the membrane-bound and soluble forms of α-agglutinin

With some exceptions, GPI-anchored proteins are attached to the plasma membrane (Cross 1990; Englund 1993). To test the location of the GPI-anchored 140 kD and >300 kD forms, and the soluble >300 kD form of α-agglutinin, the

Figure 10. a cell-specific binding of the membrane-bound 140 kD and >300 kD forms of α-agglutinin. Membrane pellet prepared from [^{35}S]methionine-labeled W303-1B[pAGα1'] cells was resuspended and treated with PI-PLC. The PI-PLC released labeled proteins were incubated with unlabeled **a** or α cells. After incubation, the cells were collected, and the bound labeled proteins were released from the cells, immunoprecipitated using anti-α-agglutinin, and analyzed by SDS-PAGE and fluorography. C, labeled α-agglutinin before incubation with cells.


preincubation with unlabelled α-agglutinin
- - - +
incubation with mating type
C α a a
>300 kD -
140 kD -

susceptibility of these forms to exogenous protease treatment of intact cells was determined. Cells were pretreated with DTT to facilitate access of exogenous protease to the plasma membrane, and then incubated with different concentrations of proteinase K. The treated cells were lysed, and fractionated into cell walls, membranes and soluble protein fractions. α-agglutinin was immunoprecipitated from each fraction. The level of a cytoplasmic enzyme, glucose-6-phosphate dehydrogenase (G-6-PDH), was also determined to monitor plasma membrane integrity. This enzyme was sensitive to proteinase K digestion (data not shown). With increasing proteinase K concentration, the cell wall form of α-agglutinin progressively disappeared (Fig. 11). Both the membrane-bound and the soluble >300 kD forms were converted to lower molecular weight species at low proteinase K concentrations and decreased in level at higher concentrations. In contrast, the level of the 140 kD α-agglutinin was not altered by increasing proteinase K concentration. Similarly, there was no reduction in the level of glucose-6-phosphate dehydrogenase, indicating that the plasma membrane of the protease-treated cells remained intact and impermeable to the protease. These results indicate that the 140 kD form occurs intracellularly, the membrane-bound >300 kD form is attached to the external face of the plasma membrane, and the soluble >300 kD form has properties of a periplasmic protein.

Kinetics of α-agglutinin secretion

A pulse-chase experiment was performed to determine the temporal relationship among various forms of α-agglutinin. Cells were pulse-labeled with [^{35}S]methionine and chased with excess unlabeled methionine, cysteine, and sulfate. The cells were lysed, and cell walls, membranes and soluble fractions were prepared and α-agglutinin was

Figure 11. Proteinase K treatment of intact cells. [^{35}S]methionine labeled W303-1B cells were pretreated with DTT and incubated with different concentrations of proteinase K. Cells were then lysed in lysis buffer, and fractionated into cell walls, membranes and soluble protein fractions. The cell walls were subsequently treated with laminarinase. α-agglutinin was immunoprecipitated from each fraction. Glucose-6-phosphate-dehydrogenase (G-6-PDH) was precipitated from the soluble fraction with anti-G-6-PDH (Sigma). The immunoprecipitates were analyzed by SDS-PAGE and fluorography. P: membrane-bound forms of α-agglutinin; S: soluble $>$300 kD form.

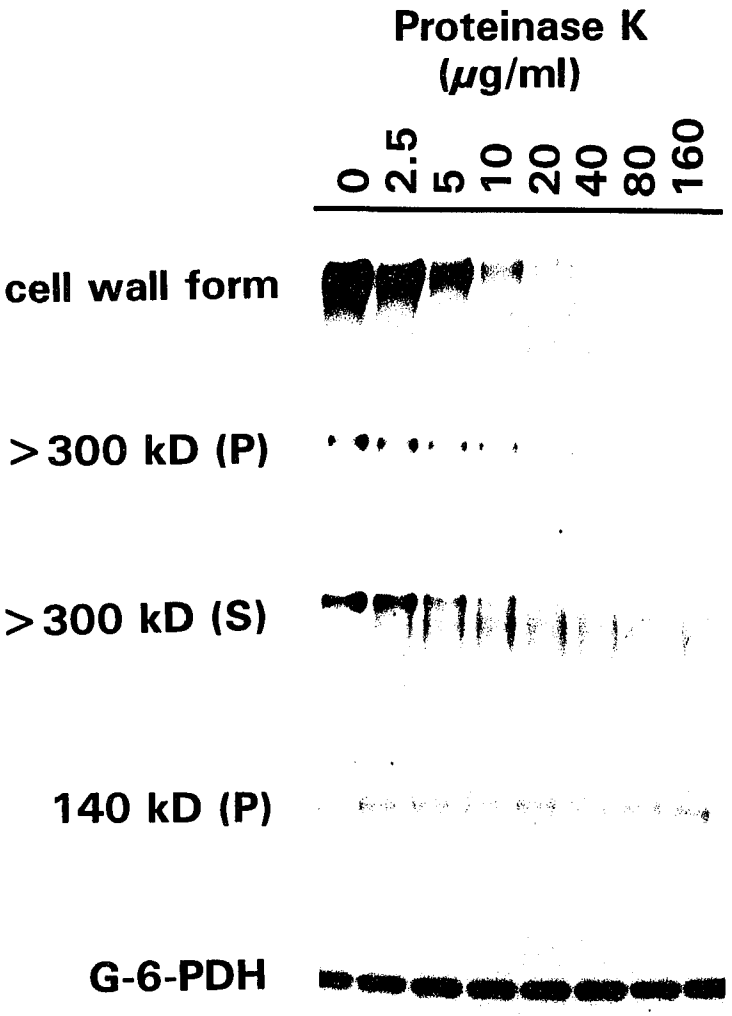
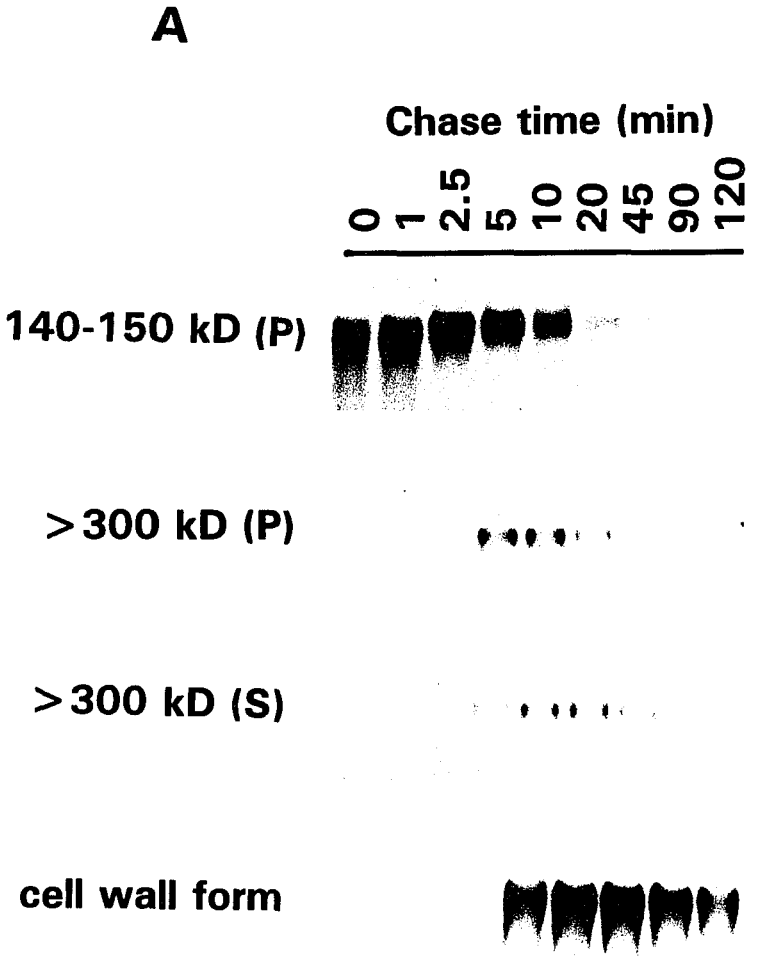
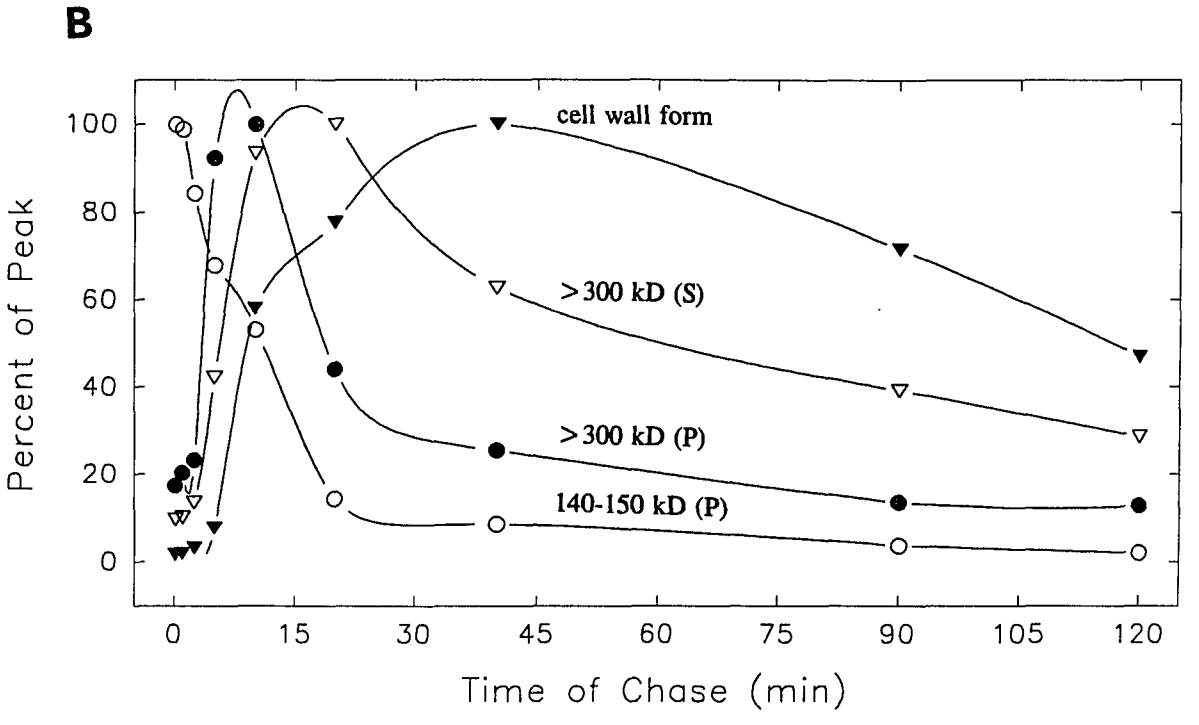

Proteinase K
(μg/ml)
0
2.5
5
10
20
40
80
160
cell wall form
>300 kD (P)
>300 kD (S)
140 kD (P)
G-6-PDH

Figure 12. Pulse-chase analysis of α-agglutinin maturation. A. W303-1B[pAGα1'] cells were pulse-labeled with [^{35}S]methionine for 2 min, and chased by addition of excess unlabeled methionine, cysteine and sulfate. Aliquots of cells were harvested at the indicated times, lysed in lysis buffer, and fractionated into cell walls, membranes and soluble protein fractions. α-agglutinin was immunoprecipitated from each fraction, and analyzed by SDS-PAGE and fluorography. P: membrane-bound forms; S: soluble $>$300 kD form. **B.** Quantitative analysis of pulse-chase results from (**A**). α-agglutinin bands were excised from the dried gels and radioactivity was determined by liquid scintillation counting. The radioactivity in each form was normalized to the maximal counts in that fraction. The maximal counts were 2800 cpm for the 140-150 kD form (o), 590 cpm for the membrane-bound $>$300 kD (●), 308 cpm for the soluble $>$300 kD form (△), and 2080 cpm for the cell wall form (▲).


A
Chase time (min)
0
1
2.5
5
10
20
45
90
120
140-150 kD (P)
>300 kD (P)
>300 kD (S)
cell wall form

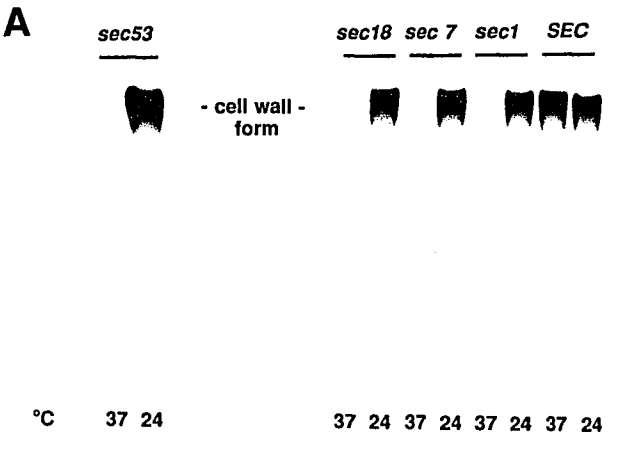

B
Percent of Peak
100
80
60
40
20
0
0
15
30
45
60
75
90
105
120
Time of Chase (min)
cell wall form
>300 kD (S)
>300 kD (P)
140-150 kD (P)

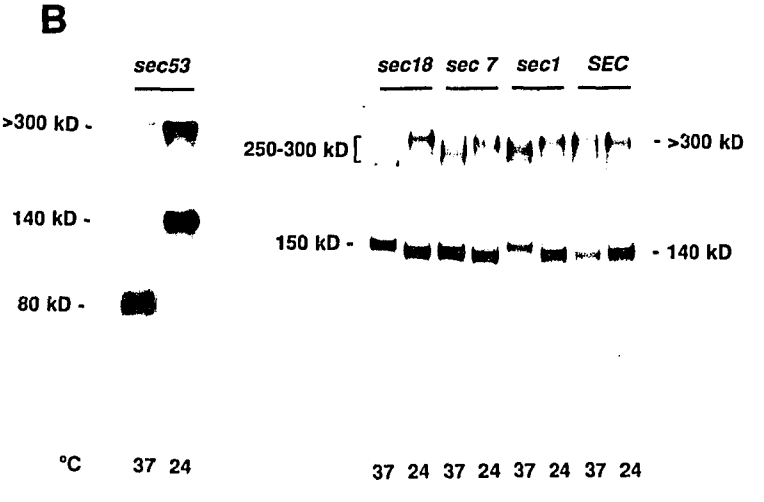
immunoprecipitated from each fraction. The 140 kD form of α-agglutinin was present immediately following the pulse. This form gradually increased its molecular size to 150 kD before disappearance by 20 min of chase (Fig. 12). The membrane-bound >300 kD form of α-agglutinin peaked at 5-10 min, and gradually disappeared. The soluble >300 kD form appeared by 5 min, reached a maximal level at 10-20 min, and gradually decreased. The cell wall form of α-agglutinin was first detected at 10 min, reached a maximal level by about 45 min. These kinetic results are consistent with the non-cell wall forms of α-agglutinin being intermediates of the mature cell wall anchored α-agglutinin.

Additional forms of α-agglutinin in *sec* mutants

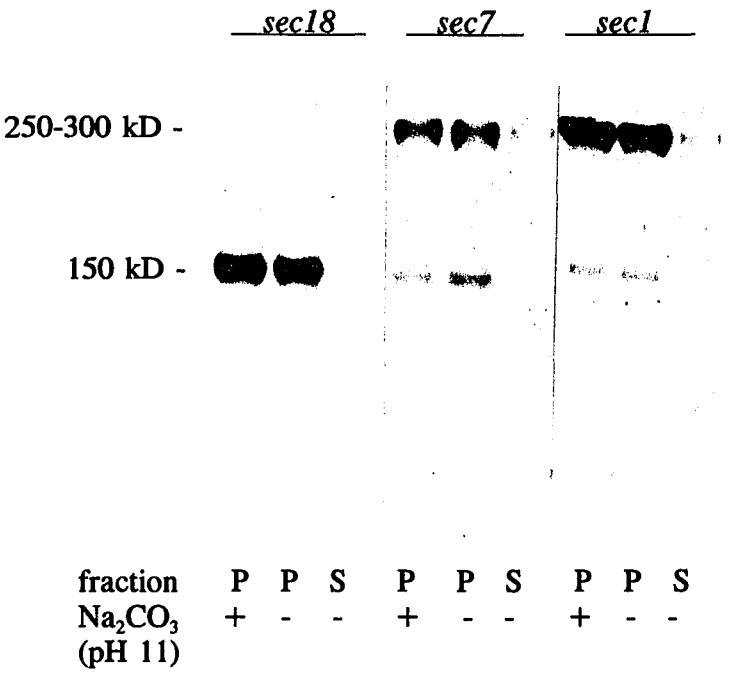
To identify additional intermediates in the transport and cell wall anchorage of α-agglutinin, several temperature-sensitive secretory mutants were tested. *sec53* mutants have temperature-sensitive mutations in phosphomannomutase, resulting in an inability to incorporate N- or O-linked saccharides or GPI anchors into glycoproteins at the restrictive temperature (Kepes and Schekman 1988; Conzelmann et al. 1990). *sec18* mutants were initially shown to be blocked in ER to Golgi transport (Novick and Schekman 1983; Kaiser and Schekman 1990; Graham and Emr 1991); more recent studies have identified a defect in NSF, a protein required for vesicle fusion, resulting in blockage in a pre-Golgi compartment (Eakle et al. 1988; Wilson et al. 1989). *sec7* mutations block protein transport from the Golgi apparatus, and the *sec1* protein is required for the fusion of the secretory vesicles with the plasma membrane (Novick et al. 1980; Novick and Schekman 1983; Esmon et al. 1981; Schekman and Novick 1982;

Figure 13. α-agglutinin forms accumulated in *sec* mutants. *sec* mutants and the wild-type strain (NY191) were preincubated at 24°C or 37°C for 30 min and were retained at the same temperature during labelling with [^{35}S]methionine. Labeled cells were lysed in SDS lysis buffer, and cell walls were separated from the SDS extracts by spinning at 13,600 x g for 5 min. α-agglutinin was immunoprecipitated from the laminarinase extracts of the cell walls or from the SDS extracts of the cells, separated by SDS-PAGE and visualized by fluorography. **A.** Laminarinase extracted cell wall α-agglutinin. **B.** SDS extracted forms of α-agglutinin from cell lysates. In both **A** and **B**, α-agglutinin from *sec53* cells was separated on 6% SDS polyacrylamide gels, and α-agglutinin from other strains was separated on 5% SDS gels. **C.** Membrane association of α-agglutinin forms from *sec* mutants. *sec* mutants were labeled with [^{35}S]methionine at restrictive temperature (37°C) and broken in lysis buffer with glass beads. The lysates were treated with (+) or without (-) 0.1 M Na_2CO_3, pH 11 for 30 min on ice, and the membranes were pelleted by ultracentrifugation. α-agglutinin in the membrane pellet (P) and in the supernatant (S) was immunoprecipitated and analyzed by SDS-PAGE and fluorography.


A
sec53
sec18
sec 7
sec1
SEC
- cell wall - form
°C
37 24
37 24 37 24 37 24 37 24


B
sec53
sec18
sec 7
sec1
SEC
>300 kD -
140 kD -
80 kD -
250-300 kD [
- >300 kD
150 kD -
- 140 kD
°C
37 24
37 24 37 24 37 24 37 24

C


sec18
sec7
sec1
250-300 kD -
150 kD -
fraction P P S P P S P P S
Na_2CO_3 + - - + - - + - -
(pH 11)

Franzusoff and Schekman 1989). These *sec* mutants were tested for blockage in α-agglutinin secretion. Incubation at the restrictive temperature (37°C) blocked transport of α-agglutinin to the cell wall in all four *sec* mutants (Fig. 13A), whereas the transport was not temperature-dependent in the wild-type strain.

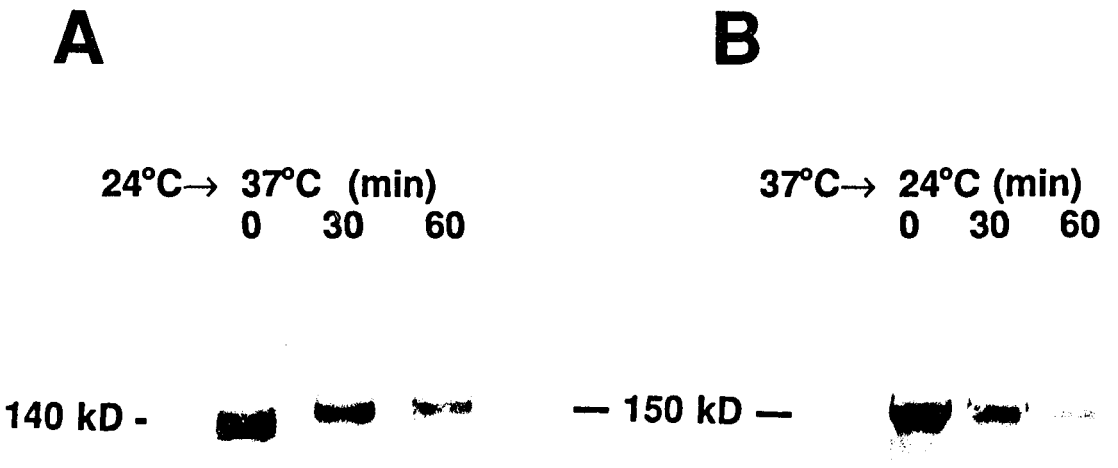
The 140 kD and the >300 kD forms of α-agglutinin present in wild-type cells were extracted by SDS from all four *sec* mutants at the permissive temperature (24°C) (Fig. 13B). However, incubation of the *sec* mutants at the restrictive temperature identified alternative α-agglutinin bands in the SDS extracts (Fig. 13B). The *sec53* mutant contained an 80 kD form of α-agglutinin. The *sec18* mutant showed a major 150 kD form and a minor 250 kD form. The *sec7* mutant showed the 140 kD form and an additional form of 250-300 kD. The *sec1* mutant accumulated the major 250-300 kD form and the minor 150 kD form. Accumulation of these forms in *sec* mutants suggests that they could be intermediates in transport of α-agglutinin.

The 150 kD form observed in the *sec18* mutant and the majority of the 250-300 kD form accumulated in the *sec7* and *sec1* mutants at restrictive temperature were sedimented with cell membranes, and were not removed from the membrane fraction by treatment with 0.1 M Na_2CO_3, pH 11, indicating that they are tightly membrane-bound (Fig. 13C).

Relationship of the 140 kD and 150 kD forms of α-agglutinin

In the pulse-chase experiment, the 140 kD form of α-agglutinin gradually increased its molecular size to 150 kD before disappearance (Fig. 12), suggesting that the 140 kD form is a precursor of the 150 kD form. To further investigate the

Figure 14. Processing of the 140 kD and 150 kD forms of α-agglutinin in a *sec18* mutant. A. *sec18* cells containing pAGα1' were grown at 24° C and shifted to 37°C in the presence of 100 ug/ml of cycloheximide. **B.** *sec18* cells were incubated at 37°C for 2 hours and then shifted back to 24°C in the presence of cycloheximide. In both **A** and **B**, at the indicated times, aliquots of cells were harvested and lysed in lysis buffer. the lysates were extracted with Triton X-114 followed by phase separation. Membrane proteins in the detergent phase of TX-114 were precipitated by trichloroacetic acid and analyzed by immunoblotting using α-agglutinin antiserum.

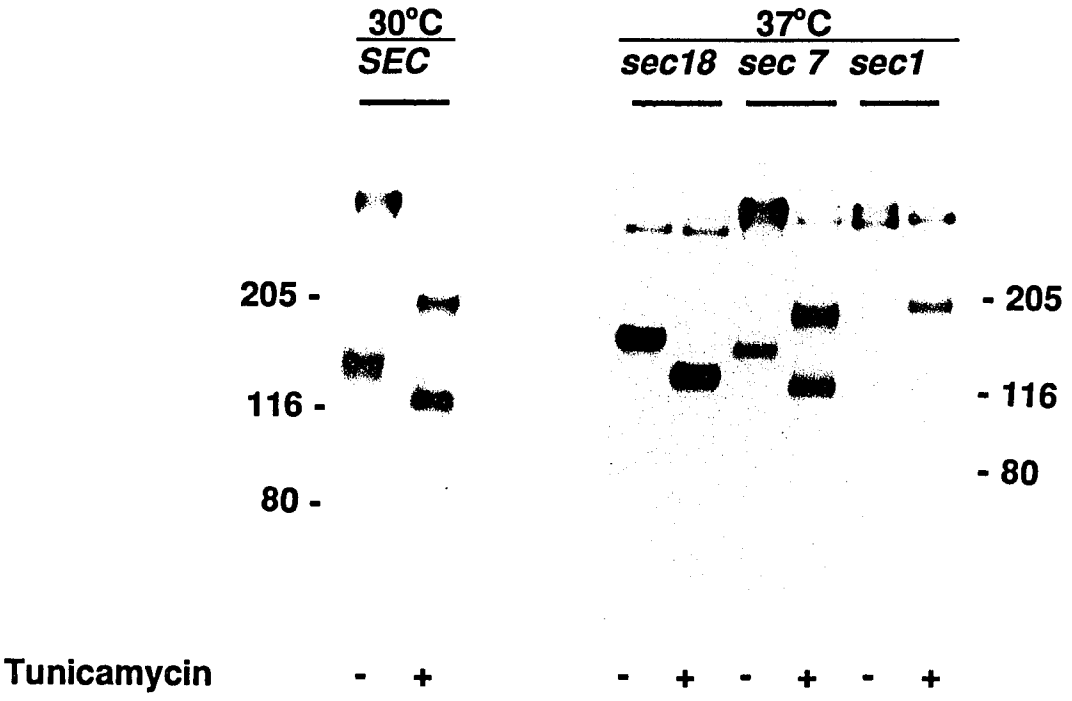

A
B
24°C→ 37°C (min)
0 30 60
37°C→ 24°C (min)
0 30 60
140 kD -
— 150 kD —

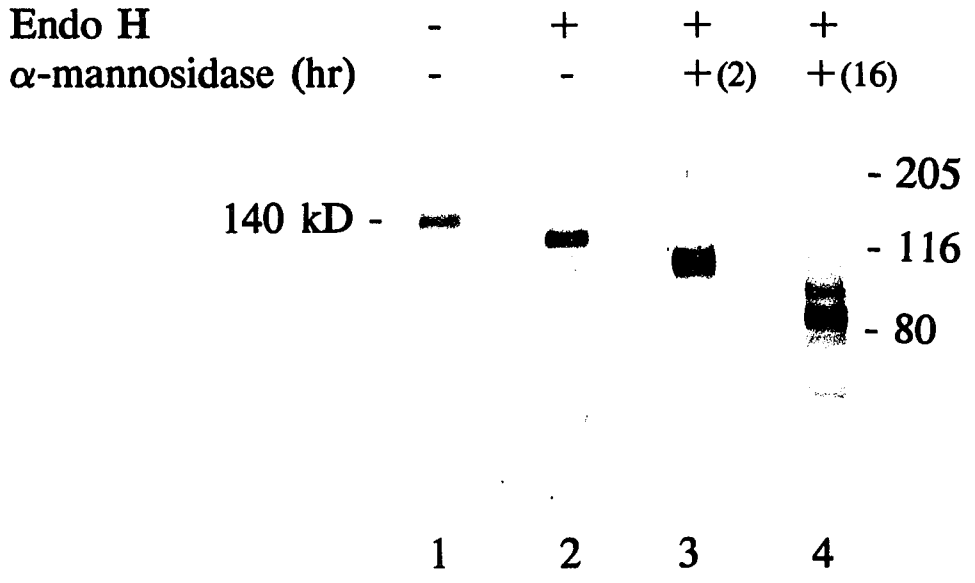
relationship of these two forms, a temperature shift experiment was carried out using a *sec18* mutant that accumulates the 150 kD form at the restrictive temperature. *sec18* cells were shifted from the permissive temperature (24°C) to the restrictive temperature (37°C) in the presence of cycloheximide to inhibit new protein synthesis. The 140 kD form present at permissive temperature was converted to the 150 kD form within half an hour after the temperature shift (Fig. 14A). In a reciprocal experiment, *sec18* cells were shifted from the restrictive to the permissive temperature in the presence of cycloheximide. The 150 kD form of α-agglutinin gradually disappeared without appearance of the 140 kD form (Fig. 14B). These results indicate that the 140 kD form of α-agglutinin is a precursor of the 150 kD form, and that the conversion to 150 kD form occurs in the secretory pathway before the *sec18* block.

Glycosylation of α-agglutinin

N-glycosylation of the non-cell wall forms of α-agglutinin was investigated by labeling wild-type cells or *sec* mutants in the presence of tunicamycin. The molecular sizes of the 140 kD and >300 kD forms of α-agglutinin were decreased to 125 kD and 200 kD, respectively, in the presence of tunicamycin (Figs. 15A and 7B). Digestion of with Endo H resulted in similar shifts in mobility for both forms (Figs. 9C, and 15B). Tunicamycin treatment of *sec* mutants at the restrictive temperature resulted in a shift of the 150 kD form in *sec18* cells to 135 kD and of the 140 kD minor form in *sec7* to 125 kD (Fig. 15A). The majority (>90%) of the 250-300 kD form in *sec7* and *sec1* mutants shifted to about 200 kD, with the protein from tunicamycin-treated *sec7* cells migrated slightly faster than that from *sec1* (Fig. 15A). Each of the *sec* mutants also

Figure 15. Analysis for glycosylation of α-agglutinin. A. *sec* mutants and the wild-type strain were labeled with [^{35}S]methionine at the indicated temperature in the absence (-) or presence (+) of tunicamycin (15 ug/ml), and lysed in SDS. α-agglutinin was immunoprecipitated from SDS extracts of the cells and analyzed by SDS-PAGE and fluorography. Tunicamycin was added 30 min prior to labelling. **B.** Membrane proteins in the detergent phase of TX-114 were treated with PI-PLC. The PI-PLC released proteins (lane 1) were treated with Endo H for 2 hr at 37°C (lane 2). The Endo H treated sample was treated with α-mannosidase for 2 hr at 25°C (lane 3) or for 16 hr at 25°C (lane 4). Proteins in the Endo H treated or Endo H and α-mannosidase double treated samples were precipitated with TCA and analyzed by immunoblotting using anti-α-agglutinin. Molecular size standards on the right are indicated in kD.


30°C
SEC
37°C
sec18
sec 7
sec1
205 -
116 -
80 -
- 205
- 116
- 80
Tunicamycin
- + - + - + - + - +


Endo H - + + +
α-mannosidase (hr) - - +(2) +(16)
140 kD -
- 205
- 116
- 80
1 2 3 4

showed minor high molecular weight bands in the presence of tunicamycin; these minor high molecular size materials were also resistant to Endo H digestion (data not shown). It is not known whether these tunicamycin/Endo H resistant bands represent additional forms of α-agglutinin or non-glycosylated proteins coimmunoprecipitated with the α-agglutinin. The size shifts in the presence of tunicamycin indicate that these non-cell wall forms of α-agglutinin are N-glycosylated.

To test whether α-agglutinin contains O-linked carbohydrates, the 140 kD form of α-agglutinin (Fig. 15B, lane 1) was treated with Endo H, resulting in the 125 kD form (lane 2), which was further treated with α-mannosidase. A 110 kD band was observed after treatment with α-mannosidase for 2 hr (lane 3). Extended incubation with α-mannosidase (16 hr) resulted in a major band of 85 kD, and two minor bands of 93 kD and 78 kD (lane 4); all of these three bands bound to concanavalin A, which has affinity for terminal mannose and glucose residues (data not shown). It is likely that these three bands resulted from incomplete removal of mannose residues from the protein, as α-mannosidase mediated proteolysis towards [^{14}C]-BSA was not detectable (data not shown). These results suggest that the α-agglutinin is also O-glycosylated.

DISCUSSION

The mechanism for cell wall attachment of *S. cerevisiae* cell adhesion glycoprotein α-agglutinin is not well understood. The results presented in this section show that the mature wall-bound α-agglutinin is synthesized with a glycosyl phosphatidylinositol (GPI) membrane anchor. GPI anchorage may provide a mechanism for proper cell wall localization of this protein.

Covalent cell wall association of the mature α-agglutinin

As stated in section I, it is unlikely that the mature cell wall α-agglutinin is attached to the plasma membrane. Several investigators have suggested that the cell wall α-agglutinin is associated with ß-glucan, based on the observation that this protein can be released from intact cells or from isolated cell walls by treatment with ß-glucanases (Hauser and Tanner 1989; Lasky and Ballou 1988; Schreuder et al. 1993), although proteolytic release mediated by contaminating proteases can not be ruled out (Zlotnik et al. 1984). Laminarinase, a preparation of ß-1,3-glucanase which did not contain detectable protease activity or GPI-cleaving activity, released a form of α-agglutinin with molecular weight of >300 kD from isolated cell walls of α strains (Fig. 5). The laminarinase-released wall α-agglutinin was not extractable by hot SDS, or by DTT treatment (data not shown) which removes disulfide-bonded wall mannoproteins and therefore increases cell wall permeability, resulting in the release of some soluble mannoproteins physically trapped within the wall (Esmon et al. 1987; De Nobel et al. 1989). Neither the inositol nor the fatty acid label was detected in the laminarinase-extracted cell wall α-agglutinin (data not shown). These results further demonstrate that

the mature α-agglutinin is covalently bound to the cell wall rather than attached to the plasma membrane by a GPI anchor.

Membrane association of some forms of α-agglutinin by a GPI anchor

Two membrane-bound forms of α-agglutinin with molecular sizes of apparent 140 kD and >300 kD, respectively, were identified in wild-type α strains. These forms were not removed from the membranes by treatment with 0.1 M Na_2CO_3, pH 11, but were quantitatively released from the membranes by treatment with PI-PLC (Figs. 7 and 8). Metabolical labeling experiments showed that these proteins could be labeled with both [^{3}H]myo-inositol and [^{3}H]palmitic acid; after PI-PLC treatment, the [^{3}H]myo-inositol label remained with the proteins while the [^{3}H]palmitic acid label was lost (Fig. 9A and 9B). These findings indicate that the 140 kD and >300 kD membrane-bound forms of α-agglutinin are covalently attached by an inositol-containing phospholipid, consistent with the presence of a GPI anchor.

Conzelmann et al (1992) have reported that two different types of lipid moieties are present in yeast GPI-anchored proteins: diacylglycerol and ceramide. It appears that proteins are first attached to a diacylglycerol-containing GPI anchor, and the lipid moiety is subsequently exchanged for a ceramide. It will be interesting to know whether the lipid moiety is different in the 140 kD ER form and the >300 kD plasma membrane form of α-agglutinin, as this will reveal whether the lipid is remodeled during transport from the ER to the cell surface. Materials have been sent to Conzelmann to try to determine the lipid composition of the α-agglutinin GPI anchor.

A pathway for processing of α-agglutinin

In addition to the GPI-anchored forms, a soluble >300 kD form was detected in

all tested wild-type α strains. Additional forms were identified in *sec* mutants at restrictive temperature. A proposed pathway for α-agglutinin processing is shown in Figure 16, and the experimental bases are summarized and discussed below.

The earliest proposed α-agglutinin intermediate was seen in a *sec53* mutant at restrictive temperature (Fig. 13). This intermediate had molecular size of 80 kD, and is likely to represent the unmodified α-agglutinin polypeptide, as *sec53* mutants block addition of O- and N-linked oligosaccharides and GPI anchors to proteins at restrictive temperature (Kepes and Schekman 1988; Conzelmann et al, 1990). The discrepancy between 80 kD and the predicted molecular size of α-agglutinin peptide (70 kD) is most likely due to anomalous migration of Ser- and Thr-rich proteins on SDS-polyacrylamide gel system (Early 1988). An α-agglutinin fusion protein expressed in *E. coli* shows a molecular size of 80 kD on SDS-gels rather than 70 kD as expected (Wojiechowicz 1990).

The 140 kD GPI-anchored form of α-agglutinin was observed in wild-type strains as well as *sec* mutants at the permissive temperature. The accumulation of this form implies that it is processed slowly and provides the rate-limiting step in the intracellular transport of α-agglutinin. This form shifted to 125 kD after Endo H treatment or after growth of cells in the presence of tunicamycin (Fig. 15), indicating that it contains 15 kD of N-linked carbohydrates. Extended α-mannosidase treatment further reduced the size of the 125 kD form to 85 kD (major band). Because O-glycosylation occurs shortly after translation and translocation in yeast, the weight removed by α-mannosidase could be O-linked mannose saccharides, as α-agglutinin contains 29% Ser and Thr, which are

potential O-glycosylation sites. Additional mannose residues linked to the core structure of the GPI anchor might also account for part of the weight removed by α-mannosidase (Fankhauser et al, 1993).

The *sec18* mutant accumulated a 150 kD membrane-bound form of α-agglutinin at the restrictive temperature (Fig. 13). The 150 kD form was susceptible to PI-PLC cleavage, and was labeled with [^{3}H]myo-inositol and [^{3}H]palmitic acid, indicating it is also GPI-anchored (data not shown). Results from the pulse-chase and temperature-shift experiments showed that the 140 kD form preceded the 150 kD form (Figs. 12 and 14). Because *sec18* blocks transport of proteins from the ER to the Golgi, the 140 kD form of α-agglutinin is likely to be modified to the 150 kD form within the ER. The size increase from 140 kD to 150 kD does not appear to include any increase in N- or O-linked carbohydrate content, as Endo H and α-mannosidase double treated products still showed a 10 kD difference in size (data not shown). The modification that causes the size increase from 140 kD to 150 kD is not known.

The *sec7* and *sec1* mutants accumulated a membrane-bound form of α-agglutinin with molecular weight of 250-300 kD at the restrictive temperature (Fig. 13). These mutations block protein transport at the level of the Golgi and secretory vesicles, respectively; therefore, this α-agglutinin form is proposed to occur in the Golgi and a post-Golgi compartment. As the 150 kD and the >300 kD forms are GPI-anchored, this form is proposed to contain a GPI anchor too (Fig. 16). Inhibition of N-glycosylation reduced the molecular size of the 250-300 kD form to about 200 kD (Fig. 15).

In addition to the 140 kD form of α-agglutinin, a >300 kD band was extracted from wild-type cells by SDS. This band was composed of a mixture of a GPI-anchored

membrane form and a soluble form (Figs. 6 and 7). The soluble >300 kD form was not labeled by [^{3}H]myo-inositol or [^{3}H]palmitic acid (Fig. 9), indicating that it was not associated with an intact GPI anchor. The loss of the GPI anchor from this form could result from proteolysis or from processing of the GPI anchor. Because neither protease mutations nor the presence of protease inhibitors in lysis buffer affected the production of this form, processing of the GPI anchor seems more likely. The absence of the >300 kD forms in the late *sec* mutant (*sec1)* at restrictive temperature suggests that they are produced in an extremely late intracellular compartment or at the cell surface. The susceptibility of the GPI-anchored >300 kD form to exogenous protease treatment of intact cells indicates that it is attached to the external face of the plasma membrane. The soluble >300 kD form had the properties of a periplasmic protein; it was sensitive to the exogenous protease digestion of intact cells (Fig. 11). The molecular size of both >300 kD forms was reduced to about 200 kD after the N-linked carbohydrates were removed (Figs. 9C and 15).

Kinetic analysis (Fig. 12) was consistent with an order of processing of: 140 kD → 150 kD → >300 kD GPI-anchored → >300 kD periplasmic → >300 kD cell wall forms of α-agglutinin. The 250-300 kD form was not detected in the kinetic analysis; therefore, it is possible that it corresponds to an aberrant product accumulated in *sec* mutants rather than an actual intermediate. The cell wall form appeared with kinetics suggesting that it corresponds to mature α-agglutinin (Terrance and Lipke 1981).

As α-agglutinin proceeds from the ER to the cell surface, the amount of N-linked carbohydrate increases (Fig. 16). However, the increase in N-linked carbohydrate content only partially accounts for the size increase from 140 kD to >300 kD. The

extension of the O-linked chains is most likely responsible for the increase from 125 kD to 200 kD. The modification that leads to the covalent wall association of α-agglutinin will be investigated in section III.

Because the mature α-agglutinin is cell wall-anchored, the GPI-anchored forms are likely to be intermediates in the cell wall localization of the glycoprotein. The role of the GPI anchor in cell wall anchorage of α-agglutinin will be explored and discussed in section III.

Figure 16. Pathway for processing of α-agglutinin. The molecular sizes of α-agglutinin forms and the N-linked carbohydrate contents are indicated. The *sec* mutants in which particular forms were detected at the restrictive temperature are indicated and the position in the secretory pathway at which the various forms are produced are inferred from the known position of the blocks in the *sec* mutants. The GPI-anchored 140 kD and >300 kD forms, the >300 kD soluble periplasmic form and the cell wall form were detected in wild-type strains and *sec* mutants at the permissive temperature. The 150 kD form accumulated in *sec18* at restrictive temperature was GPI-anchored. The 250-300 kD form detected in *sec7* and *sec1* at restrictive temperature was membrane-associated, and therefore, it is proposed to contain a GPI anchor. The periplasmic >300 kD form was not labeled by inositol or fatty acid, but could retain a portion of the GPI anchor that does not contain either of these labels. The cell wall form was covalently wall-bound, and solubilized by laminarinase treatment of the cell wall.

ER
Golgi and Secretory vesicles
Plasma Membrane
Periplasm
Cell wall
sec53
sec18
sec7,sec1
α-agglutinin form
80 kD
140 kD
GPI
150 kD
GPI
250-300 kD
(GPI)
>300 kD
GPI
>300 kD
?
>300 kD
CW
N-linked carbohydrate
15 kD
15 kD
50-100 kD
>100 kD
>100 kD

Section III

Extracellular Cross-linking between α-Agglutinin and ß-1,6-Glucan in *S. cerevisiae*

INTRODUCTION

The cell wall of *S. cerevisiae* is composed mainly of ß-glucan and mannoproteins, and a small amount of chitin (Ballou 1982; Cabib et al. 1982; Fleet 1991). However, little is known about how these wall components interact with each other to form the supramacromolecular structure of the cell wall. The cell adhesion glycoprotein α-agglutinin is covalently cell wall-bound (Fig. 5; Lasky and Ballou 1988; Weinstock and Ballou 1986; Hauser and Tanner 1989; Schreuder et al. 1993). This protein is synthesized, N- and O-glycosylated, and GPI-anchored in the ER. Subsequent passage through the Golgi apparatus to the cell surface is accompanied by elongation of N-linked, and probably O-linked carbohydrate chains. A >300 kD GPI-anchored plasma membrane form is seen about 5 min after the translation, followed by a >300 kD soluble form which has kinetic properties of a periplasmic intermediate between the plasma membrane-bound form and the mature cell wall-anchored α-agglutinin (Fig. 12, section II).

To explore the mechanism of cell wall anchorage of α-agglutinin, the possibility that this protein is cross-linked to the wall ß-1,6-glucan was tested. The results presented in this section show that the cell wall-bound form of α-agglutinin, but not the intermediate forms, is covalently associated with ß-1,6-glucan, and that the covalent association of these two components appears to be dependent on GPI anchor addition to the α-agglutinin. The cross-linkage of α-agglutinin and ß-1,6-glucan would provide a mechanism for covalent cell wall attachment of the glycoprotein.

MATERIALS AND METHODS

Strains. *S. cerevisiae KRE* gene disruption mutants *kre1::HIS3* (*MATα*), *kre6::HIS3* (*MATα*), *kre11::URA3 (MATα)* and their isogenic wild type strain SEY6210 (*MATα leu2-3,112 ura3-52 his3 lys2 trp1 suc2*) were provided by Dr. Howard Bussey. The *kre9::HIS3* strain (*MATα*) was obtained by random sporulation of a *kre9::HIS3* heterozygote strain on SEY6210 background (Spencer, 1988). The *kre9::HIS3* heterozygote strain was also provided by Dr. Howard Bussey. The *agα1-3* mutant (Lα21), which is isogenic to W303-1B (*MATα ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15*) was used to express pAGα1', pAGα1_{621*} and pAGα1_{635*}. The *AGα1* disruption mutant (*agα1::LEU2*), which was generated from W303-1B (Lipke et al. 1989), was used to express pAGα1_{621}'. X2180-1A (*MATa SUC2 mal mel gal2 CUP1*) was used as tester **a** strain in the agglutination assay.

Plasmids. The construction of pAGα1_{621*} and pAGα1_{635*} was described previously (Wojciechowicz et al. 1993). pAGα1_{621*} contains a truncation of the C-terminal 29 amino acids from *AGα1*; pAGα1_{635*} contains a replacement of the C-terminal hydrophobic sequence with a more hydrophilic sequence. The *AGα1* mutants were inserted in plasmid YEp351, which carries leucine selection marker. pAGα1_{621}' was constructed by subcloning the 4.5 kb HindIII-XbaI fragment from pAGα1_{621*} into plasmid YEp352, which has uracil selection marker. pAGα1', which contains the wild-type *AGα1*, was constructed on YEp352 (section II).

Isolation of secreted α-agglutinin from the growth medium. *agα1::LEU2* cells

expressing pAG$\alpha 1_{621}$' were grown in 300 ml leucine and uracil drop-out minimal medium at 30°C to stationary phase. The growth medium was then separated from the cells and freeze-dried. The dried material was resuspended in 5 ml of 10 mM Tris-HCl, pH 7.4, and dialyzed against 4 liters of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.1 mM PMSF overnight at 4°C. For isolation of α-agglutinin secreted by *kre* mutants, the labeling media (8 ml) were collected and dialyzed against the above buffer, and the dialysates were concentrated to 1.6 ml. α-agglutinin was immunoprecipitated from the concentrated, dialyzed media.

Immunoprecipitation. α-agglutinin was immunoprecipitated from the SDS extracts of cells or from the laminarinase extracts of cell walls as described in section II. For precipitating secreted α-agglutinin, the concentrated and dialyzed growth media were adjusted to 1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 30 ug/ml each of leupeptin, pepstatin, and antipain. To 800 ul of the material 5 ul anti-α-agglutinin antiserum (AG3) was added. The α-agglutinin immunoprecipitates were separated on 6% SDS-polyacrylamide gels, which were processed for flurography or immunoblotting.

Endo H treatment. After the immunoprecipitation, α-agglutinin was eluted from protein A-Sepharose beads with 0.1 M NaAc, pH 5.5, 3% 2-mercaptoethanol, and 0.4% SDS by boiling at 100°C for 5 min. 25 ul α-agglutinin eluate was diluted with Endo H buffer (20 mM NaAc, pH 5.5, 2 mM EDTA, 1 mM PMSF, and 30 ug/ml each of leupeptin, pepstatin, and antipain) to 100 ul. 8 ul Endo H (8 mU) was added and the mixture was incubated overnight at 35°C.

Immunoblotting. After separation on SDS-polyacrylamide gels, the α-agglutinin

was transferred to nitrocellulose membranes as described in section II. The membranes were blocked with 3% gelatin at 30°C for 2 hr, and incubated in anti-ß-1,6-glucan antibodies (1:2500 dilution in PBS containing 0.1% Tween-20) for 1 hr at room temperature, followed by incubation in goat anti-rabbit IgG for 1 hr. The immunoblots were detected with ECL Western blotting detection reagents from Amersham according to the manufacturer's instruction. The purified anti-ß-1,6-glucan antibodies (0.5 ug/ul) was provided by Dr. Frans Klis (Montijn et al. 1994). For antibody competition experiments, pustulan (ß1,6-glucan), laminarin (ß1,3-glucan) or mannan (1 mM glucose or mannose equivalent) was included in the anti-ß-1,6-glucan solution. The pustulan, laminarin or mannan was dissolved in PBS by autoclaving at 110°C for 30 min.

Other methods were described in section II.

RESULTS

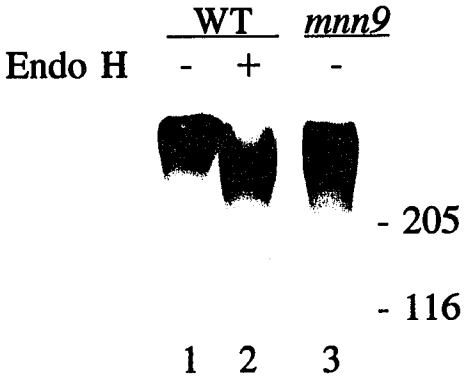
The molecular size of α-agglutinin increases during covalent association with the cell wall

The >300 kD plasma membrane-bound, the soluble periplasmic form, and the mature cell wall-bound forms of α-agglutinin all migrated on the top of 6% SDS-polyacrylamide gels (Figs. 17, 5 and 6). The apparent molecular sizes of these forms have not been determined. However, after Endo H treatment, which removes N-linked carbohydrates, the plasma membrane-bound and the soluble periplasmic forms were reduced to 200 kD (Fig. 17B, lane 2 and Fig. 9C), whereas the cell wall form was reduced to 240-300 kD (Fig. 17A, lane 2 and Fig. 9C). The apparent higher molecular size of the cell wall form than the plasma membrane-bound and the periplasmic intermediates, after N-linked carbohydrates were removed, indicates that further modification occurred in the process of covalent cell wall association.

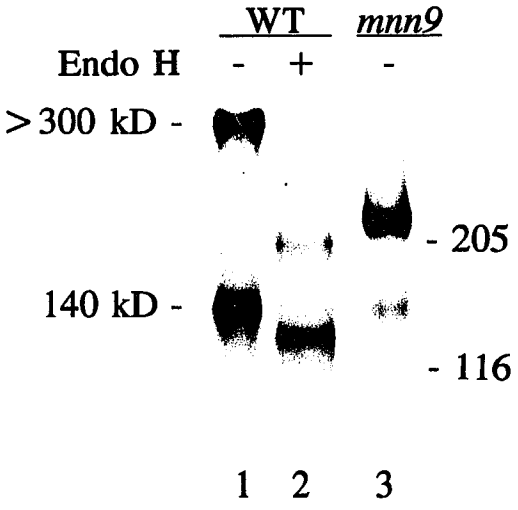
The higher molecular size of the cell wall form of α-agglutinin was seen without Endo H digestion of α-agglutinin in a *mnn9* mutant. This mutant makes mannoproteins without outer mannose polysaccharide chains added to the N-linked core oligosaccharides (Tsai et al. 1984; Tanner and Lehle 1987). Two forms of α-agglutinin with molecular weight of 140 kD and 225 kD, respectively, were isolated from the SDS extract of the *mnn9* cells (Fig. 17B, lane 3). The *mnn9* mutation did not affect the molecular weight of the 140 kD form, as this form occurs in the ER and contains only the N-linked core oligosaccharides (section II). The molecular size of the 225 kD form from *mnn9* cells was close to that of the de-N-glycosylated >300 kD α-agglutinin from

Figure 17. Higher molecular size of the cell wall α-agglutinin than the plasma membrane-bound and the periplasmic intermediates. pAGα1'-transformed W303-1B (WT) cells and LB2134 (*mnn9*) cells were labeled with [^{35}S]methionine and lysed in SDS lysis buffer. Cell walls were separated from the SDS extracts, and treated with laminarinase. α-agglutinin was immunoprecipitated from the SDS extracts of the cells or from the laminarinase extracts of the cell walls with anti-α-agglutinin, and analyzed by SDS-PAGE and fluorography. **A.** Laminarinase extracted cell wall form of α-agglutinin. **B.** SDS extracted intermediate forms of α-agglutinin. The >300 kD α-agglutinin contains a mixture of a membrane-bound form and a soluble form. The molecular size standards on the right are indicated in kD.

A


WT
mnn9
Endo H
- + -
- 205
- 116
1 2 3

B


WT
mnn9
Endo H
- + -
>300 kD -
- 205
140 kD -
- 116
1 2 3

wild-type cells (200 kD) plus the amount of N-linked core saccharides (15 kD, inferred from the amount contained in the 140 kD ER form), which is in accordance with the *mnn9* phenotype. The cell wall form of α-agglutinin from *mnn9* cells, although migrating faster than the cell wall form from wild type cells, had a molecular weight of >250 kD (Fig. 17A,lane 3). The apparent higher molecular size of the cell wall α-agglutinin than the SDS-extractable 225 kD form of α-agglutinin in *mnn9* cells again indicates that α-agglutinin is modified in some way before it becomes covalently associated with the cell wall.

Mutations in *KRE* genes specifically affect the molecular size of the cell wall form of α-agglutinin

Several genes involved in cell wall ß-1,6-glucan biosynthesis have been isolated through mutations that confer resistance to the K1 killer toxin (Meaden et al. 1990; Boone et al. 1990; Roemer and Bussey 1991; Hill et al. 1992; Brown and Bussey 1993; Brown et al. 1993). It has been shown that disruptions of *KRE1, KRE9* and *KRE11* genes reduce the level of the cell wall ß-1,6-glucan to 60%, 20% and 50%, respectively (Boone et al. 1990; Brown and Bussey 1993; Brown et al. 1993). Smaller sizes of the cell wall ß-1,6-glucan polymer are also found in these mutants. A *KRE6* disruption mutation results in reduced levels of both ß-1,3-glucan and ß-1,6-glucan in the cell wall, but does not affect the size of ß-1,6-glucan (Roemer and Bussey 1991). It was speculated that the cell wall anchorage of α-agglutinin resulted from a covalent linkage to the wall ß-1,6-glucan which was in turn cross-linked to other cell wall components. Therefore, the effect of *KRE1, KRE6, KRE9* and *KRE11* gene disruption mutations on the cell wall

anchorage and on the molecular size of α-agglutinin was investigated.

To evaluate the cell surface expression of α-agglutinin, the agglutinability of *kre* null mutants with tester **a** cells was determined first (Terrance and Lipke 1981). *kre1* and *kre11* mutants exhibited no significant decrease in agglutinability from the isogenic wild-type strain (Table I). This result indicates that α-agglutinin is normally expressed on the cell surface of *kre1* and *kre11* mutants. Since *kre6* cells are abnormally large and *kre9* cells form aggregates themselves, the agglutinability of these two mutants was difficult to quantitate using the assay, which is based on the measurement of optical density of the cells in suspension after the aggregates formed by **a** and α cells are sedimented (Terrance and Lipke 1981). However, when *kre6* or *kre9* mutant cells were mixed with tester **a** cells, larger aggregates were formed, indicating that these mutant cells are agglutinable.

The effect of *KRE* gene disruptions on α-agglutinin secretion was determined next. Wild-type cells secreted a small amount of α-agglutinin into the medium. The secreted α-agglutinin showed a molecular size of >300 kD, which was reduced to apparent 200 kD after Endo H treatment (data not shown). All four *kre* mutants secreted more α-agglutinin into the growth medium than the isogenic wild-type strain. The ratio of the secreted α-agglutinin to the covalently cell wall-bound α-agglutinin increased 2- to 3-fold in these *kre* mutants (Table I). The α-agglutinin secreted from the *kre* null mutants exhibited no mobility difference from that secreted from wild type cells (data not shown). Despite the increased secretion, a substantial amount of α-agglutinin in each *kre* mutant remained associated with the cell wall, and could only be released by

Table I Effect of *KRE* gene disruptions on cell surface expression and secretion of α-agglutinin

Genotype	AI[a]	Ratio of secreted to cell wall α-agglutinin[b]
KRE	0.70	0.076
kre1	0.68	0.210
kre6	N.D.	0.174
kre11	0.64	0.153
kre9	N.D.	0.260

[a] AI: agglutination index. The higher AI indicates a greater degree of agglutination. The agglutination assay was performed as described previously (Terrance and Lipke 1981). The tester a cells (X2180-1A) was pretreated with α-factor as described (Terrance and Lipke 1981). N.D., not determined.

[b] The *kre* mutants and their isogenic wild type strain (*KRE*) were metabolically labelled with [^{35}S]methionine for 30 min and separated from the media. The cell wall α-agglutinin was solubilized from cell walls by treatment with laminarinase, and subsequently immunoprecipitated. Under the experimental conditions, half of the total amount of the cell wall α-agglutinin was extracted from the mutant and the wild type cell walls. The secreted α-agglutinin was immunoprecipitated from the labeling media. The α-agglutinin precipitates were subjected to SDS-PAGE and fluorography. The ratio is the radioactivity (cpm) of α-agglutinin bands excised from the dried gel after fluorographic detection.

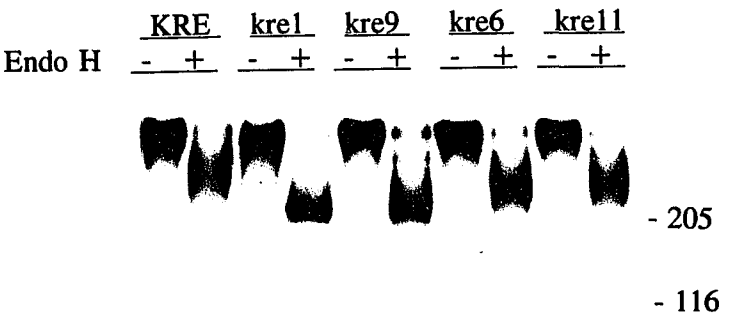
laminarinase treatment, suggesting that the majority of the α-agglutinin produced in these *kre* mutants is normally anchored in the cell wall.

It has been reported that a *kre1* mutant over-secretes proteins normally found in the growth medium (Bussey et al. 1983). It was also observed in this research that the levels of at least 7 other proteins secreted into the medium increased in the *kre1* null mutant (data not shown). Therefore, the increase in α-agglutinin secretion in *kre* mutants could be a general effect of *KRE* gene mutations on the cell wall structure. Alternatively, the effect of *KRE* gene disruptions on the molecular size of the cell wall form of α-agglutinin was determined. Before Endo H treatment, the laminarinase extracted cell wall form of α-agglutinin from the *kre* mutants and from the wild type strain migrated on the top of 6% SDS-polyacrylamide gel (Fig. 18A), and the molecular sizes could not be compared. But after Endo H, the cell wall α-agglutinin from the *kre1* and *kre9* mutants showed molecular sizes of 200-240 kD and 200-270 kD, respectively, significantly smaller than that from the wild type strain (Fig. 18A). No significant reduction of the size of the cell wall α-agglutinin was observed in the *kre6* and *kre11* mutants. A small amount of Endo H resistant material was seen in all *kre* mutants (Fig. 18A). It is not known whether this material represents Endo H-resistant form of α-agglutinin or non-glycosylated proteins coimmunoprecipitated with the α-agglutinin.

The lower molecular size of the cell wall α-agglutinin isolated from the *kre1* and *kre9* mutants could have resulted from laminarinase digestion during the extraction from the cell wall, since the mutant cell walls have an altered structure (Boone et al. 1991; Brown and Bussey 1993), and the α-agglutinin in the mutant cell walls would be more

Figure 18. Effect of *KRE* gene disruptions on the molecular size of α-agglutinin. The *kre* mutants and the isogenic wild type strain (*KRE*) were labeled with [^{35}S]methionine. The labelled cells were lysed in SDS lysis buffer, and the insoluble cell walls were separated from the SDS extracts. The cell walls were treated with laminarinase. The α-agglutinin was immunoprecipitated from the laminarinase extracts of the cell walls or from the SDS extracts of the cells, treated with (+) or without (-) Endo H, and analyzed by SDS-PAGE and fluororaphy. **A.** Laminarinase extracted cell wall form of α-agglutinin. **B.** SDS extracted intermediate forms of α-agglutinin. Samples with an equal amount of radioactivity (cpm) were applied to each lane in each gel. Molecular size standards on the right are indicated in kD.

A


KRE kre1 kre9 kre6 kre11
Endo H - + - + - + - + - +
- 205
- 116

B

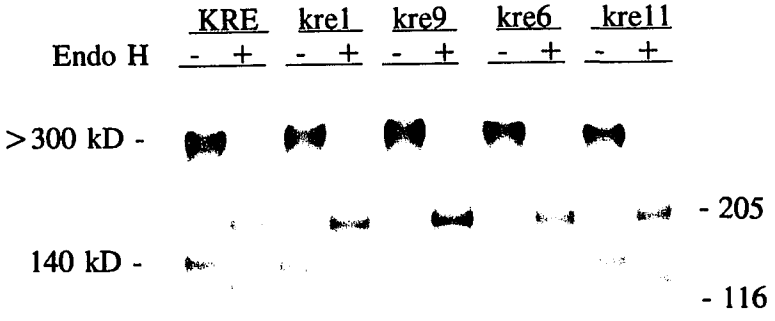
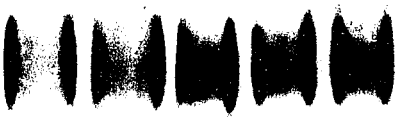

KRE kre1 kre9 kre6 kre11
Endo H - + - + - + - + - +
>300 kD -
- 205
140 kD -
- 116

Figure 19. Time course of laminarinase digestion. The wild-type strain (*KRE*) and the *kre1* mutant were labeled with [^{35}S]methionine and lysed in lysis buffer. Cell walls from the *kre1* cells were treated with laminarinase at 35°C for various periods of time as indicated. Cell walls from the wild type cells were digested with laminarinase for 2 hr, and the solubilized wall proteins were separated from the undigested cell walls (time 0 hr) and incubated at 35°C in the presence of laminarinase for various periods of time as indicated. The cell wall α-agglutinin was immunoprecipitated, treated with Endo H, and analyzed by SDS-PAGE and fluorography. Samples with an equal amount of radioactivity were loaded in each lane.

KRE wall proteins
laminarinase digest.
(hr)
0 2 4 6 16



kre1 cell walls
laminarinase digest.
(hr)
1 2 4 6 16

easily accessible to the enzyme than in the wild type cell wall. To address this question, the time course of laminarinase digestion was determined. Cell walls from *kre1* cells were treated with laminarinase for various periods of time from 1 hr to 16 hr. The extracted cell wall α-agglutinin, after Endo H treatment, showed no mobility difference over the time course (Fig. 19). In a separate experiment, the cell wall α-agglutinin was solubilized from the wild type cell wall first, and then incubated in the presence of laminarinase for different periods of time to examine whether extended laminarinase digestion would further reduce the size of the wall α-agglutinin from wild-type cells. Laminarinase treatment of the solubilized cell wall α-agglutinin for 16 hr showed the same apparent molecular size as 2 hr treatment (Fig. 19). These results imply that the lower molecular size of the cell wall α-agglutinin in *kre1* mutant is not due to the laminarinase digestion during the extraction from the cell wall.

The molecular sizes of the non-cell wall intermediate forms of α-agglutinin were unaffected by *KRE* gene disruptions. The >300 kD α-agglutinin from the *kre* mutants and from the wild type strain shifted to 200 kD after Endo H treatment (Fig. 18B). To further test whether *kre1* null mutation affects protein glycosylation, the molecular size of a plasma membrane-bound glycoprotein called Gas1p (or ggp1), which is N- and O-glycosylated, was compared (Fankhauser and Conzelmann 1991; Nuoffer et al. 1991; Vai et al. 1990). Gas1p from *kre1* cells and from wild-type cells exhibited no mobility difference (data not shown). This result further indicates that protein glycosylation is not affected by *KRE1* gene disruption. Brown and Bussey (1993) have shown that the *KRE9* disruption does not alter protein glycosylation. Thus, the lower molecular size of the cell

wall α-agglutinin in *kre1* and *kre9* mutants than in the wild type strain suggests that the cell wall α-agglutinin is covalently associated with the ß-1,6-glucan, as these mutants produce cell wall ß-1,6-glucan with reduced size.

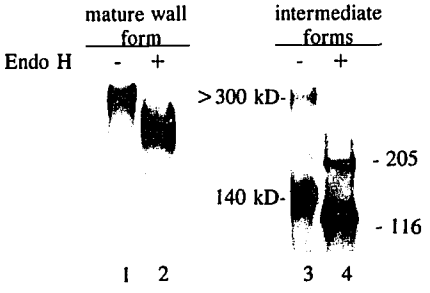
The cell wall α-agglutinin is immunoreactive with anti-ß-1,6-glucan antibodies

The possibility that the cell wall α-agglutinin was covalently linked to the wall ß-1,6-glucan was further tested using ß-1,6-glucan specific antibodies. The antibody was raised against ß-1,6-glucan-BSA conjugate, and purified using ß-1,6-glucan affinity column (Montijn et al. 1994). The cell wall α-agglutinin was immunoprecipitated from the laminarinase extract of the cell wall with anti-α-agglutinin antibodies. The α-agglutinin precipitate was analyzed by immunoblotting using anti-ß-1,6-glucan antibodies as probe. The cell wall form of α-agglutinin was recognized by the anti-ß-1,6-glucan antibodies (Fig. 20B, lane1). This recognition was competitively inhibited by pustulan (ß-1,6-glucan) (Fig. 20C, lane1), but was not inhibited by laminarin (ß 1,3-glucan) or mannan (data not shown). These results confirm that the epitope on the cell wall α-agglutinin consists of ß-1,6-glucan. The Endo H treated cell wall form still remained the reactivity with the anti-ß-1,6-glucan (Fig. 20B, lane 2). The cell wall α-agglutinin from the *kre1* mutant was less reactive with the anti-ß-1,6-glucan antibodies than that from the isogenic wild-type strain, consistent with it containing shorter ß-1,6-glucan polymer (Fig. 21).

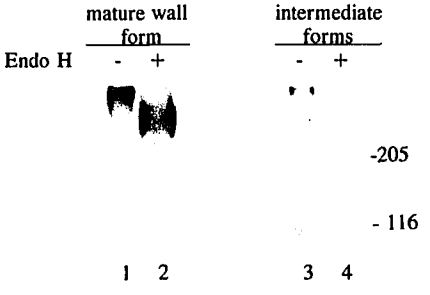
To determine whether the 140 kD and the >300 kD intermediate forms of α-agglutinin contain ß-1,6-glucan, these forms were precipitated from the SDS extract of the cells with anti-α-agglutinin antibodies, and analyzed by immunoblotting using anti-ß-1,6-glucan as probe. The 140 kD and the >300 kD forms, as well as their

Figure 20. Immunoreactivity of the cell wall α-agglutinin with anti-ß-1,6-glucan antibodies. W303-1B cells harboring pAGα1' were lysed in SDS lysis buffer. The cell wall was separated from the SDS extract, and treated with laminarinase. The cell wall α-agglutinin and the intermediate forms of α-agglutinin were immunoprecipitated with anti-α-agglutinin antibodies from the laminarinase extract of the cell wall and the SDS-extract of cells, respectively. The α-agglutinin precipitates were separated on SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. The blots were probed with anti-α-agglutinin antibodies or anti-ß-1,6-glucan antibodies, and detected with ECL Western blotting detection reagents from Amersham. **A.** α-agglutinin blot probed with anti-α-agglutinin, showing protein levels of various forms of α-agglutinin. **B.** α-agglutinin blot probed with anti-ß-1,6-glucan. **C.** as **B**, except that pustulan (ß-1,6-glucan) in a concentration of 1 mM glucose equivalent was included in the anti-ß-1,6-glucan antibody solution. Equal amounts of α-agglutinin precipitates were loaded in the corresponding lanes in **A**, **B** and **C**. Molecular size standards on the right are indicated in kD.

A. anti-α-agglutinin



B. anti-ß-1,6-glucan



C. anti-ß-1,6-glucan plus ß-1,6-glucan

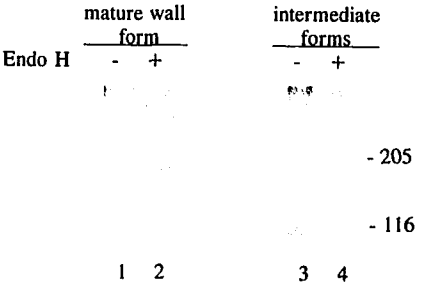
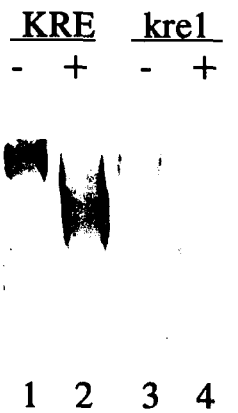


Figure 21. Effect of *KRE1* gene disruption on the immunoreactivity of the cell wall α-agglutinin with anti-ß-1,6-glucan. The *kre1* mutant and its isogenic wild-type strain (*KRE*) were lysed in SDS lysis buffer, and cell walls were collected and treated with laminarinase. The wall α-agglutinin was immunoprecipitated from the laminarinase extracts of the cell walls, separated by SDS-PAGE, and blotted onto nitrocellulose membranes. The blots were probed with anti-α-agglutinin (**A**) or anti-ß-1,6-glucan (**B**). Equal amounts of samples were loaded in the corresponding lanes in **A** and **B**.

A. anti-α-agglutinin

B. anti-ß-1,6-glucan



corresponding Endo H-treated forms did not react with the ß-1,6-glucan antibodies (Fig. 20B, lane 3 and 4), whereas these forms were strongly stained when reprobed with anti-α-agglutinin by immunoblotting (Fig. 20A, lane3 and 4). A weak band migrated on the very top of the gel was seen (Fig. 20B, lane 3). However, this material was not competed by ß-1,6-glucan (Fig. 20C, lane3), did not comigrate with the >300 kD α-agglutinin band, and was also seen in a cell extract which does not contain α-agglutinin (data not shown). Therefore, this band is non-specific. In a separate experiment, cell lysate was fractionated into membranes and soluble protein fraction by ultracentrifugation to separate the soluble periplasmic >300 kD form from the plasma membrane-bound >300 kD form of α-agglutinin. Neither of the >300 kD forms was reactive with the anti-ß-1,6-glucan (data not shown). These results indicate that the addition of ß-1,6-glucan occurs extracellularly during covalent cell wall anchorage of α-agglutinin.

GPI anchor addition is required for cell wall anchorage of α-agglutinin

Wojciechowicz et al (1993) have shown that truncation of the C-terminal 29 amino acids, which removes the hydrophobic sequence from *AGα1* (this mutant was designated as $AG\alpha1_{621}$), abolishes cell wall anchorage of α-agglutinin. Similarly, replacement of the C-terminal hydrophobic sequence of *AGα1* with a more hydrophilic sequence (this mutant was designated as $AG\alpha1_{635}$) results in 10% of the wild-type level of the cell wall α-agglutinin. Both *AGα1* mutants secrete active α-agglutinin into the medium. To test whether the loss of cell wall anchorage in $AG\alpha1_{621}$ and $AG\alpha1_{635}$ mutants resulted from a defect in GPI anchor addition to the protein, membrane

association of the 140 kD intracellular form of α-agglutinin in these mutants was analyzed. An α-agglutinin defective strain Lα21 (*MATα, agα1-3*) was transformed with a plasmid containing the wild-type *AGα1* gene or the mutated gene, and total cellular proteins were extracted with Triton X-114, followed by phase separation in TX-114. The 140 kD form of α-agglutinin from cells expressing the wild-type *AGα1* partitioned into the detergent phase of TX-114, whereas in cells expressing either $AG\alpha1_{621}$ or $AG\alpha1_{635}$, this 140 kD α-agglutinin partitioned into the aqueous phase of TX-114, indicating that the GPI membrane anchor was eliminated in these mutants (Fig. 22). Thus, the C-terminal hydrophobic sequence is essential for GPI anchor addition, and GPI anchor attachment is required for the cell wall anchorage of α-agglutinin.

To investigate the role of the GPI anchor in cross-linking of the cell wall α-agglutinin and the ß-1,6-glucan, the secreted, GPI-less $Ag\alpha1_{621}$ protein was analyzed for association with ß-1,6-glucan. The secreted $Ag\alpha1_{621}$p was precipitated with anti-α-agglutinin from the growth medium of the *agα1::LEU2* strain expressing $pAG\alpha1_{621}$'. $Ag\alpha1_{621}$p exhibited a mobility similar to that of the >300 kD plasma membrane-bound and the periplasmic intermediates from wild-type cells (Fig. 23A). After Endo H treatment, the size of the $Ag\alpha1_{621}$p shifted to 200 kD. These observations suggested that the $Ag\alpha1_{621}$p was properly glycosylated, but was not modified for cell wall anchorage. The $Ag\alpha1_{621}$p, before or after Endo H treatment, did not react with the anti-ß-1,6-glucan antibodies (Fig. 23B, lane2 and lane3). A weak, ß-1,6-glucan non-competitive signal was seen on the top of lane 2 (Fig. 23B and 23C). This signal was non-specific, as it was also seen in the growth medium of *agα1::LEU2* cells without $pAG\alpha1_{621}$' (data

not shown). These results suggest a role for the GPI anchor in cross-linking of the cell wall α-agglutinin and the ß-1,6-glucan.

Figure 22. Analysis of membrane anchorage in *AGα1* mutants. Strain Lα21 (*MATα agα1-3*) was transformed with the indicated plasmids, or with vector alone (YEp352). Cellular proteins were extracted with Triton X-114, followed by phase separation. Proteins in the detergent phase (D) and aqueous phase (A) of TX-114 were precipitated by trichloroacetic acid and analyzed by immunoblotting using anti-α-agglutinin.

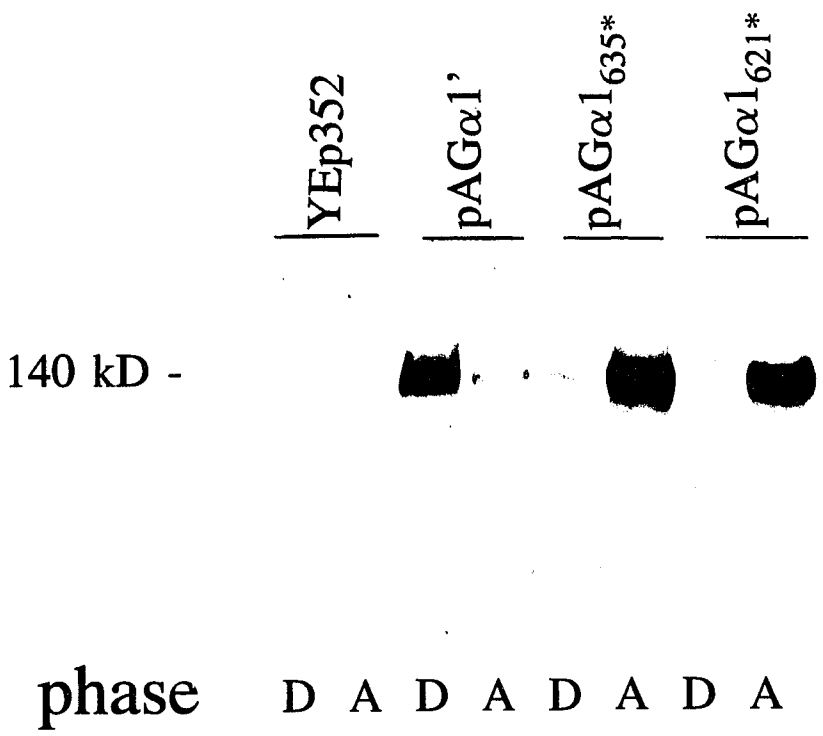
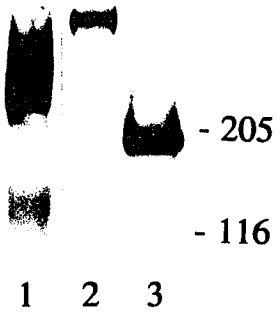
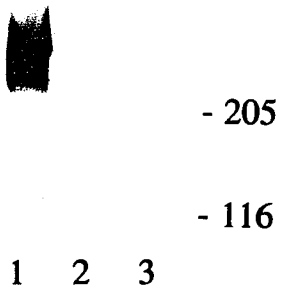

YEp352
pAGα1'
pAGα1635*
pAGα1621*
140 kD -
phase D A D A D A D A

Figure 23. Immunoblotting of secreted Agα1$_{621}$ protein with anti-ß-1,6-glucan antibodies. The C-terminal 29 amino acid truncation mutant of *AGα1* (*AGα1$_{621}$*) was expressed in *agα1::LEU2* strain. The secreted Agα1$_{621}$p was immunoprecipitated with anti-α-agglutinin from the growth medium of *agα1::LEU2* cells expressing plasmid pAGα1$_{621}$', and analyzed by immunoblotting using anti-α-agglutinin or anti-ß-1,6-glucan. **A.** Agα1$_{621}$p blot probed with anti-α-agglutinin. **B.** Agα1$_{621}$p blot probed with anti-ß-1,6-glucan. **C.** as **B,** except that pustulan (ß-1,6-glucan) was added to the anti-ß-1,6-glucan antibody solution. Lane1: Endo H treated cell wall form of α-agglutinin from wild-type cells, used as a positive reaction with the anti-ß-1,6-glucan. Lane 2: Agα1$_{621}$p before Endo H treatment. Lane 3: Agα1$_{621}$p after Endo H treatment. Equal amounts of samples were loaded in the corresponding lanes in **A, B** and **C.**

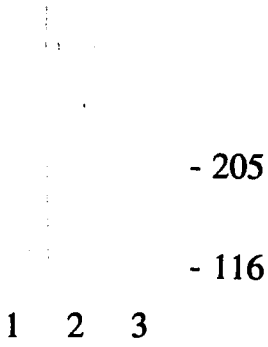
A. anti-α-agglutinin



B. anti-ß-1,6-glucan



C. anti-ß-1,6-glucan plus ß-1,6-glucan



Discussion

The results presented in this section demonstrate that α-agglutinin becomes covalently linked to the ß-1,6-glucan during the process of covalent cell wall association. The cross-linkage between these two components appears to be GPI-dependent.

Extracellular cross-linking of α-agglutinin and ß-1,6-glucan

α-agglutinin extracted from the cell wall with laminarinase exhibited higher molecular size than the plasma membrane-bound and the periplasmic intermediates (Fig. 17). The increase in size indicates that covalent modification of α-agglutinin occurs during cell wall anchorage.

The covalent association between the cell wall α-agglutinin and the ß-1,6-glucan was demonstrated by analysis of α-agglutinin size in *kre* mutants which have defects in synthesis of the cell wall ß-1,6-glucan. Disruptions of the *KRE1* and *KRE9* genes significantly reduced the molecular size of the cell wall α-agglutinin, but did not affect the size of the plasma membrane-bound and the periplasmic intermediates (Fig. 18). Since the *kre1* and *kre9* null mutants show a reduced size of the cell wall ß-1,6-glucan (Boone et al. 1990; Brown and Bussey 1993), these findings suggest that the cell wall form of α-agglutinin is covalently associated with ß-1,6-glucan. In accordance with the phenotypes of *kre6* and *kre11* null mutants, which produce cell wall ß-1,6-glucan with normal size and slightly reduced size, respectively (Roemer and Bussey 1991; Brown and Bussey 1993), there was no significant reduction of the molecular size of the cell wall α-agglutinin in these mutants (Fig. 18).

In addition to the effect on cell wall ß-1,6-glucan size, *KRE1, KRE6, KRE9* and *KRE11* gene disruptions also lead to reduced amount of the cell wall ß-1,6-glucan, with a greatest reduction reported in the *kre9* null mutant, which contains only 20 % of the wild type level of cell wall ß-1,6-glucan (Boone et al. 1990; Roemer and Bussey 1991; Brown and Bussey 1993; Brown et al. 1993). An increased secretion of α-agglutinin was observed in all four *kre* mutants, with *kre1* and *kre9* causing the most secretion of α-agglutinin into the medium (Table I). However, a substantial amount of α-agglutinin from each *kre* mutant was cell wall associated and could only be released by treatment with laminarinase (Table I). In addition, the agglutinability of *kre1* and *kre11* mutants was comparable to that of the isogenic wild-type strain (Table I). Thus, it appears that the reduced level of ß-1,6-glucan does not significantly affect the anchorage of α-agglutinin in the cell wall; this could be explained as that only a small percentage of the wall ß-1,6-glucan is used as receptors for cross-linking α-agglutinin. The increased secretion of α-agglutinin in *kre* mutants could be a general effect of *KRE* gene disruptions on the cell wall structure, as the levels of several other proteins secreted into the medium also increased in *kre1* mutant (data not shown; Bussey et al. 1983).

The cell wall α-agglutinin was immunoreactive with antibodies directed against ß-1,6-glucan, confirming the covalent association of this protein with the ß-1,6-glucan (Fig. 20). The specificity of antibody recognition was demonstrated by the competitive inhibition by ß-1,6-glucan. The observation that the cell wall α-agglutinin from *kre1* cells was less reactive with the anti-ß-1,6-glucan than that from wild-type cells further indicates that it contains shorter ß-1,6-glucan polymer.

The assembly of ß-1,6-glucan appears to initiate in the ER (Meaden et al. 1990). Some proteins are observed to be intracellularly glucosylated through ß-1,6-linkages (Montijn, unpublished data). Because the >300 kD plasma membrane-bound and the soluble periplasmic forms of α-agglutinin did not react with the anti-ß-1,6-glucan (Fig. 20), the addition of ß-1,6-glucan must occur after the protein is exterior to the plasma membrane and after the formation of the periplasmic form. Pastor et al (1984) have observed that some soluble periplasmic mannoproteins become covalently wall-associated within 15 min. This finding suggests that at least for some wall-bound mannoproteins, soluble precursors are transiently present in the periplasmic space before being covalently integrated into the wall structure. These observations are compatible with a model in which the soluble periplasmic form of α-agglutinin cross-links to the preformed cell wall ß-1,6-glucan, resulting in formation of α-agglutinin-ß-1,6-glucan complex.

Covalent association with ß-1,6-glucan would provide a mechanism for cell wall anchorage of α-agglutinin. The cell wall ß-1,6-glucan is associated with the insoluble fibers of ß-1,3-glucan (Fleet 1991; Cabib et al. 1988), and probably also associated with chitin (Roncero et al. 1988; Cabib et al. 1988). Since the cell wall α-agglutinin was isolated by treatment with laminarinase which hydrolyzes ß-1,3-linked glucan, the α-agglutinin associated ß-1,3-glucan, if there is any, would have been removed. However, as the wall ß-1,3-glucan has a polymerization of 1500 glucose residues (Manners et al. 1973a), some ß-1,3-glucan might remain associated with the α-agglutinin after short laminarinase treatments. The time course of laminarinase digestion showed that the cell wall α-agglutinin extracted by a 1 hr treatment exhibited the same molecular size as the extended digestion (16 hr) with the enzyme (Fig. 19). This result argues against the

association between α-agglutinin and ß-1,3-glucan, although such linkage can not be ruled out.

Cross-linkage between the wall α-agglutinin and the ß-1,6-glucan appears to be GPI-dependent

The bonds created in the association of α-agglutinin or other wall mannoproteins with ß-glucan are not known, but there are several possibilities. The glucan could be attached to the protein through N- or O-linked carbohydrates. However, several lines of evidence are against these possibilities. Consistent with previous reports that the glucose-containing carbohydrate chain cannot be removed from cell wall glucomannoproteins by PNGase or Endo H (Van Rinsum et al. 1991), the Endo H treated cell wall α-agglutinin was still reactive with the anti-ß-1,6-glucan (Fig. 20). It is, therefore, unlikely that the ß-1,6-glucan is attached to the N-linked chains of α-agglutinin. Furthermore, cell anchorage of α-agglutinin was not affected in a *mnn9* strain (Fig. 17), which has severely truncated N-linked mannose saccharide chains, nor was it inhibited by tunicamycin treatment of cells (data not shown; Terrance 1983). O-linked carbohydrate-mediated attachment would appear to be ruled out by the fact that ß-glucan association with peptides is not affected by ß-elimination (mild alkaline treatment), which releases O-linked chains (Van Rinsum et al. 1991). Therefore, the linkage between ß-glucan and wall mannoproteins must be novel.

It is more likely that the ß-glucan is attached to a modified GPI anchor. A C-terminal 29 amino acid truncated form of α-agglutinin, Agα1$_{621}$p, which is secreted into the medium due to a defect in GPI anchor addition, did not contain ß-1,6-glucan (Figs. 22 and 23). Except for the lack of the GPI anchor, the secreted Agα1$_{621}$p appears to be

normally processed and glycosylated, as this protein showed a mobility, before or after Endo H treatment, similar to that of the >300 kD periplasmic form of α-agglutinin from wild-type cells (Fig. 23). These observations strongly suggest that the GPI anchor plays a role in the cross-linkage between the α-agglutinin and the ß-1,6-glucan. A role for GPI anchors in cross-linking mannoproteins to the cell wall matrix has been proposed (De Nobel and Lipke 1994). Unlike N- and O-linked oligosaccharides, the reducing terminal of the glycan in the GPI anchor is oriented away from the polypeptide (Figs. 2 and 4). The removal of the phosphatidylinositol moiety from the GPI anchor would free the reducing end that has the potential to bind to other cell wall components by a transglycosylation reaction (De Nobel and Lipke 1994). The soluble >300 kD periplasmic form of α-agglutinin can not be labeled with inositol or fatty acids (Fig. 9), but preliminary results show that the ethanolamine still remains associated with this form and the mature cell wall form of α-agglutinin (data not shown). Therefore, it is likely that the >300 kD periplasmic form resulted from the cleavage of the GPI anchor between the ethanolamine phosphate and the phosphatidylinositol. The analysis of cross-linking structure will reveal whether the GPI anchor-derived glycan mediates cross-linkage between the α-agglutinin and the ß-1,6-glucan.

Section IV

Summary and Discussion

Mannoproteins form one of the two major components of the *S. cerevisiae* cell wall. They are distributed over the surface and permeate the ß-glucan network of the wall (Fleet 1991). However, only a few cell wall proteins have been characterized, and little is known about the mechanisms of cell wall anchorage. The cell adhesion glycoprotein α-agglutinin is one of the few wall proteins with known sequence and function. The mechanism of cell wall anchorage of α-agglutinin was investigated in this research. The data obtained reveal a novel mechanism which may be general to covalently anchored wall glycoproteins.

The covalent nature of cell wall association of the mature α-agglutinin has been demonstrated by the extraction of this protein from the cell wall by ß-glucanase digestion, but not by treatment with SDS or other reagents which destroy non-covalent bonds (Fig. 5; Hauser and Tanner 1989; Schreuder et al. 1993). It was shown in section II that the α-agglutinin was synthesized with a GPI anchor. Two GPI-anchored forms of α-agglutinin were identified in wild-type cells: the 140 kD ER form and the >300 kD plasma membrane form (Figs. 7, 8 and 9). GPI linkage to these two forms was demonstrated by susceptibility to PI-PLC cleavage, and metabolic labeling with both [^{3}H]myo-inositol and [^{3}H]palmitic acid. The GPI-anchored forms of α-agglutinin showed biological activity (Fig. 10). A soluble >300 kD form was also detected in wild-type cells (Fig. 7). Neither the inositol nor the palmitic acid label was detected in this soluble form (Fig. 9). The >300 kD soluble form was localized in the periplasmic space, and had kinetic properties of an intermediate between the >300 kD plasma membrane-bound form and the mature cell wall-anchored α-agglutinin (Figs. 11 and 12). Additional forms of α-agglutinin with molecular size of 80 kD, 150 kD and 250-300 kD were identified

in *sec* mutants which block protein vesicular transport at restrictive temperature (Fig. 13) (Novick et al. 1980,1981; Novick and Schekman 1983; Esmon et al. 1981; Kaiser and Schekman 1990). The 80 kD form was detected in *sec53* at restrictive temperature. Since *sec53* mutations block N- and O-glycosylation, and addition of GPI anchors to proteins, the 80 kD form is likely to represent the unmodified α-agglutinin peptide. The 150 kD form seen in the *sec18* mutant, which blocks protein transport between the ER and the Golgi at restrictive temperature, was GPI-anchored. The membrane-bound 250-300 kD form was accumulated in *sec7* and *sec1* mutants, which block protein transport at the level of the Golgi and secretory vesicles. Therefore, the 150 kD and 250-300 kD forms are likely to be intermediates between the 140 kD form and the >300 kD plasma membrane form. N- and O-glycosylation, and probably other modifications, resulted in successive increase in size of α-agglutinin during transport from the ER to the cell surface.

The mature cell wall-bound α-agglutinin had higher molecular size than the plasma membrane-bound and the soluble periplasmic intermediates, indicating that covalent modification occurred in the process of cell wall association (Fig. 17). The results presented in section III show that ß-1,6-glucan plays a role in the covalent modification. The cell wall α-agglutinin was immunoreactive with anti-ß-1,6-glucan specific antibodies (Fig. 20). The cell wall α-agglutinin from *kre* mutants, which have reduced size of the cell wall ß-1,6-glucan, exhibited lower molecular size and less immunoreactivity with the anti-ß-1,6-glucan than the cell wall α-agglutinin from wild-type cells (Figs. 18 and 21). These results demonstrate a covalent association of the cell

wall α-agglutinin and the ß-1,6-glucan. The >300 kD plasma membrane-bound and the periplasmic intermediates did not react with the anti-ß-1,6-glucan (Fig. 20). This finding indicates that the addition of the ß-1,6-glucan occurs extracellularly, and after the formation of the periplasmic form of α-agglutinin.

The requirement of GPI anchor addition for cell wall anchorage of α-agglutinin was demonstrated by the effects of C-terminal truncation mutations of *AGα1*. Truncations of the C-terminal hydrophobic domain from *AGα1* eliminated GPI anchor addition, and therefore, resulted in the loss of cell wall attachment and secretion of α-agglutinin into the medium (Fig. 22; Wojciechowicz et al. 1993). The secreted, GPI-less $Ag\alpha1_{621}p$ was not associated with the ß-1,6-glucan, suggesting that covalent association with ß-1,6-glucan mediates cell wall anchorage of α-agglutinin, and that the cross-linkage between these two components is dependent on addition of a GPI anchor to the α-agglutinin.

The data obtained in this research provide the experimental support for a novel mechanism of cell wall anchorage of α-agglutinin first proposed by Lipke and Kurjan (1992) (Fig. 24). The GPI-anchored forms of α-agglutinin (140 kD, 150 kD, 250-300 kD and >300 kD forms) are intermediates during transport to the cell wall. After the protein is localized in the plasma membrane, the GPI anchor is processed by loss of the fatty acid and inositol components. The processing of the GPI anchor allows release of α-agglutinin from the membrane, resulting in a soluble periplasmic form (soluble >300 kD form), which is subsequently cross-linked to the wall ß-1,6-glucan and becomes covalently associated with the cell wall. The cross-linkage between the α-agglutinin and the ß-1,6-glucan might occur through a transglycosylation reaction mediated by the C-

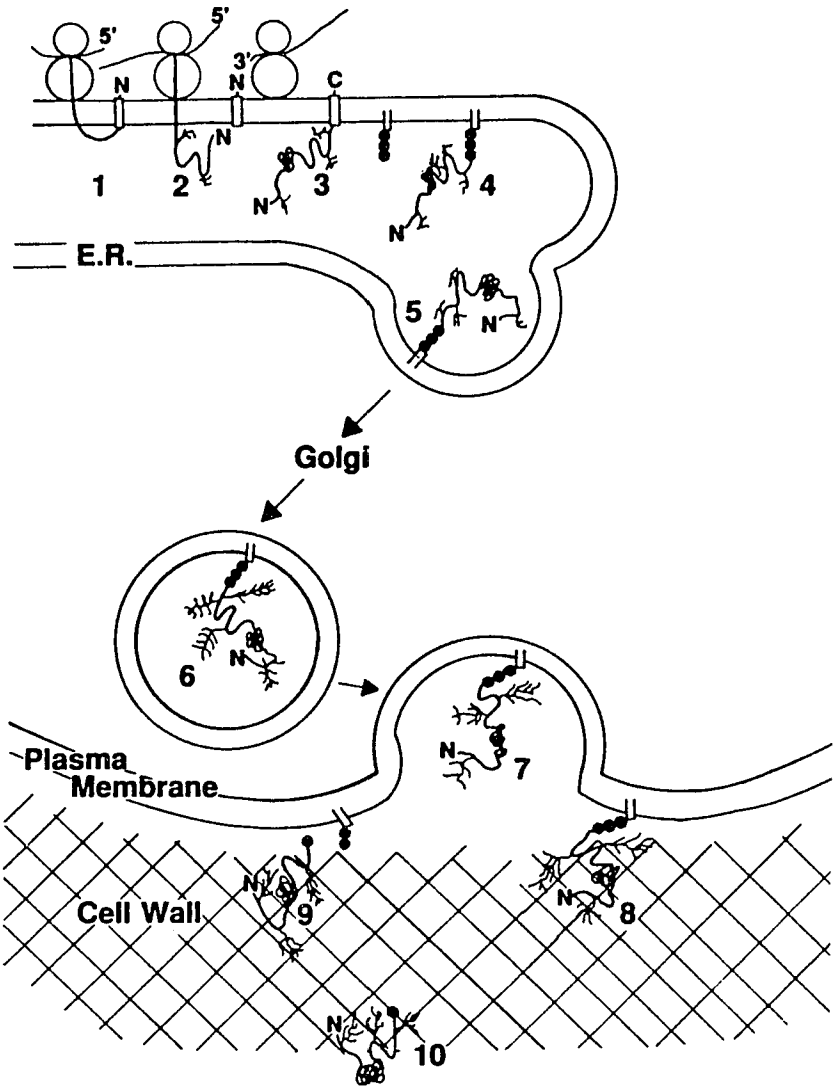
terminal, GPI-derived glycan of the protein. The periplasmic precursor of α-agglutinin could be linked to a preformed ß-1,6-glucan polymer which is already cell wall-anchored by association with insoluble ß-1,3-glucan or chitin, or to a soluble ß-1,6-glucan molecule in the periplasmic space and the complex is then integrated into the wall. Since no soluble α-agglutinin-ß-1,6-glucan complex was detected, cross-linking of the periplasmic precursor of α-agglutinin with cell wall-anchored ß-1,6-glucan would be more likely.

Cell surface attachment of **a**-agglutinin is likely to involve a similar mechanism. The gene encoding for the cell surface anchorage subunit of **a**-agglutinin (*AGA1*) has a C-terminal hydrophobic domain, characteristic of the signal sequences for GPI anchor addition (Fig. 3). Truncations of the C-terminal hydrophobic sequence from *AGA1* result in loss of cell surface **a**-agglutinin and secretion of active **a**-agglutinin into the medium (Roy et al. 1991). Several other genes coding for potential cell wall proteins also contain the C-terminal hydrophobic signal for GPI anchor addition (Boone et al. 1990; Teunissen et al. 1993; Jue and Lipke, unpublished data). Therefore, the proposed GPI anchor-mediated mechanism for cell wall anchorage of the α-agglutinin may be general to a number of covalently wall-bound proteins.

The GPI anchor addition is necessary for cell wall anchorage of α-agglutinin, but not sufficient to specify cell wall location. Two known GPI-anchored proteins from *S. cerevisiae*, Gas1p and a cAMP-binding protein, are attached to the plasma membrane rather than cell wall (data not shown; Nuoffer et al. 1991; Muller and Bandlow 1991; Muller et al. 1992). There must be, therefore, a signal that determines further transfer

of GPI-anchored proteins from the plasma membrane to the cell wall. It will be interesting to define the signal sequence that further localizes α-agglutinin to the cell wall. Identification and characterization of the enzyme involved in the release of the α-agglutinin, and probably other wall proteins from the plasma membrane before covalent integration into the cell wall structure will be of great interest, as it may provide a potential target for anti-fungal drugs with specificity in the inhibition of cell wall biosynthesis.

Figure 24 . Model for cell wall localization of α-agglutinin. A schematic diagram is shown on the left, and observed forms of α-agglutinin are indicated on the right. The α-agglutinin is synthesized, N- and O-glycosylated and GPI-anchored in the ER (1-4). The α-agglutinin with the GPI anchor-bound is then transported through the secretory pathway to the plasma membrane (5-8). The N- and O-linked carbohydrate chains are extended in the Golgi apparatus. The cell wall anchorage involves processing of the GPI anchor to produce a soluble periplasmic intermediate (9), which is subsequently cross-linked to the wall ß-1,6-glucan, resulting in covalent cell wall association (10). The cross-linking of α-agglutinin and the ß-1,6-glucan is proposed to occur through transglycosylation mediated by the C-terminal, GPI anchor-derived glycan of α-agglutinin. The diagram on the left was from Lipke and Kurjan (1992).



α-agglutinin form

80 kD

↓

140 kD

↓

150 kD

↓

250-300 kD

↓

>300 kD

↓

>300 kD (soluble)

↓

cell wall form

Section V

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