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# A Pathway for Cell Wall Anchorage of *Saccharomyces cerevisiae* α-Agglutinin

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***Saccharomyces cerevisiae* α-agglutinin is a cell wall-anchored adhesion glycoprotein. The previously identified 140-kDa form, which contains a glycosyl-phosphatidylinositol (GPI) anchor (D. Wojciechowicz, C.-F. Lu, J. Kurjan, and P. N. Lipke, Mol. Cell. Biol. 13:2554–2563, 1993), and additional forms of 80, 150, 250 to 300, and >300 kDa had the properties of intermediates in a transport and cell wall anchorage pathway. N glycosylation and additional modifications resulted in successive increases in size during transport. The 150- and 250- to 300-kDa forms were membrane associated and are likely to be intermediates between the 140-kDa form and a cell surface GPI-anchored form of >300 kDa. A soluble form of >300 kDa that lacked the GPI anchor had properties of a periplasmic intermediate between the plasma membrane form and the >300-kDa cell wall-anchored form. These results constitute experimental support for the hypothesis that GPI anchors act to localize α-agglutinin to the plasma membrane and that cell wall anchorage involves release from the GPI anchor to produce a periplasmic intermediate followed by linkage to the cell wall.**

The sexual agglutinins of *Saccharomyces cerevisiae* are complementary cell surface glycoproteins expressed by **a** and α haploid cells. Agglutinin levels are increased following treatment with the pheromone produced by cells of the opposite mating type (39, 48, 50), and binding between the α-agglutinin on α cells and the **a**-agglutinin on **a** cells causes aggregation. The aggregation facilitates cell fusion to form the diploid zygote (7). **a**-Agglutinin consists of an anchorage subunit (Aga1p) and a binding subunit (Aga2p), and α-agglutinin consists of a single subunit (Agα1p) (25). The *AGα1* gene contains a domain with significant sequence similarity to members of the immunoglobulin superfamily (49). Because many members of this superfamily are cell adhesion proteins, this homology suggests that a mechanism of cell adhesion may be conserved in yeasts and multicellular eukaryotes (25, 46).

Transport of the agglutinins to the cell surface occurs through the secretory pathway (40, 41). All three agglutinin structural genes contain N-terminal hydrophobic sequences that are likely to act as signal sequences that direct the proteins into the secretory pathway (3, 19, 24, 33). The C termini of *AGα1* and *AGA1* are also hydrophobic (24, 33) and resemble the addition signals for glycosyl-phosphatidylinositol (GPI) anchors (8, 12, 14). Such anchors have been shown or inferred to be present on several other yeast proteins (2, 5, 6, 18, 26, 30, 42). A 140-kDa form of α-agglutinin has been demonstrated to be GPI anchored to membranes, and the C-terminal hydrophobic domain is essential for membrane association of this form and cell surface localization of mature α-agglutinin (49).

Because mature α-agglutinin is anchored in the cell wall rather than to the plasma membrane (1, 19, 23, 35, 51), we have speculated that GPI anchorage of α-agglutinin to membranes may represent a transient stage during transport to the cell wall. To explore the mechanism of cell wall anchorage, we have characterized intermediates in intracellular transport and cell wall anchorage of α-agglutinin.

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## MATERIALS AND METHODS

**Strains and plasmids.** 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 secretion mutants NY432 (*MATα ura3-52 sec18-1*), NY761 (*MATα his4-619 sec7-1*), and NY4 (*MATα his4-619 sec1-1*) and their parental wild-type strain NY191 (*MATα his4-619*) were provided by 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 Robert S. Fuller, and PRY95 [*MATα alg4-4* (*sec53*) *ura3-52*] was provided by Peter Orlean. Plasmid pAGα1′ contains the full-length α-agglutinin gene *AGα1* in YEp352 (49).

**Metabolic labeling of cells.** Metabolic labelings of cells with myoinositol and palmitic acid were done as described previously (49) except that W303-1B cells harboring pAGα1′ were resuspended to a density of $3 \times 10^7$ cells per ml, and [$^3$H]myoinositol (20 mCi/mmol; American Radiolabeled Chemicals) or [$^3$H]palmitic acid (52.4 mCi/mmol; du Pont) was added to 10 or 33 μCi/ml of cell suspension, respectively.

For labeling with [$^{35}$S]methionine, cells were grown in low-sulfate minimal medium to exponential phase, collected, and resuspended to $7 \times 10^7$ cells per ml in sulfate-free medium (32). Labeling was initiated by adding TRAN$^{35}$S-LABEL (ICN Biochemicals) to 15 to 20 μCi/ml of cell suspension and continued for 20 to 30 min. Synthetic **a**-factor (a gift of Fred Naider) was added to 50 ng/ml 10 min prior to the labeling. 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 cells were collected, washed twice with 30 mM Tris-HCl (pH 7.4)–1 mM phenylmethylsulfonyl fluoride (PMSF)–1 mM EDTA, and stored at −70°C.

**Cell lysis and preparation of cell walls, membranes, and soluble protein fraction.** Labeled cells were broken with glass beads in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 30 μg each of leupeptin, pepstatin, and antipain per ml) as described previously (49). Cell walls were collected by spinning at 1,000 × *g* for 5 min at 4°C. The low-speed supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant (soluble protein fraction) was removed, and the pellet (membrane fraction) was suspended in 50 mM Tris-HCl (pH 7.4) containing 2% sodium dodecyl sulfate (SDS) and protease inhibitors as in the lysis buffer. In some experiments, the labeled cells were broken in SDS lysis buffer (lysis buffer containing 2% SDS) and then heated for 5 min at 95°C. The insoluble material (cell wall) was separated from the SDS extract by spinning at 13,600 × *g* for 5 min.

**Laminarinase treatment of isolated cell walls.** The cell wall fraction was extracted twice with hot SDS lysis buffer by heating for 5 min at 95°C each time and washed three times with cold 1 M NaCl containing 1 mM PMSF and three times with 1 mM PMSF in distilled water. The washed cell walls from $1.5 \times 10^8$ cells were treated with 0.3 U of laminarinase (Sigma) in 60 μl of 50 mM sodium acetate (pH 5.5) containing 3 mM PMSF, 2 mM EDTA, and 90 μg each of leupeptin, pepstatin, and antipain per ml at 35°C for 4 h. After treatment, the insoluble material was removed by spinning at 13,600 × *g* for 5 min, and the supernatant was analyzed by immunoprecipitation.

**Treatment of membranes with PI-PLC.** Membranes were prepared from $5 \times 10^8$ W303-1B[pAGα1′] cells. The 1,000 × *g* supernatant was centrifuged at 13,600 × *g* for 15 min at 4°C, and the pellet was resuspended in 400 μl of lysis buffer and split into two portions. One portion was treated with phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* (>90% purity by polyacrylamide gel electrophoresis; provided by Martin Low) for 90 min at 35°C. The other portion of the membrane suspension received mock treatment without the enzyme. Following this incubation, membranes were resedimented by ultracentrifugation. Proteins from the supernatant and the membranes were analyzed by immunoprecipitation.

**Proteinase K treatment of intact cells.** Proteinase treatment was carried out as described by Nuoffer et al. (30), with some modifications. W303-1B cells were labeled with [$^{35}$S]methionine and incubated in 100 mM dithiothreitol (DTT) for 30 min at room temperature (20°C). The cells were incubated with various concentrations of proteinase K, harvested, washed, and lysed in lysis buffer. Cycloheximide (25 μg/ml) was included in both DTT treatment and protease treatment buffers. The cell wall, membrane, and soluble protein fractions were prepared as described above, and α-agglutinin was immunoprecipitated.

**Binding of α-agglutinin to intact cells.** Total 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 adding sodium acetate buffer (pH 5.5) to 100 mM. Cycloheximide (20 μg/ml, final concentration) and bovine serum albumin (BSA; 500 μg/ml, final concentration) were added to the supernatant prior to the incubation with **a** or α cells.

A total of $10^8$ unlabeled **a** cells (X2180-1A) or α cells (X2180-1B) were preincubated with 2 mg of BSA per ml in 50 mM sodium acetate (pH 5.5), 25 μg of cycloheximide per ml, and 1 mM PMSF for 30 min at 30°C, and 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 and 2 mM PMSF. The released labeled proteins were analyzed by immunoprecipitation.

**Immunoprecipitation and immunoblotting.** The soluble protein fraction, SDS extract of membranes, or laminarinase extract of cell walls prepared from $10^8$ labeled cells was adjusted to 1% Triton X-100–0.2% SDS–50 mM Tris-HCl (pH 7.4)–150 mM NaCl–5 mM EDTA–1 mM PMSF–30 μg each of leupeptin, pepstatin, and antipain per ml in a final volume of 700 μl, to which 4 μl of anti-α-agglutinin-antiserum AG3 was added (49). The mixture was incubated overnight at 4°C. For antibody competition experiments, the same amount of antiserum was incubated overnight with purified unlabeled α-agglutinin (120 U). Immune complexes were pelleted by addition of protein A-Sepharose beads, and α-agglutinin was eluted from the beads as described previously (49). Immunoblotting of α-agglutinin was done as described previously (48) except that antibody AG3 was used. Proteins were separated on 6% polyacrylamide gels containing SDS unless otherwise stated (22). The gels were fixed, soaked in 1 M sodium salicylate (4), dried, and exposed to Kodak X-Omat XAR film at −70°C for fluorography.

## RESULTS

**Cell wall anchorage of α-agglutinin.** Mature α-agglutinin is extractable from intact cells by lytic glucanases (19, 35), which contain a mixture of β-glucanases and proteases (51). Schreuder et al. (35) have reported extraction of α-agglutinin from cell walls with laminarinase, a semipurified β1,3-glucanase. These results imply a covalent linkage of α-agglutinin to the cell wall glucan, although liberation of the α-agglutinin could be through action of proteases associated with the glucanases (data not shown) (44, 51).

To extract mature cell wall α-agglutinin, cells were mechanically lysed, and cell walls were collected and extracted with hot SDS to remove contaminating membrane proteins and noncovalently associated cell wall proteins (35). A >300-kDa form of α-agglutinin was extracted by a single SDS treatment (Fig. 1, lane 1), indicating that it was not covalently linked to the cell wall. The remaining cell wall material was treated with laminarinase in the presence of protease inhibitors. Additional >300-kDa α-agglutinin was released from α cells by laminarinase (lanes 4 and 5).

That the laminarinase-extractable α-agglutinin had been covalently bound to the wall was established by several controls. Multiple extractions of isolated walls with hot SDS or with DTT and EDTA released only 30% of the cell wall-associated α-agglutinin. The remaining 70% could be released only by digestion with laminarinase. No laminarinase-mediated proteolysis of $^{14}$C-labeled BSA or of soluble α-agglutinin was detectable under these conditions (data not shown). Laminarinase treatment did not release GPI-bound forms of α-agglutinin from isolated membranes (data not shown) (49). The cell wall form of α-agglutinin was present at a somewhat higher level in α cells containing the high-copy-number plasmid pAGα1′ (lane 4 versus lane 5) and was not detected in **a** cells (lane 6) or when the antiserum was preincubated with purified α-agglutinin (lane 3). These results indicate a covalent linkage of a portion of the >300-kDa form of α-agglutinin with the cell wall β-glucan and are consistent with previous reports of the cell wall linkage of α-agglutinin (19, 23, 35, 44).

**Forms of α-agglutinin in *sec* mutants.** To identify potential

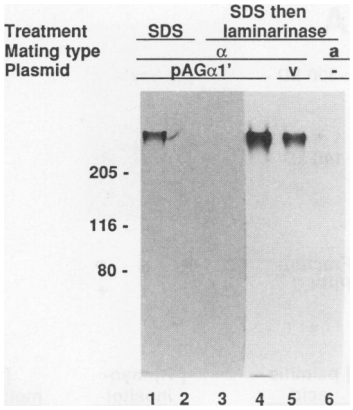


FIG. 1. Cell wall form of α-agglutinin. Yeast strains W303-1A (**a**) and W303-1B (α) were labeled with [$^{35}$S]methionine and broken in lysis buffer with glass beads. Cell walls were collected, extracted twice with hot SDS, and subsequently treated with laminarinase. α-Agglutinin was immunoprecipitated from the SDS extracts or laminarinase extracts of the cell walls by using antiserum AG3 and were analyzed by polyacrylamide gel electrophoresis and fluorography. Lane 1, first SDS extract; lane 2, second SDS extract; lanes 3 to 6, laminarinase extracts (lane 3 is the same as lane 4 except that the antiserum was preincubated with purified unlabeled α-agglutinin). Molecular size standards on the left are indicated in kilodaltons. v, vector; pAGα1′, high-copy-number *AGα1* plasmid.

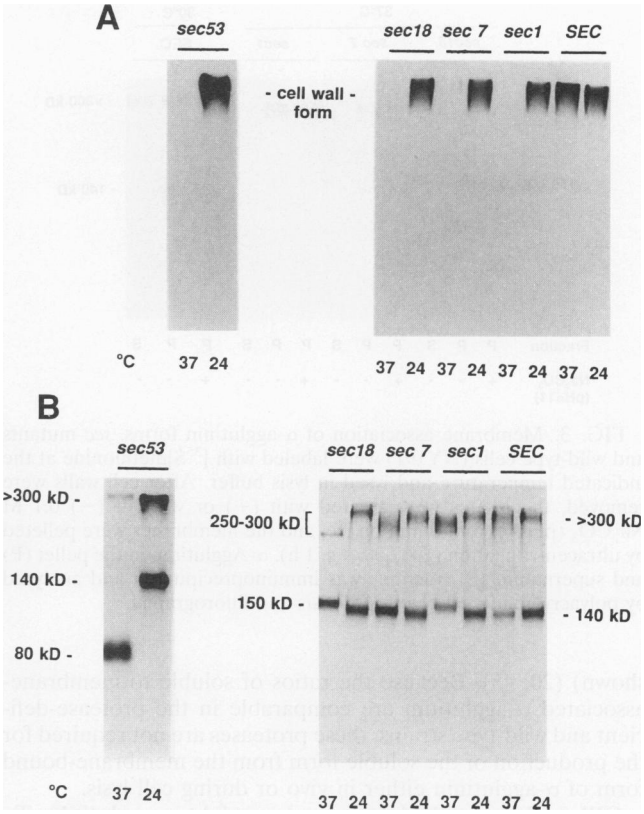


FIG. 2. α-Agglutinin forms accumulated in *sec* mutants. (A) *sec* mutants and the wild-type strain (NY191) were preincubated at 24 or 37°C for 30 min and were retained at the same temperature during labeling with [$^{35}$S]methionine. After lysis in SDS lysis buffer, the insoluble cell walls were collected and treated with laminarinase. α-Agglutinin was immunoprecipitated from the laminarinase extracts, separated on SDS-polyacrylamide gels, and visualized by fluorography. (B) α-Agglutinin from SDS extracts of *sec* mutant and wild-type cells was immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. In both panels, α-agglutinin from *sec53* cells was separated on 6% polyacrylamide gels, and α-agglutinin from other strains was separated on 5% polyacrylamide gels.

intermediates in transport and cell wall anchorage of α-agglutinin, we used temperature-sensitive *sec* mutants that block secretory protein transport at specific steps at the restrictive temperature. *sec53* mutants have a temperature-sensitive phosphomannomutase, resulting in an inability to incorporate N- or O-linked saccharides or GPI anchors into glycoproteins at the restrictive temperature (5, 32). *sec18* mutants were initially shown to be blocked in endoplasmic reticulum (ER)-to-Golgi transport (17, 29); the defect in this mutant is in NSF, a protein required for vesicle fusion, resulting in blockage in a pre-Golgi compartment (10, 17, 47). *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 (13, 15, 27–29, 34). Incubation at the restrictive temperature blocked transport of α-agglutinin to the cell wall in all four *sec* mutants (Fig. 2A), whereas transport was not temperature dependent in the wild-type strain.

A 140-kDa form of α-agglutinin was extracted by SDS from the wild-type strain and all of the *sec* mutants at the permissive temperature (Fig. 2B). This form was previously shown to be released from the lipid phase of a Triton X-114 partition by PI-PLC treatment and could be metabolically labeled with myoinositol and palmitate (49), indicating that it is GPI anchored. A >300-kDa form of α-agglutinin was also extracted by SDS treatment of the wild type or the *sec* mutants grown at the permissive temperature (Fig. 2B).

Labeling of the *sec* mutants at the restrictive temperature identified alternative bands in the SDS extracts (Fig. 2B). The *sec53* mutant contained an 80-kDa form of α-agglutinin. The *sec18* mutant showed a major 150-kDa form and a minor 250-kDa form (Fig. 2B). The *sec7* mutant showed the 140-kDa form and an additional major form that migrated between 250 and 300 kDa. The *sec1* mutant accumulated forms of 250 to 300 kDa and 150 kDa (Fig. 2B). Accumulation of these forms in *sec* mutants suggests that they could be intermediates in transport of α-agglutinin.

**Membrane association of various forms of α-agglutinin.** To investigate whether the different forms of α-agglutinin expressed by wild-type cells and *sec* mutants were membrane associated, cell membranes were separated from soluble proteins by ultracentrifugation (100,000 × *g*, 1 h). The 140-kDa GPI-anchored form, the 150-kDa form, and at least 90% of the 250- to 300-kDa form sedimented with membranes and were not removed from the membrane fraction by treatment with 0.1 M $Na_2CO_3$ (pH 11) (Fig. 3), indicating that they are tightly membrane bound (16). In contrast, the >300-kDa form was found in both the soluble and membrane fractions (Fig. 3); about 40 to 60% of this form distributed to the soluble fraction in experiments with several wild-type strains, and the ratio did not vary in the presence or absence of the high-copy-number pAGα1′ (data not shown). The soluble >300-kDa protein exhibited no obvious mobility difference from the sedimentable >300-kDa protein either on 6% polyacrylamide gels (Fig. 3 and 4B) or on 4 to 15% gradient polyacrylamide gels (data not shown). About 50% of the >300-kDa form was soluble in two mutants with reduced protease activity: a *pep4/prb1* double mutant, which lacks vacuolar proteinase A and proteinase B, and a *kex2* mutant, which is deficient in a Golgi-associated protease that cleaves some protein precursors (data not

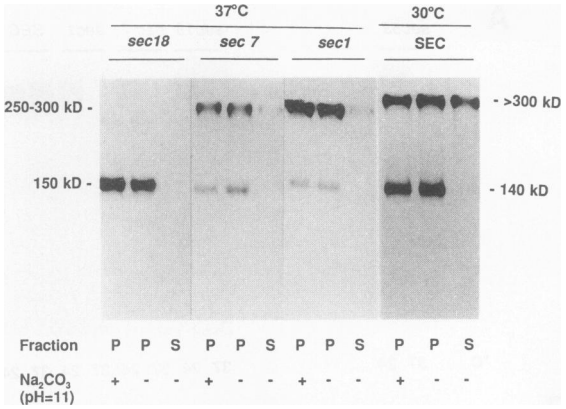


FIG. 3. Membrane association of α-agglutinin forms. *sec* mutants and wild-type cells (NY191) were labeled with [$^{35}$S]methionine at the indicated temperature and lysed in lysis buffer. After cell walls were removed, the lysates were treated with (+) or without (−) 0.1 M $Na_2CO_3$ (pH 11) for 30 min on ice, and the membranes were pelleted by ultracentrifugation (100,000 × *g*, 1 h). α-Agglutinin in the pellet (P) and supernatant (S) fractions was immunoprecipitated and analyzed by polyacrylamide gel electrophoresis and fluorography.

shown) (20, 45). Because the ratios of soluble to membrane-associated α-agglutinin are comparable in the protease-deficient and wild-type strains, these proteases are not required for the production of the soluble form from the membrane-bound form of α-agglutinin either in vivo or during cell lysis.

**GPI anchorage of high-molecular-weight α-agglutinin.** To determine whether membrane association of the sedimentable >300-kDa form of α-agglutinin (Fig. 3) was mediated by a GPI anchor, the susceptibility of this protein to cleavage by PI-PLC was tested. Total membranes were isolated, resuspended, treated with bacterial PI-PLC, and resedimented by ultracentrifugation. The majority of the 140- and >300-kDa proteins were released from the membrane fraction to the soluble fraction by PI-PLC treatment (Fig. 4A). The phospholipase C resistance of the remainder of the α-agglutinin was apparently not due to modification of the α-agglutinin, as Triton X-114-extracted 140-kDa form of α-agglutinin was quantitatively released to the aqueous phase by phospholipase C (49).

GPI-anchored proteins, including the 140-kDa form of α-agglutinin, can be metabolically labeled with inositol and fatty acids (14, 49). W303-1B[pAGα1′] cells were labeled with [$^3$H]myoinositol or [$^3$H]palmitic acid, and cell membranes and soluble protein fractions were separated by ultracentrifugation. Both inositol- and palmitate-labeled 140- and >300-kDa forms of α-agglutinin were immunoprecipitated from the membrane fractions (Fig. 4B). 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 both the membrane-bound >300-kDa form and the 140-kDa form of α-agglutinin are covalently attached by an inositol-containing phospholipid, consistent with the presence of a GPI anchor.

In contrast to the membrane-bound forms, no labeled α-agglutinin was immunoprecipitated from the soluble fraction from either the myoinositol or the palmitic acid labeling (Fig. 4B). This fraction contained about 60% of the >300-kDa α-agglutinin, as determined by [$^{35}$S]methionine labeling (Fig. 4B). The soluble >300-kDa form of α-agglutinin therefore does not have an intact GPI anchor. Similarly, no myoinositol- or palmitate-derived label was detected in the cell wall forms of α-agglutinin (data not shown).

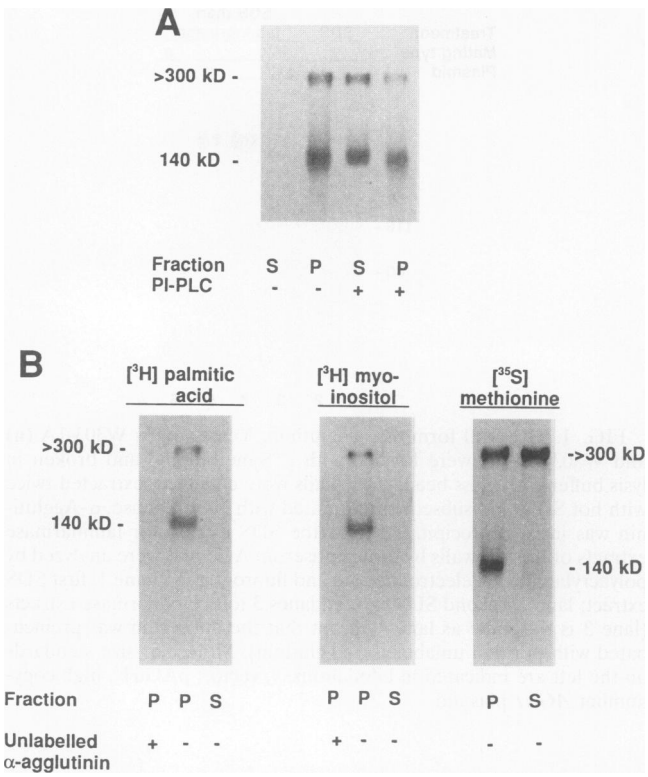


FIG. 4. Testing for the linkage of a GPI anchor to α-agglutinin forms. (A) Release of α-agglutinin from membranes by PI-PLC treatment. The membrane pellet prepared from [$^{35}$S]methionine-labeled W303-1B[pAGα1′] was resuspended in lysis buffer, treated without (−) or with (+) bacterial PI-PLC, and resedimented by ultracentrifugation. α-Agglutinin in the supernatant (S) and membrane pellet (P) was analyzed by immunoprecipitation and fluorography. (B) Incorporation of [$^3$H]myoinositol and [$^3$H]palmitic acid into α-agglutinin. W303-1B[pAGα1′] cells were metabolically labeled with [$^3$H]myoinositol, [$^3$H]palmitic acid, or [$^{35}$S]methionine and lysed in lysis buffer. After cell walls were removed, the lysate was fractionated into supernatant (S) and membrane pellet (P) by ultracentrifugation. α-Agglutinin was immunoprecipitated and analyzed by polyacrylamide gel electrophoresis and fluorography.

**Activity of the 140- and >300-kDa forms of α-agglutinin.** Secreted proteins are folded and assembled in the ER shortly after synthesis and translocation; monomeric proteins are often active at this stage (31). If the 140- and >300-kDa 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 (38, 39). To test for activity, $^{35}$S-labeled membrane-bound α-agglutinin was released with PI-PLC and incubated with **a** or α cells and then eluted from the cells with SDS. The labeled 140- and >300-kDa proteins were able to bind to **a** cells but not to α cells (Fig. 5). Unlabeled α-agglutinin competed for binding of both proteins to **a** cells (Fig. 5). This selective binding indicated that both forms of α-agglutinin were biologically active. The activity of the 140-kDa form of α-agglutinin is consistent with the previous finding that complete N-linked oligosaccharide chains are not required for the activity of α-agglutinin (38). In a similar experiment, the 125- and 200-kDa GPI-linked forms of α-agglutinin from tunicamycin-treated cells were also active (data not shown).

**N glycosylation of α-agglutinin.** Labeling of wild-type cells in the presence of tunicamycin, which inhibits N glycosylation,

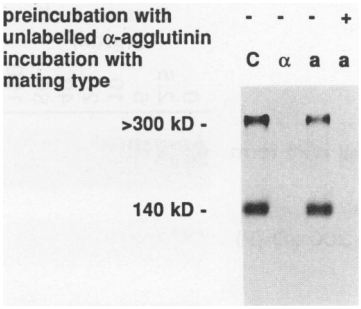


FIG. 5. **a**-Cell-specific binding of the 140- form and >300-kDa forms of α-agglutinin. PI-PLC-released membrane proteins prepared from [$^{35}$S]methionine-labeled W303-1B[pAGα1′] cells were incubated with unlabeled **a** or α cells. The cells were collected, and the bound labeled proteins were released from the cells, immunoprecipitated, separated on a polyacrylamide gel, and fluorographed. C, labeled α-agglutinin before incubation with cells.

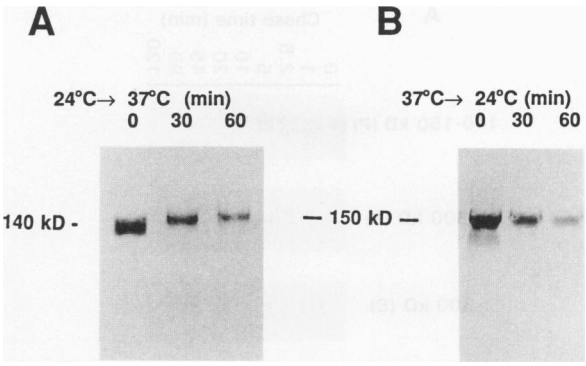


FIG. 7. Processing of the 140- and 150-kDa forms of α-agglutinin in a *sec18* mutant. (A) *sec18* cells containing pAGα1′ were grown at 24°C and shifted to 37°C in the presence of 100 μg of cycloheximide per ml. (B) *sec18* cells were incubated at 37°C for 2 h and then shifted back to 24°C in the presence of cycloheximide. In both panels, at the indicated times, aliquots of cells were harvested and lysed. Total protein was extracted with Triton X-114 followed by phase separation (16), and membrane proteins in the detergent phase were precipitated by trichloroacetic acid and analyzed by immunoblotting with antiserum AG3. The >300-kDa forms of α-agglutinin segregate to the aqueous phase in Triton X-114 and were not observed in this experiment (49).

decreased the molecular sizes of the 140- and >300-kDa forms of α-agglutinin to 125 and about 200 kDa, respectively (Fig. 6). Digestion of the 140- and >300-kDa forms with endo-*N*-acetylglucosaminidase H resulted in similar shifts in mobility for both forms (data not shown). Tunicamycin treatment of *sec* mutants at the restrictive temperature resulted in a shift of the 150-kDa form in *sec18* cells to 135 kDa and of the 140-kDa minor form in *sec7* to about 125 kDa (Fig. 6). More than 90% of the 250- to 300-kDa form in *sec7* and *sec1* mutants shifted to about 200 kDa, with the protein from tunicamycin-treated *sec7* cells being slightly smaller than that from *sec1* cells (Fig. 6). Each of the *sec* mutants also showed minor high-molecular-weight bands in the presence of tunicamycin. These bands probably represent other proteins that coprecipitate with α-agglutinin, since they are not reactive in immunoblots of the precipitated α-agglutinin. The sizes of α-agglutinin in the presence of tunicamycin indicate that the 140- and >300-kDa forms of α-agglutinin contain N-linked carbohydrates but that additional modifications are also present.

**Relationship of the 140- and 150-kDa forms of α-agglutinin.** Temperature shift experiments were done to investigate the relationship of the 140-kDa form of α-agglutinin expressed by wild-type strains and by the *sec* mutants at the permissive temperature and the 150-kDa form accumulated by the *sec18* mutant cells at the restrictive temperature. *sec18* cells shifted from the permissive temperature to the restrictive temperature in the presence of cycloheximide to inhibit new protein synthesis. The 140-kDa α-agglutinin was detected immediately after the temperature shift and was converted to the 150-kDa form within 30 min (Fig. 7A). In a reciprocal experiment, *sec18* cells were shifted from the restrictive to the permissive temperature in the presence of cycloheximide. The 150-kDa form of α-agglutinin gradually disappeared without appearance of the 140-kDa form (Fig. 7B). These results indicate that the 140-kDa form of α-agglutinin is a precursor of the 150-kDa form and that the conversion to 150-kDa form occurs in the secretory pathway before the *sec18* block.

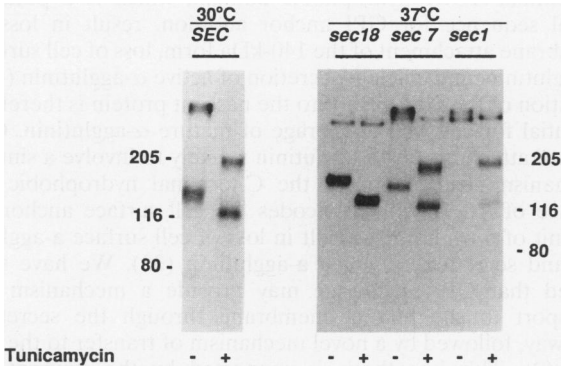


FIG. 6. Analysis for N glycosylation of α-agglutinin. *sec* mutants and the wild-type strain (NY191) were labeled with [$^{35}$S]methionine at the indicated temperature in the absence (−) or presence (+) of tunicamycin (15 μg/ml). α-Agglutinin was immunoprecipitated from SDS extracts of the cells and analyzed by polyacrylamide gel electrophoresis and fluorography. Tunicamycin was added 30 min prior to labeling (39). Molecular sizes are indicated in kilodaltons.

**Kinetics of α-agglutinin secretion.** A pulse-chase experiment was performed to determine the temporal relationship among the forms of α-agglutinin detectable in wild-type cells. Cells were pulse-labeled with [$^{35}$S]methionine and chased with excess unlabeled methionine, cysteine, and sulfate. The cells were broken, the cell walls, membranes, and soluble fractions were isolated, and α-agglutinin was immunoprecipitated and analyzed by polyacrylamide gel electrophoresis. The 140-kDa form of α-agglutinin was present immediately following the pulse and was gradually converted to the 150-kDa form, most of which disappeared by 20 min (Fig. 8). The membrane-bound >300-kDa protein peaked at 5 to 10 min and gradually disappeared. The soluble >300-kDa protein appeared by 5 min, reached a maximal level at 10 to 20 min, and gradually decreased. The cell wall form of α-agglutinin was also first detected at 10 min and reached a maximal level by about 45 min. These kinetic results are consistent with the non-cell wall forms of α-agglutinin present in wild-type cells being intermediates of mature cell wall anchored α-agglutinin.

**Cellular location of the >300-kDa forms of α-agglutinin.** The blockage of α-agglutinin transport before formation of the >300-kDa forms in the *sec1* mutant at the restrictive temperature suggested that these forms were localized at the cell surface. The susceptibility of these forms to exogenous protease treatment of intact cells was therefore tested. Cells were pretreated with DTT to facilitate access of exogenous protease

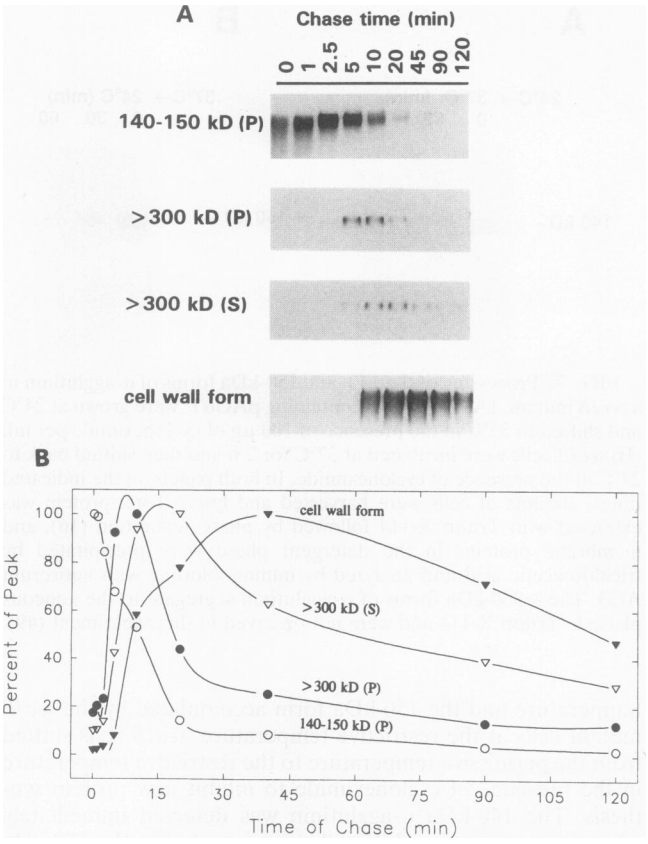


FIG. 8. Pulse-chase analysis of α-agglutinin maturation. (A) W303-1B[pAGα1′] cells were pulse-labeled with [$^{35}$S]methionine for 2 min and chased by addition of excess unlabeled methionine, cysteine, and sulfate. Aliquots of cells were harvested at the indicated times and lysed in lysis buffer. Cell walls were collected and treated with laminarinase. The lysate was fractionated into supernatant and membrane pellet by ultracentrifugation. α-Agglutinin was immunoprecipitated and analyzed by polyacrylamide gel electrophoresis and fluorography. P, membrane-bound forms; S, soluble >300-kDa form. (B) Quantitative analysis of pulse-chase results from panel A. Bands were excised, 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 2,800 cpm for the 140- to 150-kDa form (○), 590 cpm for the membrane-bound >300-kDa form (●), 308 cpm for the soluble >300-kDa form (▽), and 2,080 cpm for the cell wall form (▼).

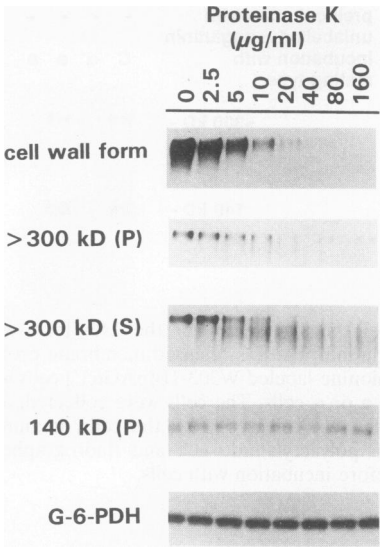


FIG. 9. Proteinase K treatment of intact cells. [$^{35}$S]methionine-labeled W303-1B cells were pretreated with DTT and incubated with different amounts of proteinase K. Cells were then lysed in lysis buffer, cell walls, membranes, and soluble protein fraction were prepared, and α-agglutinin was immunoprecipitated from each fraction. Glucose-6-phosphate dehydrogenase (G-6-PDH) was precipitated from the soluble fraction by using anti-G-6-PDH. The immunoprecipitates were analyzed by polyacrylamide gel electrophoresis and fluorography. P, membrane-bound forms of α-agglutinin; S, soluble >300-kDa form.

to the plasma membrane, incubated with different concentrations of proteinase K, lysed, and fractionated into cell wall, membrane, and soluble protein fractions. The level of glucose-6-phosphate dehydrogenase was also assayed to monitor plasma membrane integrity. This enzyme is sensitive to proteinase K (data not shown). With increasing proteinase K concentration, the cell wall α-agglutinin progressively disappeared (Fig. 9). Both the membrane-bound and soluble >300-kDa forms were converted to lower-molecular-weight species at low proteinase K concentrations and decreased in level at higher concentrations. These results suggest that the >300-kDa forms of α-agglutinin are exposed on the external face of the plasma membrane. In contrast, 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. The level of the 140-kDa α-agglutinin was not altered by increasing proteinase K concentrations, consistent with the intracellular localization of this form.

## DISCUSSION

The mechanism of attachment of cell wall proteins is not well understood. The sexual agglutinins are well-characterized cell wall proteins, for which functional assays are available, and can be used to study the attachment process. The release of mature α-agglutinin from cell walls by β-glucanase but not by SDS extraction suggests that it is covalently bound to the glucan moiety of the wall (Fig. 1) (35).

We previously identified an intracellular 140-kDa form of α-agglutinin that contains a GPI anchor. Truncations of the C-terminal *AGα1* hydrophobic domain, which is a putative signal sequence for GPI anchor addition, result in loss of membrane attachment of the 140-kDa form, loss of cell surface α-agglutinin, and efficient secretion of active α-agglutinin (49). Addition of the GPI anchor to the nascent protein is therefore essential for cell wall anchorage of mature α-agglutinin. Cell surface attachment of **a**-agglutinin is likely to involve a similar mechanism; truncations of the C-terminal hydrophobic sequence of *AGA1*, which encodes the cell surface anchorage subunit of **a**-agglutinin, result in loss of cell surface **a**-agglutinin and secretion of active **a**-agglutinin (33). We have suggested that GPI anchorage may provide a mechanism for transport to the plasma membrane through the secretory pathway, followed by a novel mechanism of transfer to the cell wall (9). This hypothesis is supported by the experiments reported here; a summary of the proposed pathway is shown in Fig. 10.

The earliest proposed α-agglutinin intermediate was seen at the restrictive temperature in a *sec53* mutant, which is blocked in addition of O-linked, N-linked, and GPI oligosaccharides (5). The apparent molecular weight of 80,000 is close to the

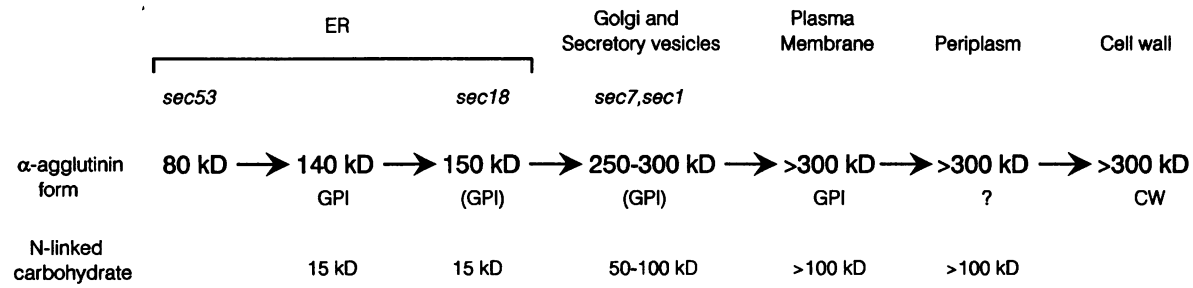


FIG. 10. Model for processing of α-agglutinin. The apparent sizes of α-agglutinin forms are indicated. The size contributed by N-linked carbohydrate was determined by a comparison of the apparent sizes with and without tunicamycin treatment. The 140- and >300-kDa forms were detected in wild-type strains and *sec* mutants at the permissive temperature. The *sec* mutants in which particular forms were detected at the restrictive temperature are indicated, and the positions of all forms within the secretory pathway are inferred from the known positions of the blocks in the *sec* mutants. The >300-kDa band includes membrane-bound, soluble periplasmic, and cell wall-anchored forms as described in the text. The 140- and >300-kDa membrane-linked forms have been demonstrated to contain GPI anchors. Because the 150- and 250- to 300-kDa forms are proposed to be intermediate between the two demonstrated GPI-anchored forms, we propose that they also contain GPI anchors. The periplasmic >300-kDa form was not labeled by inositol or fatty acid but might retain a portion of the GPI anchor that does not contain either of these labels. The >300-kDa cell wall-associated form (CW) is likely to correspond to mature α-agglutinin.

predicted size of 68 kDa of the *AGα1* product without the N-terminal signal sequence, because the high Ser and Thr content of *AGα1* should increase the apparent size in the Laemmli gel system (11). The size of a fusion protein of α-agglutinin expressed in *Escherichia coli* was similar (47a). This 80-kDa form is likely to represent the unmodified peptide and therefore to occur within the ER.

The 140-kDa 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 in wild-type cells implies that it is processed slowly and provides the rate-limiting step in the intracellular transport of α-agglutinin. The size shift of this form after growth in the presence of tunicamycin indicates that it is N glycosylated. Because O glycosylation also occurs shortly after translation and translocation in yeast cells, O glycosylation is also likely to contribute to the apparent size increase from the 80-kDa form (5, 21, 37).

The *sec18* mutant accumulated a 150-kDa membrane-bound form of α-agglutinin at the restrictive temperature (Fig. 2B). This form was also observed in the pulse-chase experiment with wild-type cells (Fig. 8). The 140-kDa form precedes the 150-kDa form (Fig. 7 and 8), suggesting that the 140-kDa form of α-agglutinin is modified to produce the 150-kDa form. Because *sec18* blocks transport of proteins from the ER to the Golgi complex, this modification is likely to occur within the ER (Fig. 10).

The *sec7* and *sec1* mutants accumulated membrane-bound forms of α-agglutinin with molecular sizes of 250 to 300 kDa at the restrictive temperature (Fig. 2B). These mutations block protein transport at the level of the Golgi complex and secretory vesicles, respectively; therefore, these α-agglutinin forms are proposed to occur in the Golgi and a post-Golgi compartment. The slightly higher average mobility of the α-agglutinin form present in *sec7* cells in comparison with wild-type and *sec1* cells (Fig. 2B) is consistent with its containing somewhat less carbohydrate due to arrest in an earlier secretory compartment. A similar underglycosylation has been observed for invertase in a *sec7* mutant (15).

In addition to the 140-kDa form of α-agglutinin, a >300-kDa 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 (Fig. 3 and 4B). The absence of these forms and the cell wall form in the late *sec* mutant (*sec1*) at the restricted temperature suggests that they are produced in an extremely late intracellular compartment or at the cell surface. The membrane-linked >300-kDa form is GPI anchored; it was released from the membrane by a PI-PLC and was labeled by both inositol and fatty acid. The susceptibility of this form to exogenous protease treatment of intact cells indicates that it is exposed at the external face of the plasma membrane. The soluble >300-kDa form had the properties of a periplasmic protein; it was sensitive to the exogenous protease digestion of intact cells (Fig. 9). This form was not labeled by inositol or fatty acid (Fig. 4B), indicating that it was not associated with an intact GPI anchor. The failure to label cell wall α-agglutinin with palmitate or inositol is consistent with this result.

Kinetic analyses (Fig. 7 and 8) were consistent with an order of processing of 140-kDa → 150-kDa → >300-kDa GPI-anchored → >300-kDa periplasmic → >300-kDa cell wall forms of α-agglutinin. Because the 150- and 250- to 300-kDa forms were membrane associated and had the kinetic properties of intermediates between the 140- and >300-kDa GPI-anchored forms, we propose that the 150- and 250- to 300-kDa forms are also GPI anchored. The 250- to 300-kDa 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 (39).

Incubations with tunicamycin allowed determination of the sizes of the N-linked carbohydrate present on each of the forms (Fig. 10). The amounts of N-linked carbohydrate increased as the proteins proceeded to later stages of the secretory pathway, as would be predicted for intermediates in transport. At early steps of the proposed pathway, there were significant increases in size that were not affected by tunicamycin; the 80-kDa → 140-kDa increment included only 15 kDa of N-linked carbohydrate, the 140-kDa → 150-kDa increment did not include any increase in N-linked carbohydrate content, and the 150-kDa → 250- to 300-kDa increment included an increase of only 35 to 85 kDa in N-linked carbohydrate. Possible modifications to explain the size differences include addition and modification of the GPI anchor, extension of O-linked carbohydrate, addition of tunicamycin-insensitive, glucose-containing N-linked oligosaccharide, and phosphorylation (5, 9, 25a, 36, 43).

The forms of α-agglutinin identified in this study can be ordered in a pathway for cell wall localization (Fig. 10). The

properties of these forms constitute the first experimental support for an anchorage mechanism that we have proposed (9, 25). GPI anchored forms have the properties of intermediates in processing of α-agglutinin, and four of the intermediates (140 kDa, 150 kDa, membrane-bound >300 kDa, and soluble >300 kDa) were observable in wild-type cells. Two of these forms were extracellular, a location probably unique to intermediates in localization of cell wall mannoproteins. We have also begun to characterize the modifications of α-agglutinin that accompany intracellular transport and anchorage, including the initial GPI addition, extent of N glycosylation, and possible modification of the GPI anchor. Further characterization to determine the mechanism of transfer from the plasma membrane to the cell wall is of considerable interest (9). This mechanism could potentially be involved in localization of other wall proteins as well as the **a**- and α-agglutinins.

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