Glycosyl Phosphatidylinositol-dependent Cross-linking of α-Agglutinin and β1,6-Glucan in the Saccharomyces cerevisiae Cell Wall

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Abstract. The cell adhesion protein α-agglutinin is bound to the outer surface of the *Saccharomyces cerevisiae* cell wall and mediates cell–cell contact in mating. α-Agglutinin is modified by addition of a glycosyl phosphatidylinositol (GPI) anchor as it traverses the secretory pathway. The presence of a GPI anchor is essential for cross-linking into the wall, but the fatty acid and inositol components of the anchor are lost before cell wall association (Lu, C.-F., J. Kurjan, and P. N. Lipke, 1994. A pathway for cell wall anchorage of *Saccharomyces cerevisiae* α-agglutinin. *Mol. Cell. Biol.* 14:4825–4833.). Cell wall association of α-agglutinin was accompanied by an increase in size and a gain in reactivity to antibodies directed against β1,6-glucan. Several *kre* mutants, which have defects in synthesis of cell wall β1,6-glucan, had reduced molecular size of cell wall α-agglutinin. These findings demonstrate that the cell wall form of α-agglutinin is covalently associated with β1,6-glucan. The α-agglutinin biosynthetic precursors did not react with antibody to β1,6-glucan, and the sizes of these forms were unaffected in *kre* mutants. A COOH-terminal truncated form of α-agglutinin, which is not GPI anchored and is secreted into the medium, did not react with the anti–β1,6-glucan. We propose that extracellular cross-linkage to β1,6-glucan mediates covalent association of α-agglutinin with the cell wall in a manner that is dependent on prior addition of a GPI anchor to α-agglutinin.

THE cell wall of *Saccharomyces cerevisiae* consists of two major components: β glucans and mannoproteins (Ballou, 1982; Cabib et al., 1982; Fleet, 1991; Klis, 1994). The glucan is composed of two classes: the β1,3-glucan and β1,6-glucan. The β1,3-glucan consists mainly of linear β1,3-linked glucan with some branching through β1,6 linkage. This class of glucan has an average of 1,500 glucose residues per molecule and forms insoluble fibers (Manners et al., 1973*a*). The β1,6-glucan contains predominantly β1,6-linkages with some β1,3-linked branch points, and has a degree of polymerization of approximately 140 glucose residues (Boone et al., 1990; Manners et al., 1973*b*). The β1,3-glucan and the β1,6-glucan are cross-linked to each other, and association of β1,6-glucan with chitin has been suggested (Mol and Wessels, 1987; Surarit et al., 1988). Several genes involved in cell wall β1,6-glucan biosynthesis have been isolated through mutations that confer resistance to

the K1 killer toxin, which binds to cell wall β1,6-glucan (Meaden et al., 1990; Boone et al., 1990; Roemer and Bussey, 1991; Hill et al., 1992; Brown and Bussey, 1993; Brown et al., 1993). *kre1*, *kre9*, and *kre11* mutations reduce the level of alkali-insoluble cell wall β1,6-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 β1,6-glucan polymer are also found in these mutants. A *kre6* disruption results in reduced levels of both β1,3-glucan and β1,6-glucan in the cell wall, but does not affect the size of β1,6-glucan (Roemer and Bussey, 1991; Roemer et al., 1993, 1994). Disruption of *KRE5* reduces β1,6-glucan content to near zero and compromises growth ability (Meaden et al., 1990).

The wall mannoproteins are usually of high molecular weight, carrying large amounts of N- and/or O-linked mannose polysaccharides (Ballou, 1982, 1988; Fleet, 1991). The basis of cell wall association of mannoproteins are not well understood. Some mannoproteins can be extracted from the cell wall by SDS whereas others are extracted only after digestion of the cell wall with β-glucanase (Pastor et al.,

1984; Valentin et al., 1984; De Nobel et al., 1990; Mrsa et al., 1992). The SDS-extractable mannoproteins appear to be non-covalently associated with the cell wall. β-Glucanase extractable mannoproteins have been shown to contain glucose residues, and it has been proposed that this class of mannoproteins is cross-linked to the wall β-glucan (Shibata et al., 1983; Pastor et al., 1984; Van Rinsum et al., 1991). Some wall mannoproteins have covalently associated β1,6-glucan (Montijn et al., 1994).

α-Agglutinin is a cell surface mannoprotein expressed by α cells. It binds to a-agglutinin expressed on the surface of a cells to mediate direct cell–cell contact during the mating process (Cross et al., 1988; Terrance and Lipke, 1981; Lipke and Kurjan, 1992). Mature α-agglutinin is cell wall anchored and can only be extracted by β-glucanase treatment (Lasky and Ballou, 1988; Hauser and Tanner, 1989; Schreuder et al., 1993; Lu et al., 1994). We have previously shown that the α-agglutinin is synthesized with a glycosyl phosphatidylinositol (GPI)[1] anchor (Wojciechowicz et al., 1993). GPI-linked biosynthetic precursors have been identified, including a 140-kD ER form and a >300-kD plasma membrane form (Wojciechowicz et al., 1993; Lu et al., 1994). A soluble >300-kD form, which has characteristics of a periplasmic intermediate between the >300-kD plasma membrane form and the cell wall form, has also been detected (Lu et al., 1994). A model for GPI anchor–mediated cell wall integration of α-agglutinin has been proposed based on the structure of GPI anchors. Release from the GPI anchor would produce a periplasmic intermediate which subsequently would be linked to the cell wall glucan via a transglycosylation reaction (De Nobel and Lipke, 1994; Lu et al., 1994).

To explore the mechanism of the cell wall anchorage of α-agglutinin, we tested whether this protein is cross-linked to the cell wall β-glucan. We show that the β1,3-glucanase-extractable cell wall form of α-agglutinin is covalently linked to β1,6-glucan, but the biosynthetic intermediates are not associated with glucan. The implications of these results for the mechanism of mannoprotein linkage to the cell wall are discussed.

Materials and Methods

Yeast Strains and Plasmids

S. cerevisiae strain W303-1B (MATα ade2-1 can1-100 ura3-1 leu2-3,112 trp1-1 his3-11,15) was used to express plasmid pAGα1, which contains the full length α-agglutinin gene AGα1 in YEp352 (Wojciechowicz et al., 1993). Plasmid pAGα1₆₂₁′ was constructed by subcloning the 4.5-kb HindIII-XbaI fragment from plasmid pAGα1₆₂₁* (Wojciechowicz et al., 1993) into YEp352. The agα1::LEU2 strain is isogenic to W303-1B and was previously described (Lipke et al., 1989). LB2134-2B (MATα mnn9 SUC2 mal mel gal2 CUP1) was obtained from Yeast Genetics Stock Center. X2180-1A (MATα SUC2 mal mel gal2 CUP1) was used as the tester a strain in the agglutination assay. The KRE 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 described previously (Boone et al., 1990; Roemer and Bussey, 1991; Brown et al., 1993). The kre::HIS3 and kre9::HIS3 strains (MATα) were obtained by random sporulation of a KRE5/kre5::HIS3 and KRE9/kre9::HIS3 heterozygous diploids, respectively, in the SEY6210 background (Spencer and Spencer, 1988).

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1. Abbreviations used in this paper: endo H, endo-N-acetylglucosaminidase; GPI, glycosyl phosphatidylinositol.

Metabolic Labeling of Cells

Labeling with [35S]methionine was performed as described (Lu et al., 1994). Briefly, exponentially growing cells were collected and resuspended at a density of 7 × 10⁷ cells per ml in sulfate-free medium. TRAN35S-LABEL (ICN Biochemicals, Irvine, CA) was added to 20 μCi/ml, and the suspension was incubated for 30 min at 30°C. Synthetic a-factor (a gift of Dr. Fred Naider, College of Staten Island, Staten Island, NY) was added to 50 ng/ml cell suspension 10 min before labeling. At the end of the labeling the cells were collected and washed as described (Lu et al., 1994). The medium was processed as described below.

Isolation of Cell Walls and Laminarinase Treatment

Labeled or unlabeled cells were broken with glass beads in 2% SDS lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 30 μg/ml each of leupeptin, pepstatin, and antipain, followed by heating at 100°C for 5 min. The insoluble cell walls were separated from the SDS extract by spinning at 13,600 g for 5 min, and extracted once with SDS lysis buffer by heating for 5 min at 100°C. The cell wall pellet was then washed, and treated with laminarinase as described (Lu et al., 1994).

Isolation of Secreted α-Agglutinin from the Growth Medium

agα1::LEU2[pAGα1₆₂₁′] cells were grown in 300 ml synthetic medium without leucine and uracil at 30°C to stationary phase. The growth medium was separated from the cells and freeze-dried. The dried material was redissolved 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 isolating secreted α-agglutinin from the kre mutants, labeled growth media (8 ml) were dialyzed in the above buffer, and the dialysates were concentrated to 1.6 ml. α-Agglutinin was immunoprecipitated from the concentrated, dialyzed media.

Purification of Anti-β1,6-Glucan Antibodies

The anti-β1,6-glucan antiserum was raised against bovine serum albumin-β1,6-glucan conjugates in rabbit (Montijn et al., 1994) and was purified using affinity chromatography. Epoxy-activated Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, NJ) was used to couple β1,6-glucan via direct coupling of free oxirane groups to the hydroxyl groups of the sugars. 360 mg of pustulan (β1,6-glucan) was dissolved in 21 ml of distilled water by autoclaving for 40 min at 110°C, and incubated with swollen Sepharose gel (from 3 mg dried beads) at 37°C for 20 h with shaking. After the coupling, excess β1,6-glucan was removed, and the gel was used to pack the column. The antiserum was treated with ammonium sulfate to separate albumins from the immunoglobulins (Harlow and Lane, 1988). The precipitate was redissolved in PBS (Harlow and Lane, 1988), and applied on the column with a flow rate of 0.5 ml/min. The column was washed with at least 10-bed volumes of PBS. The bound antibodies were eluted with 0.1 M glycine, pH 2.5, and dialyzed against distilled water. The purified antibody solution was concentrated and stored at −20°C.

Immunoprecipitation

α-Agglutinin was immunoprecipitated from SDS extracts of cells or from laminarinase extracts of the cell walls of 7.5 × 10⁷ plasmid-containing cells or 5 × 10⁸ cells without plasmid as described previously (Lu et al., 1994). For immunoprecipitating secreted α-agglutinin, concentrated and dialyzed 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 μg/ml each of leupeptin, pepstatin, and antipain. To 800 μl of the material 5 μl anti-α-agglutinin antiserum (AG3) was added. The antiserum was pre-adsorbed with heat-killed a cells as described (Wojciechowicz and Lipke, 1989). The α-agglutinin immunoprecipitates were analyzed on 6% SDS-polyacrylamide gels, which were processed for fluorography (Lu et al., 1994) or immunoblotting. For fluorography, the gels were soaked in sodium salicylate, dried, and exposed to Kodak X-omat XAR film at −70°C for 2 d. The α-agglutinin from a constant number of cells was loaded in each lane, except for Fig. 2, in which a constant amount of radioactivity was loaded.

Treatment with Endo N-Acetylglucosaminidase H

After immunoprecipitation, α-agglutinin was eluted from protein A beads with 0.1 M sodium acetate, pH 5.5, 3% 2-mercaptoethanol, and 0.4% SDS

by boiling at 100°C for 5 min. 25 μl α-agglutinin eluate was diluted with endo H buffer (20 mM sodium acetate, pH 5.5, 2 mM EDTA, 1 mM PMSF, and 30 μg/ml each of leupeptin, pepstatin, and antipain) to 100 μl. 8 μl of recombinant endo N-acetylglucosaminidase H (endo H; Boehringer-Mannheim Biochemicals, Indianapolis, IN) (8 mU) was added and the mixture was incubated overnight at 35°C.

Immunoblotting

After separation on SDS-polyacrylamide gels, proteins were transferred to nitrocellulose membranes overnight at 40 V. The membranes were blocked with 3% gelatin at 30°C for 2 h, and incubated with the purified anti-β1,6-glucan antibodies (0.5 μg/μl) (1:2,500 dilution in PBS containing 0.1% Tween-20) for 1 h at room temperature. The blots were then incubated in peroxidase-conjugated anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) (1:2,500 dilution in PBS containing 0.2% Tween 20) for 45 min at room temperature. The immunoblots were visualized with ECL Western blotting detection reagents (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instruction. For antibody competition experiments, pustulan (β1,6-glucan), laminarin (β1,3-glucan), or mannan with a concentration of 1 mM glucose or mannose equivalent was added to the anti-β1,6-glucan solution. The pustulan, laminarin, and mannan were dissolved in PBS by autoclaving at 110°C for 30 min.

Results

The Molecular Size of α-Agglutinin Increases during Covalent Association with the Cell Wall

We have previously identified three forms of α-agglutinin with molecular sizes >300 kDa: two SDS-soluble forms and a cell wall form. The SDS extracts contain a GPI-anchored form and a form that has lost the fatty acid and inositol moieties from the anchor. These forms are predicted to be intermediates in the cell wall anchorage process (Lu et al., 1994).

Cell wall α-agglutinin was isolated from SDS-extracted cell walls by treatment with laminarinase, which contains β1,3-glucanase as the predominant activity (Fig. 1 A, lane 1) (Van Rinsum et al., 1991; Lu et al., 1994). Laminarinase-mediated proteolysis of α-agglutinin is not detectable under the experimental conditions (Lu et al., 1994). The >300-kD intermediate forms as well as a 140-kD intracellular form of α-agglutinin were immunoprecipitated from the SDS extract of the cells (Fig. 1 B, lane 1). The apparent molecular sizes of the cell wall form and the SDS-soluble forms have not been determined previously; all three forms migrated near the top of 6% SDS-polyacrylamide gels. However, after treatment with endo H, which removes N-linked saccharides, the large forms that are extracted with SDS were reduced to about 200 kD (Fig. 1 B, lane 2), whereas the endo H-treated cell wall form showed a molecular weight of 240–300 kD (Fig. 1 A, lane 2). The apparent higher molecular size of the cell wall form compared with the intermediate forms after endo H treatment, indicates that further modification occurs in the process of covalent cell wall association.

mnn9 cells make mannoproteins without outer mannose polysaccharide chains added to the N-linked core saccharides (Tsai et al., 1984; Tanner and Lehle, 1987). In an *mnn9* mutant, an increase in molecular size of the cell wall form of α-agglutinin relative to precursors was seen even in the absence of endo H treatment (Fig. 1). The SDS extract of the *mnn9* cells contained forms of α-agglutinin with molecular weights of 140 and 225 kD (Fig. 1 B, lane 3). The size of the 140-kD form, which occurs in the ER (Lu et al., 1994), was not affected by the *mnn9* mutation. In accordance with
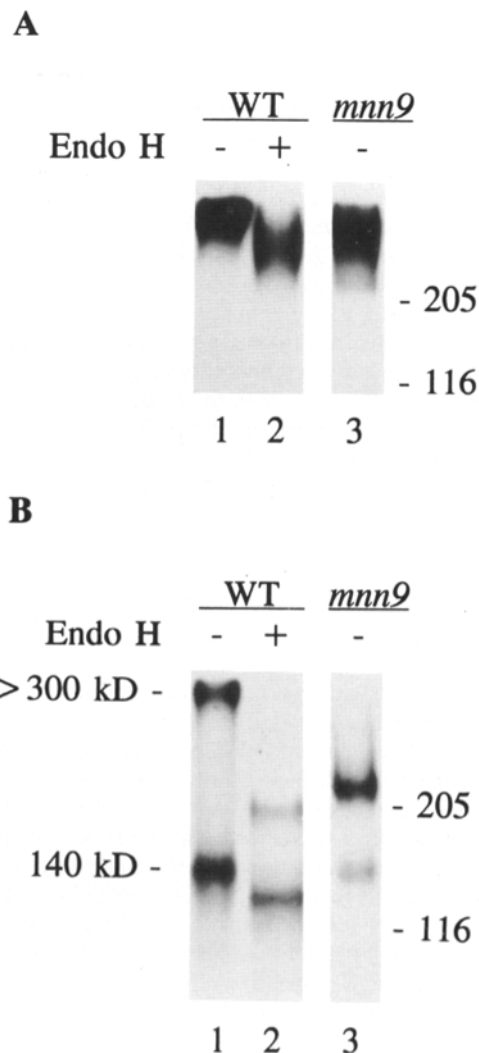
A

B

Figure 1. Molecular size of the cell wall, plasma membrane-bound, and periplasmic forms of α-agglutinin. W303-1B[pAGα1'] cells (*WT*) and LB2134-2B (*mnn9*) cells were labeled with [³⁵S]methionine and broken in SDS lysis buffer. α-Agglutinin was immunoprecipitated from the SDS extracts of the cells and from the laminarinase extracts of the cell walls using anti-α-agglutinin antiserum, and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. (*A*) Laminarinase-extracted cell wall form of α-agglutinin. (*B*) SDS-extracted forms of α-agglutinin (biosynthetic intermediates; Lu et al., 1994). The >300-kD α-agglutinin contains a soluble >300 form and a membrane-bound >300-kD form (Lu et al., 1994). The molecular size standards on the right are labeled in kD.

the *mnn9* phenotype, the molecular size of the 225-kD form from *mnn9* cells was close to that of the de-N-glycosylated soluble large forms of α-agglutinin (200 kD). The cell wall form of α-agglutinin from *mnn9* cells, although migrating faster than the cell wall form from wild type cells, had apparent molecular size of >250 kD (Fig. 1 A, lane 3). The apparent higher molecular size of the cell wall form (>250 kD) compared to the SDS-extractable 225-kD α-agglutinin in *mnn9* cells again indicated that the α-agglutinin was modified before or during anchorage in the cell wall.

kre Mutations Affect Cell Wall Anchorage and Molecular Size of Cell Wall α-Agglutinin

Some β1,3-glucanase-extractable cell wall proteins are covalently linked to β1,6-glucans (Montijn et al., 1994). We speculated that the cell wall anchorage of α-agglutinin resulted from a covalent linkage to the wall β1,6-glucan which was in turn cross-linked to other cell wall components. Therefore, *krel, kre5, kre6, kre9*, and *krel1* gene disruption mutants, which have defects in synthesis of β1,6-glucan, were tested for effects on α-agglutinin.

Assays for functional cell surface α-agglutinin revealed no significant decrease in the agglutinability of the *krel::HIS3* and *krel1::URA3* mutants relative to the isogenic wild-type strain (Table I). This result indicated that α-agglutinin is expressed on the cell surface of *krel::HIS3* and *krel1::URA3* mutants. Since the *kre5::HIS3* and *kre6::HIS3* mutant cells are abnormally large and the *kre9::HIS3* cells formed aggregates, the agglutinability of these two mutants was difficult to quantify using our assay, which measures the optical density of the cell suspension after the aggregates are sedimented (Terrance and Lipke, 1981). However, when *kre6:: HIS3* or *kre9::HIS3* mutant cells were mixed with tester **a** cells, larger aggregates were formed, indicating that these mutant cells are agglutinable.

We next tested the effect of *KRE* gene disruptions on α-agglutinin secretion. Wild type strain SEY6210 secreted a small amount of α-agglutinin into the medium. The secreted α-agglutinin had molecular size >300 kD, which was reduced to 200 kD after endo H treatment (data not shown). The *kre* mutants secreted more α-agglutinin into the growth medium than the wild-type strain. The fraction of the secreted α-agglutinin to the laminarinase-extracted cell wall α-agglutinin increased two- to threefold in the *krel::HIS3, kre6::HIS3, kre9::HIS3*, and *krel1::URA3* mutants (Table I).

kre5 mutants show a severe deficiency in β1,6-glucan syn-

Table I. Effect of kre Gene Disruptions on Cell Surface Expression and Secretion of α-Agglutinin

Genotype	AI*	Fraction of α-agglutinin secreted into medium‡
KRE	0.70	.07
krel::HIS3	0.68	.21
kre5::HIS3	ND	.82
kre6::HIS3	ND	.15
kre9::HIS3	ND	.20
krel1::URA3	0.64	.13

* AI, agglutination index. Higher AI indicates a greater degree of agglutination. The agglutination assay was performed as described previously (Terrance and Lipke, 1991). The tester **a** cells (X2180-1A) were pretreated with α-factor as described (Terrance and Lipke, 1981). ND, not determined.

‡ The *kre* mutants and their isogenic wild type strain (*KRE*) were metabolically labeled with [³⁵S]methionine for 30 min and separated from the media. The cell wall α-agglutinin was solubilized from SDS-extracted 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, as determined by time courses of release of α-agglutinin. The secreted α-agglutinin was immunoprecipitated from the labeling media. The α-agglutinin precipitates were subjected to SDS-polyacrylamide gel electrophorresis followed by fluorography. α-Agglutinin bands were excised from the gel and quantitated by liquid scintillation counting. The fraction of secreted α-agglutinin was defined as the radioactivity of α-agglutinin in the culture medium divided by the sum of the α-agglutinin radioactivity in the medium and that extracted from cell walls. Cell wall α-agglutinin radioactivity was corrected for the efficiency of extraction from the cell wall.

thesis, a growth defect, and aberrant morphology (Meaden et al., 1990). Two *kre5::HIS3 MAT*α strains were obtained by sporulation of a heterozygous diploid. These strains secreted the majority of their α-agglutinin into the growth medium (Table I). Analysis of the cell walls showed <10% of wild type levels of α-agglutinin in the walls (data not shown). Thus, the severe defect in glucan synthesis in the *kre5::HIS3* strains was accompanied by a failure to anchor most of the α-agglutinin into the cell wall.

The α-agglutinin secreted from the *kre* mutants exhibited no mobility difference from that secreted from the wild type cells (data not shown). Despite the increased secretion, a substantial amount of α-agglutinin in *kre* mutants (other than *kre5::HIS3*) remained associated with the cell wall, and could only be released by laminarinase treatment, suggesting that the majority of the α-agglutinin produced in the *krel:: HIS3, kre6::HIS3, kre9::HIS3*, and *krel1::URA3* mutants is anchored in the cell wall.

The increase in α-agglutinin secretion could be a general effect of *kre* mutations on cell wall structure. A *krel* mutant oversecretes proteins normally found in the growth medium (Bussey et al., 1983), and analysis of the proteins in the culture supernatant from wild type and *krel* cells showed increased levels of at least seven other proteins in the *krel* null mutant compared to the wild type strain (data not shown).

To determine whether anchorage of α-agglutinin was specifically affected by the *kre* mutations, we assayed the molecular size of the α-aggutinin from the cell walls of the mutants. Before endo H treatment, the laminarinase-extracted cell wall form of α-agglutinin from the mutants and from the wild type strain migrated on the top of 6% SDS-polyacrylamide gels, and the molecular sizes could not be compared. After endo H treatment, the cell wall α-agglutinin from *krel::HIS3* and *kre9::HIS3* mutants showed molecular sizes of 200–240 and 200–270 kD, respectively, significantly smaller than that from the isogenic wild type strain (Fig. 2 *A*). A slight size reduction of the cell wall α-agglutinin was observed in *kre6::HIS3* and *krel1::URA3* mutants (Fig. 2 *A*). This reduction was observed in several preparations of cell wall α-agglutinin from these mutants. A small amount of endo H resistant material was seen in extracts from all *kre* mutants. It is not clear whether this material represents an endo H–resistant form of α-agglutinin or non-glycosylated proteins that remain associated with α-agglutinin during immunoprecipitation.

The altered cell wall structure of the *krel* mutant is likely to make the cell wall glucans more accessible to added enzymes; therefore the reduced molecular size of the cell wall α-agglutinin from the *krel::HIS3* mutant could have resulted from more extensive digestion by laminarinase during the extraction from the cell wall (Boone et al., 1990). To address this possibility, time courses of laminarinase digestion were performed. Cell walls from *krel::HIS3* cells were treated with laminarinase for various periods of time from 1–16 h. The extracted cell wall α-agglutinin, after endo H treatment, showed no mobility difference over the time course (Fig. 2 *B*). We also monitored laminarinase digestion of α-agglutinin isolated from wild type cells. The cell wall α-agglutinin was solubilized from the wild type cell wall in an initial 2-h incubation, and then was reincubated in the presence of laminarinase to examine whether extended laminarinase digestion would further reduce the size of the wild type cell wall

α-agglutinin. A 16-h incubation with laminarinase yielded α-agglutinin of the same molecular size as that originally isolated (Fig. 2 B). These results imply that the lower molecular size of the cell wall α-agglutinin in the *krel::HIS3* mutant relative to wild type is not due to incomplete laminarinase digestion during the extraction from the wall of wild type cells.

The molecular sizes of the non-cell wall intermediates of α-agglutinin were unaffected by *kre* disruptions. The SDS-extracted >300-kD α-agglutinin from the wild type and mutant strains had an apparent molecular size of 200 kD after endo H treatment (Fig. 2 C). To further test whether the *krel::HIS3* mutation affects only cell wall proteins, the plasma membrane-bound GPI-anchored glycoprotein Gas1p or Ggp1p was immunoprecipitated from the membrane fractions (Nuoffer et al., 1991; Vai et al., 1990; Fankhauser and Conzelmann, 1991). Gas1p from the *krel* mutant and from the wild-type strain exhibited no mobility difference (data not shown). Therefore the *krel::HIS3* mutation altered processing of cell wall-associated α-agglutinin, but did not affect processing of a membrane-associated GPI-anchored glycoprotein.

Cell Wall α-Agglutinin Is Immunoreactive with Anti–β1,6-Glucan Antibodies

The possibility that the cell wall α-agglutinin was covalently linked to cell wall β-glucan was further tested using specific anti–β1,6-glucan antibodies. The antibodies were raised against β1,6-glucan serum albumin conjugate (Montijn et al., 1994) and purified using a β1,6-glucan affinity column. Cell wall α-agglutinin was immunoprecipitated from the laminarinase extract of the cell wall with anti–α-agglutinin antibodies, and the precipitate was analyzed by immunoblotting with anti–β1,6-glucan antibodies. The cell wall form of α-agglutinin was recognized by the anti–β1,6-glucan antibodies both before and after treatment with endo H (Fig. 3 B, lanes 1 and 2). This recognition was competitively inhibited by pustulan (β1,6-glucan) (Fig. 3 C, lanes 1 and 2), but not by laminarin (β1,3-glucan) or mannan (data not shown). These results indicate that the epitope on the cell wall α-agglutinin consists of β1,6-glucan. Cell wall α-agglutinin from the *krel::HIS3* cells was less reactive with the anti–β1,6-glucan antibodies than that from the isogenic wild type strain (Fig. 4), consistent with a reduced size of β1,6-glucan in the mutant.

To determine whether the non-cell wall forms of α-agglutinin contained β1,6-glucan, the soluble precursors were precipitated from the SDS extract of wild type cells with anti–α-aggutinin, and immunoblotted with anti–β1,6-glucan antibodies. The 140 kD and the >300-kD forms, as well as their corresponding endo H-treated forms, did not react with the β1,6-glucan antibodies (Fig. 3 B, lanes 3 and 4), whereas they were strongly stained when reprobed with the α-agglutinin antibodies (Fig. 3 A, lanes 3 and 4). A weak, immunoreactive band migrated on the very top of the gel (Fig. 3 B, lane 3). However, this band is non-specific, because it was not competed by β1,6-glucan (Fig. 3 C, lane 3), did not comigrate with the >300-kD α-agglutinin band, and was also seen in an a cell extract which does not contain α-agglutinin (data not shown). Because the SDS extracts contain two >300-kD forms of α-agglutinin, the cells were
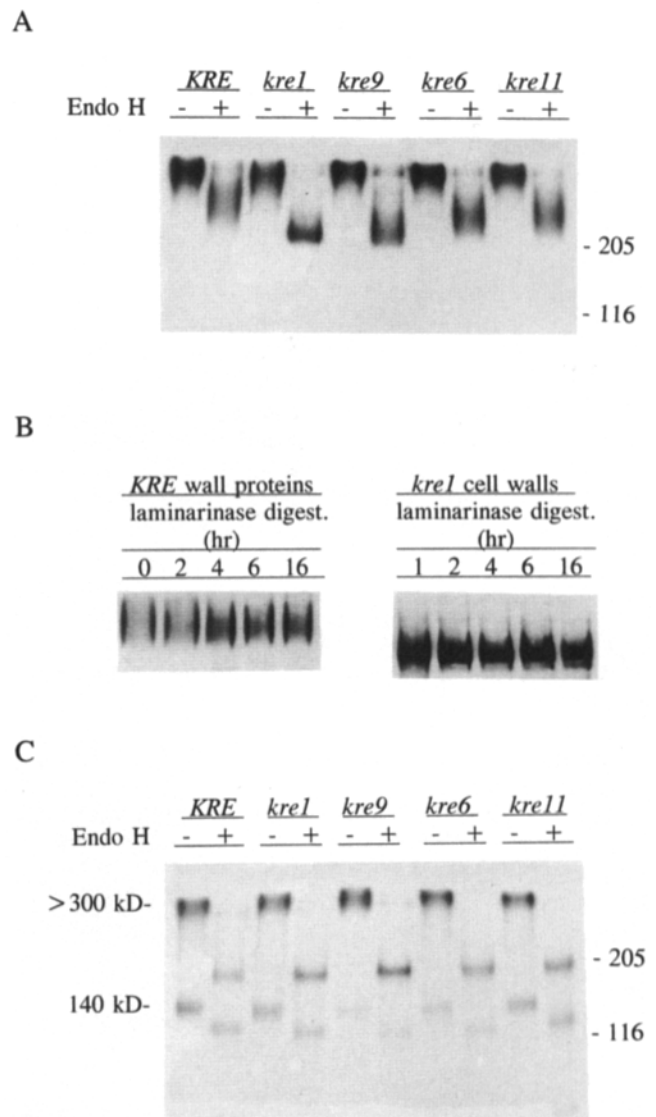
Figure 2. Effect of KRE gene disruptions on the molecular size of α-agglutinin. *kre* mutants and their isogenic wild type strain were labelled with [³⁵S]methionine, lysed, and cell walls were sedimented and treated with laminarinase. (*A*) Laminarinase-extracted cell wall form of α-agglutinin. The cell walls were treated with laminarinase for 4 h. α-Agglutinin was immunoprecipitated from the laminarinase extracts of the cell walls. (*B*) Time course of laminarinase digestion. The cell wall from the *krel* cells was treated with laminarinase for the indicated time. The cell wall from the wild type cells (*KRE*) was digested with laminarinase for 2 h. The solubilized wall proteins from each strain were separated from the undigested cell wall (time 0 h), and incubated in the presence of laminarinase for various periods of time at 35°C. The cell wall α-agglutinin was immunoprecipitated and treated with endo H. (*C*) SDS-extracted intermediate forms of α-agglutinin. α-agglutinin was immunoprecipitated from SDS extracts of *kre* and wild type cells. α-Agglutinin precipitates were analyzed by SDS–polyacrylamide gel electrophoresis followed by fluorography. Equal amounts of radioactivity (cpm) were applied to each lane in each gel. Molecular size standards on the right are indicated in kD, and >300- and 140-kD intermediate forms of α-agglutinin are marked on the left.
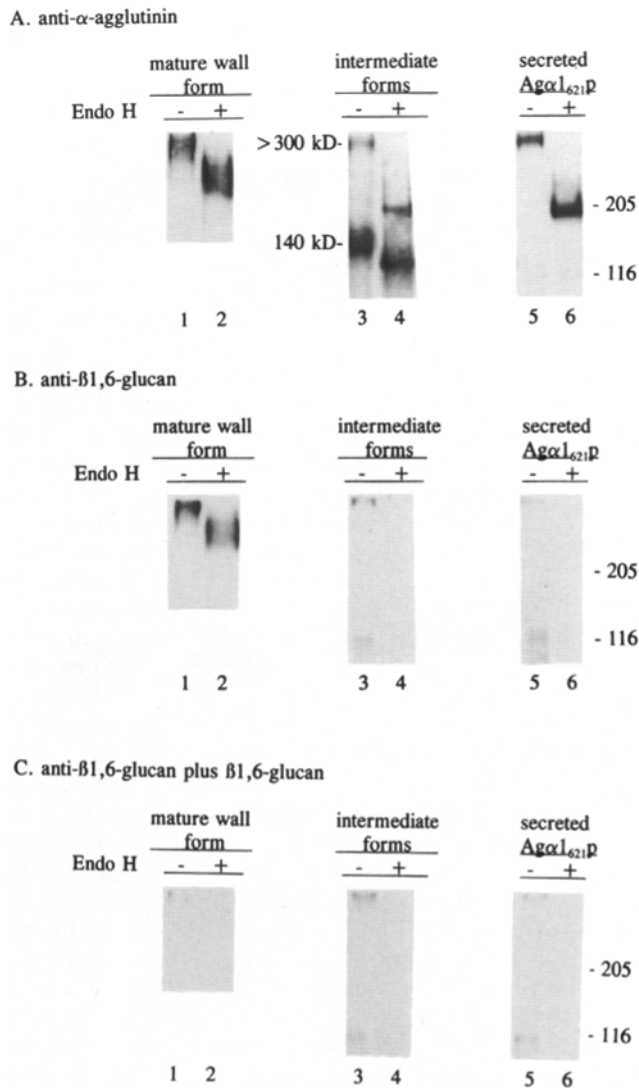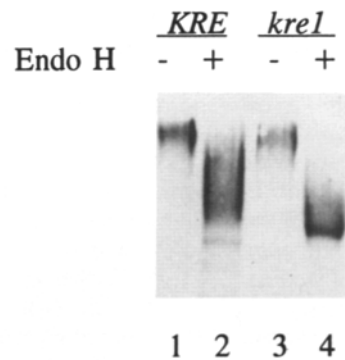
A. anti-α-agglutinin

A. anti-α-agglutinin

B. anti-β1,6-glucan

B. anti-β1,6-glucan

C. anti-β1,6-glucan plus β1,6-glucan

Figure 3. Reaction of α-agglutinin with antibodies to β1,6-glucan. W303-1B[pAGα1'] cells were lysed in SDS lysis buffer. The wall α-agglutinin and the intermediate forms were immunoprecipitated with anti-α-agglutinin antibodies from the laminarinase extracts of the cell walls and the SDS extracts of cells, respectively. The COOH-terminal truncated form of α-agglutinin (Agα1$_{621}$p) was immuno-precipitated from the growth medium of an agα1::LEU2[pAGα1$_{621}$'] strain. (*A*) α-Agglutinin probed with anti-α-agglutinin. (*B*) α-Agglutinin blot probed with anti-β1,6-glucan. (*C*) As in *B*, except that pustulan (β1,6-glucan, 1 mM glucose equivalent) was included in the anti-β1,6-glucan antibody solution. Equal amounts of α-agglutinin precipitate were loaded in the corresponding lanes in *A*, *B*, and *C*.

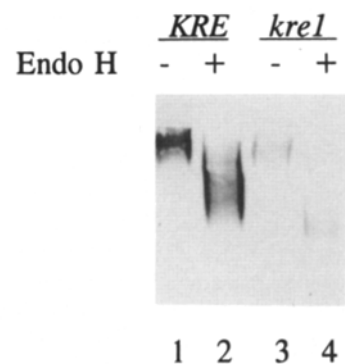*Figure 4.* Effect of *kre1* gene disruption on immunoreactivity of the cell wall α-agglutinin with anti-β1,6-glucan. The *kre1* mutant and its isogenic wild-type strain (*KRE*) were lysed in SDS lysis buffer, and the cell walls were collected and treated with laminarinase. The wall α-agglutinin was immunoprecipitated with anti-α-agglutinin, separated on SDS–polyacrylamide gels, and blotted onto nitrocellulose membranes. The blots were probed with anti-α-agglutinin (*A*) or anti-β1,6-glucan (*B*). Equal amounts of samples were loaded in the corresponding lanes in *A* and *B*.

lysed and fractionated into membrane and soluble protein fractions by ultracentrifugation, which separates the soluble periplasmic >300-kD form from the plasma membrane-bound >300-kD form (Lu et al., 1994). Neither >300-kD form was reactive with the anti-β1,6-glucan (data not shown). These results indicate that the addition of β1,6-glucan occurs at the cell surface after formation of the soluble >300-kD form of α-agglutinin.

A truncation of the COOH-terminal 29–amino acid residues from Agα1p (Agα1$_{621}$p) eliminates cell surface attach-

ment and allows secretion of active α-agglutinin into the medium (Wojciechowicz et al., 1993). The loss of cell surface attachment in the Agα1$_{621}$p apparently results from a defect in GPI anchor addition (Wojciechowicz et al., 1993). We therefore determined whether the *AGα1$_{621}$* gene product was modified by addition of β1,6-glucan. Agα1$_{621}$p was precipitated with anti-α-agglutinin from the growth medium of an agα1::LEU2[pAGα1$_{621}$'] strain. Agα1$_{621}$p exhibited a mobility similar to that of the SDS–extracted >300-kD α-agglutinin from wild-type cells (Fig. 3 *A*, lane 5). After endo H treatment, the size of the Agα1$_{621}$p shifted to 200 kD (Fig. 3 *A*, lane 6). Agα1$_{621}$p did not react with the anti-β1,6-glucan, either before or after endo H treatment (Fig. 3 *B*, lanes 5 and 6). A very weak, β1,6-glucan non-competitive signal was seen at the top of the gel (Fig. 3, *B* and *C*, lane 5). This signal was non-specific, as it was also found in the growth medium of agα1::LEU2 cells without pAGα1$_{621}$' (data not shown). This result suggests that the

GPI anchor is necessary for cross-linkage of the cell wall α-agglutinin and the β1,6-glucan.

Discussion

Three extracellular forms of α-agglutinin have been identified: a GPI-anchored form bound to the plasma membrane, a soluble periplasmic form which has lost the fatty acid and inositol from the GPI anchor, and the mature form that is covalently bound to the cell wall (Lu et al., 1994). We have proposed that the plasma membrane form is released to produce the periplasmic form, which then becomes linked to the cell wall. To examine the mechanism of cell wall attachment, we have further characterized these forms, which were previously indistinguishable in SDS gel analysis due to their large size (>300 kD). After endo H treatment to release N-linked carbohydrate, the cell wall form had a higher molecular size than the plasma membrane-bound and periplasmic forms. In mnn9 cells, which are defective in addition of outer chain N-linked carbohydrate, a similar molecular size difference was detected even in the absence of endo H treatment. This size difference indicates that covalent modification of α-agglutinin occurs either immediately before or during cell wall attachment.

Two approaches demonstrated that α-agglutinin is covalently modified by addition of β1,6 glucan in the process of cell wall anchorage. β1,6-glucan–specific antibodies specifically reacted with cell wall α-agglutinin but not with the plasma membrane or periplasmic forms of α-agglutinin. α-agglutinin was also characterized in kre mutants, which are altered in β1,6-glucan structure and/or levels in the cell wall. Mutations in kre1 and kre9 affect late steps in synthesis of cell wall β1,6 glucan, and show a reduced size and altered structure (Boone et al., 1990; Brown and Bussey, 1993). In these mutants, cell wall α-agglutinin had markedly reduced size after endo H treatment (200–240 and 200–270 kD for kre1 and kre9, respectively, versus 240–300 kD for the wild-type). Cell wall α-agglutinin from kre1 cells was less reactive with anti-β1,6-glucan antibodies in comparison to the wild-type, consistent with the shorter β1,6-glucan polymers found in kre1 mutants.

In addition to the alterations in β1,6-glucan structure discussed above, kre1, kre5, kre6, kre9, and kre11 mutants show reduced amounts of cell wall β1,6-glucan (Brown et al., 1993). The tested kre mutants showed increased secretion of α-agglutinin with a concomitant reduction in the fraction of α-agglutinin bound to the cell wall. The increase in secretion of α-agglutinin was most marked in the strain with the most severe phenotype.

Covalent association of α-agglutinin with β1,6-glucan would provide a mechanism to anchor the glycoprotein to the cell wall matrix. The bonds created during the association of α-agglutinin or other mannoproteins with cell wall glucan are not known, but the structures of the components suggest several possibilities. Glucan could be attached to α-agglutinin through N- or O-linkage or through linkage to the GPI anchor.

Several lines of evidence argue against N- or O-linkage of the glucan to the peptide. Endo H–treated cell wall α-agglutinin remained reactive with the anti-β1,6-glucan antibodies; therefore the β1,6-glucan is not attached to endo H–sensitive N-linked chains. Anchorage of α-agglutinin and bulk man-

noproteins is not affected in a mnn9 mutant, which has severely truncated mannosyl side chains, or by tunicamycin treatment (Wojciechowicz et al., 1993; Lu et al., 1994). In addition, the a-agglutinin anchorage subunit Agalp contains no consensus N-glycosylation sites and appears to contain only O-linked carbohydrate mannosyl chains (Lipke and Kurjan, 1992); therefore unless a- and α-agglutinins use different mechanisms of cell wall attachment, linkage cannot be through a conventional N-linked carbohydrate chain. Attachment through O-linked carbohydrate is not consistent with the alkali resistance of the β-glucan association with mannoprotein (Van Rinsum et al., 1991).

Recently, Schreiner et al. (1994) have reported a direct linkage of β-glucose to asparagine in mammalian laminin. A similar linkage in α-agglutinin would be resistant to endo H and to tunicamycin, and so cannot be ruled out. However, it is not apparent how formation of such a linkage would be dependent on GPI anchor addition in α-agglutinin.

The current results are consistent with β1,6-glucan attachment to a modified GPI anchor. A COOH-terminal truncation of α-agglutinin that eliminates GPI anchor attachment results in secretion of α-agglutinin into the medium (Wojciechowicz et al., 1993) and a lack of recognition by anti-β1,6-glucan antibodies (Fig. 3 A). The periplasmic form of α-agglutinin lacks the inositol and fatty acid components of the GPI anchor but preliminary results indicate that at least the ethanolamine moiety of the GPI anchor remains (Lu, C. F., and P. N. Lipke, unpublished results). Removal of the phosphatidylinositol component of the GPI anchor would leave the COOH-terminal glycan attached to the protein, including ethanolamine phosphate and at least one carbohydrate residue (De Nobel and Lipke, 1994). The reducing end of the GPI-derived glycan would remain with the protein and could become attached to cell wall β1,6-glucan by a transglycosylation reaction (De Nobel and Lipke, 1994). The structure of Candida albicans mannoproteins is consistent with this hypothesis; β1,6-glucan linkage to mannoproteins is through a phosphodiester linkage (Kapteyn et al., 1994), as is the linkage between GPI anchors and proteins. Further support for this hypothesis comes from construction of chimeric proteins (Van Berkel et al., 1994). Addition of the COOH-terminal 30–amino acid residues of α-agglutinin to the secreted marker protein α-galactosidase is sufficient to β-glucosylate and anchor the α-galactosidase in the cell wall of S. cerevisiae. This 30-residue α-agglutinin sequence contains the GPI addition signal, but only a few potential O-glycosylation sites, and no Asn residues.

Processing and assembly of α-agglutinin and β1,6-glucan are complex processes. The cellular locations of the products of KRE and related genes imply that β1,6 glucan assembly is initiated in the ER and proceeds during transport through the secretory pathway (Brown et al., 1993). Krelp appears to be a GPI anchored extracellular protein; therefore final assembly of the glucan may be extracellular. α-Agglutinin is also synthesized in the ER and altered by GPI addition and N- and O-glycosylation in the secretory pathway (Lu et al., 1994). A GPI-anchored form of α-agglutinin of >300 kD is membrane associated and extracellular (Lu et al., 1994). Neither this GPI-anchored α-agglutinin form nor the periplasmic form reacted with anti-β1,6-glucan antibodies and neither form showed size alterations in kre mutants (Figs. 1 and 2). Therefore these forms were not modified by glucan

addition, although glucan is covalently bound to the mature cell wall form. We propose that the periplasmic form of α-agglutinin, and possibly other cell wall mannoproteins, is cross-linked to preformed β1,6-glucan, perhaps by trans-glycosylation of the GPI anchor-derived glycan, and then becomes integrated into the growing cell wall by linkage of β1,6-glucan to β1,3-glucan polymers. The data we have presented suggests that the cross-linking reactions are extracellular, and occur within the periplasmic space and cell wall.

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