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Cell Surface Anchorage and Ligand-Binding Domains of the *Saccharomyces cerevisiae* Cell Adhesion Protein α-Agglutinin, a Member of the Immunoglobulin Superfamily

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α-Agglutinin is a cell adhesion glycoprotein expressed on the cell wall of *Saccharomyces cerevisiae* α cells. Binding of α-agglutinin to its ligand a-agglutinin, expressed by a cells, mediates cell-cell contact during mating. Analysis of truncations of the 650-amino-acid α-agglutinin structural gene *AGα1* delineated functional domains of α-agglutinin. Removal of the C-terminal hydrophobic sequence allowed efficient secretion of the protein and loss of cell surface attachment. This cell surface anchorage domain was necessary for linkage to a glycosyl phosphatidylinositol anchor. A construct expressing the N-terminal 350 amino acid residues retained full a-agglutinin-binding activity, localizing the binding domain to the N-terminal portion of α-agglutinin. A 278-residue N-terminal peptide was inactive; therefore, the binding domain includes residues between 278 and 350. The segment of α-agglutinin between amino acid residues 217 and 308 showed significant structural and sequence similarity to a consensus sequence for immunoglobulin superfamily variable-type domains. The similarity of the α-agglutinin-binding domain to mammalian cell adhesion proteins suggests that this structure is a highly conserved feature of adhesion proteins in diverse eukaryotes.

Cell surface glycoproteins are involved in cell-cell adhesion in many eukaryotic systems. In the yeast *Saccharomyces cerevisiae*, haploid cells of **a** and α mating types express adhesion glycoproteins called **a**-agglutinin and α-agglutinin, respectively. Binding between these complementary cell wall proteins mediates aggregation of cells during mating (for reviews of agglutinin structure and function, see references 26 and 50). The agglutinins facilitate mating under conditions that do not promote cell-cell contact (28, 35, 38).

Both of the *S. cerevisiae* agglutinins and their structural genes have been characterized (26). **a**-Agglutinin contains a high proportion of O-linked carbohydrate and consists of two subunits, a core subunit (Aga1), which mediates cell surface attachment, and a binding subunit (Aga2), which interacts with α-agglutinin (5, 25, 35, 51). α-Agglutinin is composed of a single polypeptide encoded by the *AGα1* gene (16, 28). Treatment of α-agglutinin with endoglycosidase H (endo H) indicates that it is composed of about 50% N-linked carbohydrate. The endo H-treated protein retains some carbohydrate, which is likely to be O linked, although it is possible that it is endo H-resistant N-linked carbohydrate (5, 16, 40, 44).

Both agglutinins are transported to the cell surface through the secretory pathway. Consistent with this finding, all three agglutinin structural genes, *AGα1*, *AGA1*, and *AGA2*, initiate with prototypical signal sequences for proteins that are transported through the secretory pathway (5, 16, 28, 35, 42, 43). *AGα1* and *AGA1* genes also terminate with hydrophobic sequences that are reminiscent of signal sequences for addition of glycosyl phosphatidylinositol (GPI) anchors (14). Therefore, we have speculated that GPI anchors are involved in cell surface localization of both agglutinins (26).

To determine functional domains of α-agglutinin, we have tested the effect of C-terminal truncations of *AGα1*. Our results delineate two independent domains. The C-terminal domain is required for cell surface anchorage, and the N-terminal half contains the binding domain of α-agglutinin. This N-terminal domain shows sequence and structural similarity to the immunoglobulin fold sequences found in many mammalian adhesion proteins, suggesting that the mechanism of adhesion mediated by glycoprotein-glycoprotein interactions is highly conserved.

MATERIALS AND METHODS

Yeast strains. The *agα1-3* mutant (Lα21), which is isogenic to wild-type strain W303-1B (*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-2 can1-100*), was used to express the wild-type and truncated *AGα1* constructs, and the *agα1::LEU2* strain used for immunoblots contains a disruption constructed in W303-1B (28). Bioassays utilized tester strain X2180-1A (*MATa SUC2 mal mel gal2 CUP1*) (41). W303-1B and temperature-sensitive *sec* mutants NY431 (*MATα ura3-52 sec18-1*), NY432 (*MATα ura3-52 sec18-1*), and NY191 (*MATα his4-619*) (provided by Peter Novick) were used for GPI anchor analyses.

Construction of *AGα1* truncations. The individual truncations (described below) were produced by elimination of various restriction sites within the *AGα1* coding sequences by cleaving with the restriction enzyme, filling in the overhang with Klenow DNA polymerase or trimming back the

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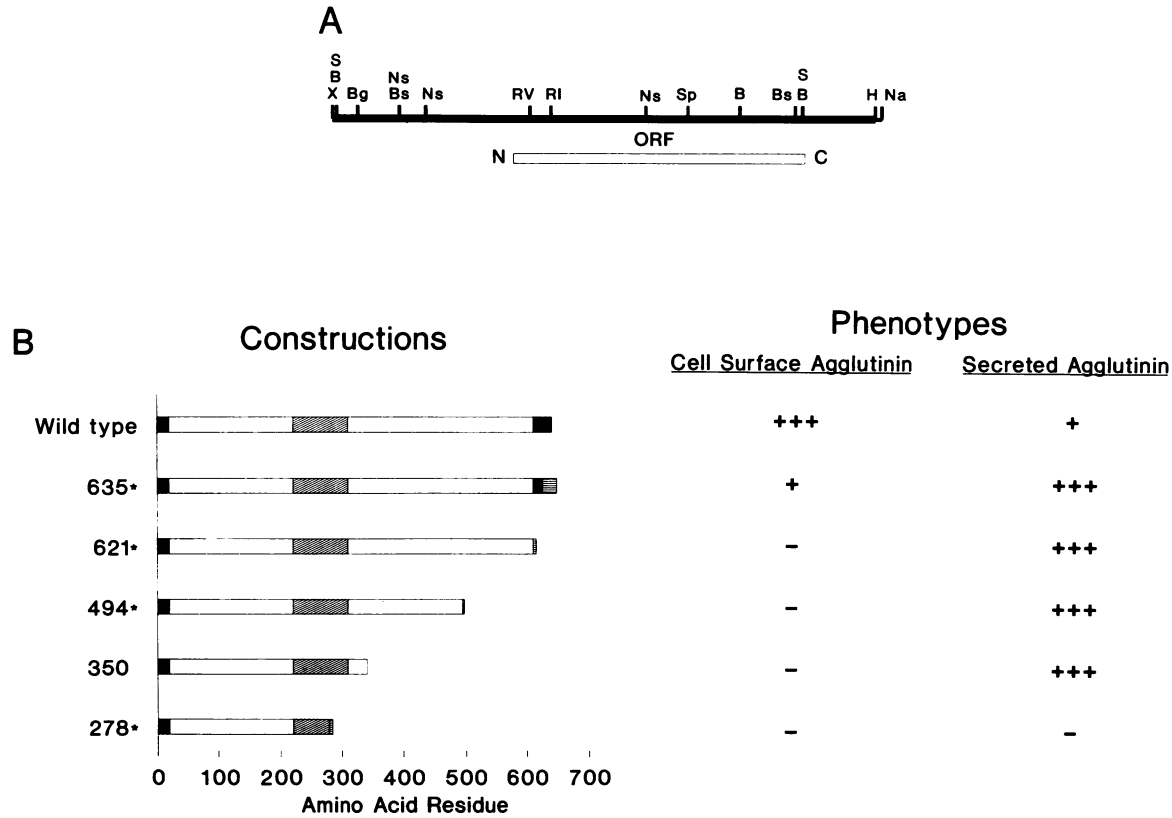

FIG. 1. Structures and phenotypes of *AGα1* truncations. (A) Restriction map indicating sites that were used for subcloning and modification to produce truncations as described in Materials and Methods. Yeast sequences are shown as a thick line; a plasmid-derived sequence is represented by a thin line on the right between the *Hin*dIII and *Nar*I sites. ORF, *AGα1* open reading frame; N, N terminus; C, C terminus; X, *Xba*I; B, *Ban*II; S, *Sac*I; Bg, *Bgl*II; Ns, *Nsi*I; RV, *Eco*RV; RI, *Eco*RI; Sp, *Spe*I; Bs, *Bsp*HI; H, *Hin*dIII; Na, *Nar*I. (B) Wild-type and truncated *AGα1* products that were expressed from the high-copy-number plasmid YEp351 in strain Lα21. Hydrophobic N and C termini are indicated by black shading, the proposed immunoglobulin fold-like domain between residues 220 and 310 is indicated by diagonal stripes, and additional C-terminal amino acids resulting from frameshifts are indicated by horizontal stripes. In single-letter code, the resulting amino acids are as follows: $AG\alpha1_{635*}$, VVRSFFCFSRTCFSKTGTVNLVH; $AG\alpha1_{621*}$, HDFNL; $AG\alpha1_{494*}$, ALL; and $ag\alpha1_{278}$, YNLYPLM. The phenotypes of the wild-type construct and the truncation mutants are summarized.

overhang with T4 polymerase, and religating (31). Plasmids with the restriction site eliminated were initially identified by loss of the restriction site and confirmed by double-stranded DNA sequencing (30, 39).

Relevant restriction sites in *AGα1* are shown in Fig. 1A. A 4.5-kb *Xba*I-*Hin*dIII *AGα1* fragment was subcloned from plasmid pH27 (28) into YEp351 to produce pAGα1 and into YEp352 (17) to form pAGα1′. The *Spe*I site was eliminated from pAGα1 to produce $pAG\alpha1_{350}$ (for nomenclature of the truncations, see Results). The *Eco*RV-*Nar*I fragment was subcloned into pRA10 (30) to produce pCL4. The *Sac*I site was eliminated to produce pCL8. The *Spe*I-*Hin*dIII fragment from pCL8 was subcloned into *Spe*I-*Hin*dIII-cleaved pAGα1 to create $pAG\alpha1_{635*}$. Elimination of the *Sac*I site in pCL8 also eliminated one of two *Ban*II sites within *AGα1*. The *Ban*II site in pCL8 was eliminated to produce pCL10. The *Spe*I-*Hin*dIII fragment from pCL10 was subcloned into *Spe*I-*Hin*dIII-cleaved pAGα1 to create $pAG\alpha1_{494*}$. The *Nsi*I site within *AGα1* in pCL4 was eliminated to produce pCL4N. *AGα1* was reconstructed in this plasmid by subcloning the *Xba*I-*Eco*RI fragment from pAGα1 into pCL4N. The *Bgl*II-*Nar*I fragment from pCL4N was subcloned into pAGα1 to produce $pAG\alpha1_{278*}$. The *Bsp*HI site in *AGα1* in plasmid pH27 (28) was eliminated to produce pH27B. The *Spe*I-*Hin*dIII fragment from pH27B was subcloned into pAGα1 to produce $pAG\alpha1_{621*}$.

Preparation of cell extracts. Extracts of W303-1B and Lα21 were prepared from 1-liter cultures (optical density at 660 nm of 0.8). Periplasmic proteins were released as previously described (21). For cell extracts, the cells were pelleted and resuspended in 50 ml of 100 mM sodium acetate (pH 5.5)–0.03% Triton X-100–1 mM *p*-chloromercuribenzoate. The resuspended cells were disrupted with glass beads as described previously (40), using a Bead Beater. The cell mixture was centrifuged at 28,000 × *g* for 10 min, and the supernatant (crude cell extract) was harvested. Protein quantitation of crude extract was determined by using the BCA Protein Assay (Pierce Chemical Co., Rockford, Ill.). For Western immunoblotting, 5 μg of cell extract was deglycosylated overnight at room temperature in 10 mM sodium acetate (pH 5.5)–1 mM phenylmethylsulfonyl fluoride (PMSF) with 10^{-4} U of endo H (Boehringer Mannheim).

Partial purification of *AGα1* peptides from culture supernatants. Lα21 transformants were grown in 200 ml of synthetic leucineless medium overnight at room temperature to stationary phase. The cells were centrifuged, and the supernatants were harvested. Aliquots of 100 ml were dialyzed overnight against 4 liters of 10 mM sodium acetate (pH 5.5)

at 4°C. The dialyzed material was chromatographed on a DEAE-Sephadex column (1-ml bed volume). The column was washed with 10 mM sodium acetate (pH 5.5) and eluted with 2 ml of 250 mM NaCl–10 mM sodium acetate (pH 5.5). α-Agglutinin activity was bound and eluted efficiently.

Immunological methods. Rabbit antisera were produced by injection of purified deglycosylated α-agglutinin (AG1), a fusion peptide of AGα1 expressed in *Escherichia coli* (AG2) (28), or purified secretion product from $AG\alpha1_{350}$ (AG3). Western blotting in Fig. 2B was done as described previously (49), using material representing 4 U of α-agglutinin activity from partially purified cell culture supernatants that had been deglycosylated with endo H as described above. Immunoblots for lipid-bound forms of α-agglutinin used the material extracted from 2×10^8 cells.

Metabolic labeling of cells and immunoprecipitation. For [^{3}H]myoinositol labeling, *sec18*[pAGα1′] cells were grown in inositol-free minimal medium at 24°C. Exponentially growing cells were centrifuged and resuspended in the same medium to a density of 7×10^7/ml. The cell suspension (15 ml) was preincubated at the labeling temperature for 30 min before addition of 0.3 mCi of [^{3}H]myoinositol (11.9 Ci/mmol; Dupont) and **a**-factor (final concentration of 50 ng/ml), and labeling was continued for 2 h at 24°C.

Labeling with [^{3}H]palmitic acid (51.6 mCi/mmol; Amersham) was done in the presence of **a**-factor for 2 h, using 50 μCi/ml to label 20 ml of cell suspension.

[^{35}S]methionine (ICN Biomedicals) labeling was carried out as described previously (45), with a 30-min labeling period initiated 10 min after addition of **a**-factor.

Labelings were terminated by adding NaN_3 to 10 mM and PMSF to 1 mM and chilling the culture on ice. The cells were collected and washed. Aliquots of 3.5×10^8 cells were resuspended in 150 μl of 2% sodium dodecyl sulfate (SDS)–50 mM Tris (pH 7.4)–5 mM PMSF, and cells were broken by vortexing with glass beads. Insoluble material was removed by centrifugation at 10,000 × *g* for 5 min. Supernatant (100 μl) was diluted to 1 ml with lysis buffer containing 1% Triton X-100 to a final concentration of 0.2% SDS. Antiserum against α-agglutinin fusion protein (AG2; 10 μl) (49) or against the N-terminal half of α-agglutinin AG3 (1 μl) was added to 100 μl of diluted lysate, and the mixture was incubated overnight at 4°C.

For antibody competition experiments, the same amount of antiserum was preincubated with partially purified unlabeled α-agglutinin (>50% purity, 250 U [40]). Immune complexes were precipitated by addition of protein A-Sepharose beads (15% suspension in lysis buffer, 20 μl per μl of antiserum) and incubation for 2 h at 4°C, with agitation. The beads were pelleted and washed as described previously (1). The protein antigen was released from the beads by heating at 97 to 98°C in 50 μl of Laemmli sample buffer for 4 min and separated by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS (24). The gel was fixed, soaked in En^{3}Hance (Dupont), dried, and exposed to Kodak X-Omat XAR-5 film at −70°C for fluorography.

Preparation of membrane proteins. Cells were grown at 30°C in minimal medium (Difco yeast nitrogen base plus 2% glucose) supplemented as appropriate. The pheromones **a**-factor (provided by Fred Naider) and α-factor (Sigma) were added to exponentially growing cells (cell density of 10^7/ml) to a final concentration of 15 ng/ml unless otherwise stated, and cell growth was continued for 1 or 2 h. Cells were then collected by centrifugation, washed twice in 10 mM Tris (pH 7.4)–1 mM PMSF and resuspended in lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 30 μg each of leupeptin, pepstatin, and antipain per ml). This cell suspension was mixed with equal volume of cold glass beads (0.5 mm; Sigma) and broken by vortexing four times for 1 min each time, with 1-min cooling intervals on ice. At least 90% of the cells were broken, as determined by phase-contrast microscopy. Cell walls were removed by centrifugation at 500 × *g* for 5 min, and Triton X-114 (precondensed as described by Bordier [3]) was added to the supernatant to a final concentration of 2% (vol/vol) to solubilize membrane proteins. After incubation on ice for 1 h, the insoluble material was removed by spinning at 10,000 × *g* for 5 min at 4°C. The supernatant was warmed to 35°C, and phases were separated by centrifugation at 10,000 × *g* for 20 s at room temperature. The detergent phase (membrane protein fraction) was reextracted three times by the addition of a 10-fold volume of lysis buffer to remove remaining water-soluble proteins. Membrane proteins in the Triton X-114 detergent phase were diluted in a fivefold volume of lysis buffer and precipitated by addition of trichloroacetic acid to a final concentration of 10% (vol/vol). The precipitated proteins were washed three times in cold acetone and resuspended in Laemmli sample buffer for SDS-PAGE (24).

PI-PLC treatment. Membrane proteins in the Triton X-114 detergent phase were treated with phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* (>90% purity by SDS-PAGE; provided by Martin Low, Columbia University) as described previously (7). After the incubation, phases were separated and the aqueous and detergent phases were extracted once more as described elsewhere (3, 8). Bovine serum albumin was added to the reextracted aqueous phase as carrier protein (final concentration of 100 μg/ml). Proteins in both aqueous and detergent phases were then precipitated by trichloroacetic acid and subjected to SDS-PAGE.

Agglutinin assays. The bioassay for α-agglutinin activity has been described previously (40). Cell surface agglutinin was assayed by a quantitative enzyme-linked immunoassay as described previously (49).

RESULTS

C-terminal truncations of AGα1 allow secretion of active α-agglutinin. To investigate the functional domains of α-agglutinin, frameshift mutations in *AGα1* were constructed to yield C-terminal truncated products. The truncations were expressed from a high-copy-number plasmid (YEp351) in the α *agα1-3* mutant Lα21, which expresses no measurable α-agglutinin activity or immunoreactive peptides (28). In our nomenclature for the truncations ($AG\alpha1_{x*}$), x indicates the number of amino acids from the 650-amino-acid *AGα1* open reading frame remaining in the construct and an asterisk indicates that additional amino acids are encoded by the altered reading frame and are present at the C terminus of the truncated protein; the extra amino acids are specified in the legend to Fig. 1.

Because the agglutinins are transported to the cell surface through the secretory pathway (42, 43) and we had proposed that the C terminus of the *AGα1* is involved in cell surface attachment (28), we investigated whether the truncated proteins were secreted into the growth medium. Intact or endo H-treated α-agglutinin binds to the **a**-agglutinin present on the surface of **a** cells (40, 41); this binding masks **a**-agglutinin and inhibits **a**-cell agglutinability. The presence of active, i.e., binding-competent, α-agglutinin was therefore assayed by measuring the inhibition of **a**-agglutinability (41)

TABLE 1. Cell surface and secreted α-agglutinin

Transforming plasmid[a]	Cell surface α-agglutinin		α-Agglutinin activity secreted (U/ml)[b]
	Agglutinability index[c]	Enzyme immunoassay substrate hydrolyzed (10^{-5} μmol/min)[d]	
pAGα1	0.64	0.17	0.18
pAGα1$_{635*}$	0.17	0.02	3.30
pAGα1$_{621*}$	0.03	ND	2.70
pAGα1$_{494*}$	0.09	ND	2.80
pAGα1$_{350}$	0.02	0.00	3.40
pAGα1$_{278*}$	0.03	0.00	<0.02
YEp351	0.00	ND	<0.1

[a] Strain Lα21 (*MATα agα1*) was transformed with the indicated plasmids. Transformants were tested for agglutinability with *MAT***a** cells, and culture supernatants were tested for α-agglutinin activity.
[b] Activity reflects inhibition of **a**-cell agglutinability by specific masking of cell surface **a**-agglutinin, as described previously (40, 41). Each active form of α-agglutinin bound reversibly to **a** cells but not to α cells (data not shown).
[c] The fraction of cells that were agglutinable (41). A value of <0.10 indicates an agglutination defect; a value of >0.30 indicates agglutinability.
[d] The relative amount of bound anti-α-agglutinin was determined by incubating cells with antibody AG1 and then with alkaline phosphatase-conjugated secondary antibody and was quantitated by enzyme assay (49). ND, not determined.

by culture supernatants from *agα1* cells expressing the truncations. The four truncations retaining at least 350 amino acids of the *AGα1* sequence showed secretion of active α-agglutinin into the culture medium (Fig. 1; Table 1).

The secreted activity represents authentic α-agglutinin. Functional analysis of α-agglutinin domains would be facilitated by use of soluble forms of secreted protein; however, it was first necessary to determine whether the secreted activity represented authentic α-agglutinin. Truncated α-agglutinin peptides were partially purified from culture supernatants, treated with endo H, and analyzed by immunoblotting using antiserum AG2, which was raised against a fusion protein containing amino acids 128 to 356 of *AGα1* (28). Preimmune serum did not react with any species in the extract (data not shown). Antiserum AG2 recognized α-agglutinin species in crude extracts of wild-type cells but not in the *agα1::LEU2* cells (Fig. 2A) or **a** cells (data not shown). The multiple bands seen in the α-cell extract are commonly observed for purified α-agglutinin and have been previously characterized as α-agglutinin breakdown products (16, 40).

The AG2 antibody detected single immunoreactive peptides in supernatants derived from the truncations *AGα1*$_{621*}$, *AGα1*$_{494*}$, and *AGα1*$_{350}$ (Fig. 2B). The sizes of the detected peptides increased with the proportion of the gene remaining in the truncation (Fig. 2B; Table 2). The *AGα1*$_{635*}$ truncation showed a peptide similar in size to the *AGα1*$_{621*}$ peptide as well as smaller peptides. The smaller peptides probably represent degradation products, as is also seen for α-agglutinin purified from the cell surface (Fig. 2A) (16, 40). Overexpression of the wild-type *AGα1* gene allowed some α-agglutinin secretion (Table 1), and resulted in an array of peptides similar to that seen with the *AGα1*$_{635*}$ truncation (data not shown). The amount of activity secreted by each truncation was about two to three times the amount of α-agglutinin normally expressed on the cell surface (49). The α-agglutinin activity in the culture supernatants had the dose response, reversible binding, and pH dependence of authentic α-agglutinin (40, 41). These results indicate that the secreted proteins correspond to truncated forms of α-agglutinin that retain full binding activity.

The C-terminal hydrophobic domain is involved in cell surface attachment. The secretion of the truncated α-agglutinins suggested that all of the frameshift mutations removed a domain involved in cell surface attachment. A defect in cell surface attachment should also result in a loss of cell surface α-agglutinin, which was assayed by testing cellular agglutinability and by an enzyme-linked immunoassay using anti-α-agglutinin antibody AG1 (49). Cells expressing *AGα1*$_{621*}$, *AGα1*$_{494*}$, or *AGα1*$_{350}$ truncations were not agglutinable, and no cell surface α-agglutinin was detectable in enzyme immunoassay of the strain expressing *AGα1*$_{350}$ (Fig. 1; Table 1). Although the *AGα1*$_{635*}$ strain efficiently secreted the truncated α-agglutinin into the growth medium, some α-agglutinin remained surface associated; this strain showed a low level of residual agglutinability, and the *AGα1*$_{635*}$ peptide was expressed on the cell surface at 10% of the level of wild-type α-agglutinin (Table 1). Based on two- to threefold overproduction of the truncated protein from the plasmid, these data indicate that >95% of the AGα$_{635*}$ peptide was released into the medium. The observation that wild-type and *AGα1*$_{635*}$ forms of α-agglutinin show multiple bands

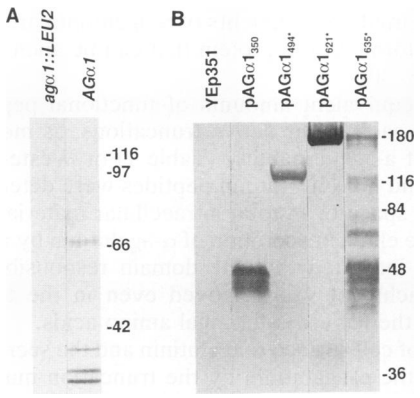

FIG. 2. Western analysis of wild-type and truncated α-agglutinin. (A) Antibody to the AGα1 fusion protein (amino acids 128 to 356) isolated from *E. coli* was tested for recognition of α-agglutinin peptides. Five micrograms of crude cell extracts was treated with endo H and then subjected to gel electrophoresis and immunoblotting. Peptides are detectable in crude cell extract from wild-type α cells (W303-1B) but not from the isogenic *agα1::LEU2* disruption mutant. (B) For strains expressing active truncations, peptides were isolated from culture supernatants by ion-exchange chromatography. Four units of each peptide and an equivalent amount of mock-purified YEp351 supernatant were deglycosylated with 10^{-4} U of endo H and subjected to SDS-PAGE followed by immunoblotting using antibody AG2. Marker sizes are indicated in kilodaltons.

TABLE 2. Predicted and observed molecular weights of secreted α-agglutinin peptides

Product	Peptide mol wt[a]	Observed mol wt[b]	Observed − expected
AGα1	70,000	180,000[c]	110,000
AGα1$_{635*}$	70,000	180,000[c]	110,000
AGα1$_{621*}$	67,000	180,000	113,000
AGα1$_{494*}$	53,000	120,000	67,000
AGα1$_{350}$	37,000	45,000	8,000
agα1$_{278*}$	29,500	32,000	2,500

[a] Calculated from the deduced amino acid sequence.
[b] Determined by SDS-PAGE of endo H-treated peptides followed by immunoblotting.
[c] Size of largest peptide.

implies that multiple fragments of α-agglutinin are generated from those forms of the protein that can be anchored in the cell wall (16, 40).

Roughly equivalent amounts of functional peptide were secreted by each of the active truncations, as measured by inhibition of **a**-agglutinability (Table 1) or Western blotting (Fig. 2B), and no α-agglutinin peptides were detected in the periplasmic space or in total intracellular material (data not shown). The efficient secretion of α-agglutinin by all of these truncations indicated that the domain responsible for cell surface attachment was removed even in the truncations eliminating the fewest C-terminal amino acids.

The loss of cell surface α-agglutinin and the secretion of all or most of the α-agglutinin by the truncation mutants indicated that a cell surface attachment domain had been eliminated. In $AG\alpha1_{635*}$, about half of the hydrophobic C terminus (15 amino acids) was replaced by a more hydrophilic sequence (23 amino acids; mean hydrophobicity of 0, as opposed to +2 in the native sequence [9, 23]) (Fig. 1). In $AG\alpha1_{621*}$, the entire hydrophobic C terminus (29 amino acids) was replaced by a 5-amino-acid polar sequence. This anchorage domain therefore correlated with the C-terminal hydrophobic domain.

A membrane-bound form of α-agglutinin. The C-terminal domain implicated in cell surface attachment has the characteristics of GPI anchor signal sequences (14). If a GPI anchor were involved in localization to the cell wall, then a membrane-bound form of α-agglutinin should be present within the secretory pathway, although the anchor might not be retained on the mature cell wall-bound protein. We therefore tested for membrane-bound forms of α-agglutinin. Cells were grown and treated with **a**-factor to increase expression of α-agglutinin (41, 49), and protein was extracted in SDS. Two soluble forms of α-agglutinin with apparent molecular sizes of 140 and >300 kDa were detected in the α strain (Fig. 3A). These proteins were present at higher levels in a strain carrying pAGα1′ and were not detected in the **a** strain or when the antiserum was preincubated with purified α-agglutinin. The 140-kDa species partitioned to the detergent phase of a Triton X-114 extract, as do GPI-anchored proteins (Fig. 3B) (3, 7, 8). The 300 kDa form partitioned to the aqueous phase of the extraction and may be an intermediate in cell wall anchorage (29).

To determine whether the detergent-soluble α-agglutinin was peripherally or tightly associated with the membranes, isolated cell membranes were treated with 75 mM $NaCO_3$ (pH 10.0) to release peripheral membrane proteins (8, 15) and subsequently pelleted by ultracentrifugation. The observation of similar amounts of 140-kDa form in the control and carbonate-treated samples demonstrates that this form of α-agglutinin is tightly associated with the membrane (Fig. 3C). GPI-anchored proteins have similar properties (8).

The 140-kDa protein can be metabolically labeled with myoinositol and palmitic acid. Although there is a wide variation in the fatty acid composition of GPI anchors, palmitate is reported to be efficiently incorporated into proteins as inositol-containing phospholipid in *S. cerevisiae* (7). To investigate whether the 140-kDa membrane protein contains covalently bound inositol and fatty acid, [^{3}H]myoinositol and [^{3}H]palmitic acid were used to metabolically label *sec18*[pAGα1′] α cells (11, 13, 32, 33). Cell lysates were prepared and immunoprecipitated. The anti-α-agglutinin antiserum precipitated a 140-kDa tritiated protein from both labelings (Fig. 4A). As previously reported, most cellular proteins were not detectably labeled (reference 8 and data not shown). These results indicate that the membrane-bound α-agglutinin contains both inositol and palmitic acid.

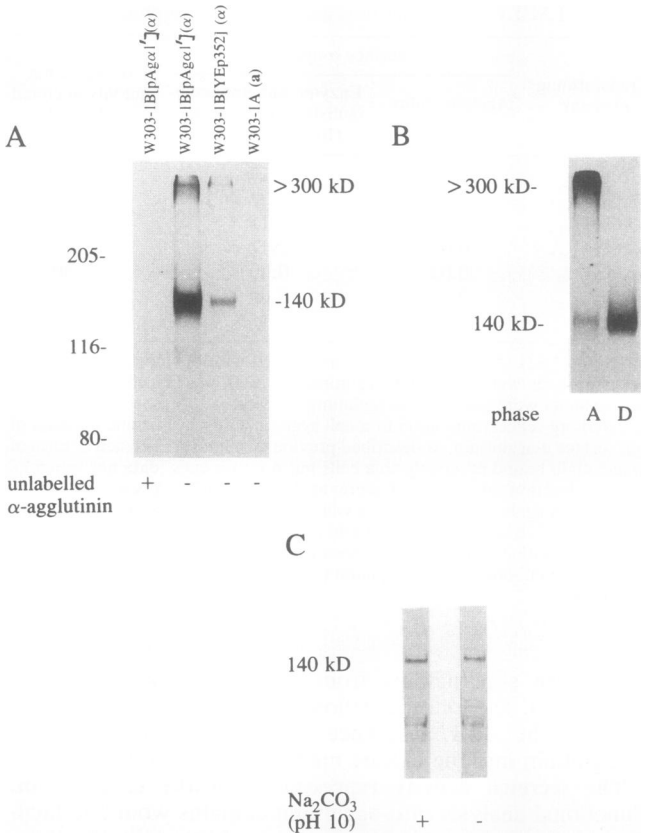

FIG. 3. Membrane-bound form of α-agglutinin. (A) Extractable forms of α-agglutinin in **a** and α cells. ^{35}S-labeled α-agglutinin was extracted with SDS, immunoprecipitated, electrophoresed, and fluorographed. In the left lane, the antibody was preincubated with purified unlabeled α-agglutinin. (B) Triton X-114 partitioning of soluble α-agglutinin. ^{35}S-labeled α-agglutinin was immunoprecipitated from the aqueous phase (lane A) or the detergent phase (lane D) of the Triton X-114 extract, electrophoresed, and fluorographed. (C) Tight association of the 140-kDa protein with membrane fraction. Cells were broken with glass beads in lysis buffer (pH 7.4), and the cell wall fraction was removed by low-speed centrifugation. The membranes in the supernatant were treated with Na_2CO_3, pH 10 (lane +), or in lysis buffer (lane −) for 30 min on ice and pelleted by ultracentrifugation. The membrane pellets were dissolved in 2% SDS and analyzed by immunoblotting.

Treatment of GPI-anchored proteins with PI-PLC releases the protein from the membrane (7, 8, 14). When [^{3}H]inositol-labeled α-agglutinin was treated with PI-PLC, the labeled α-agglutinin was released from the detergent to the aqueous phase (Fig. 4B). A similar PI-PLC digestion also resulted in the loss of palmitate-labeled α-agglutinin from the detergent phase (Fig. 4B). Labeled material was not immunoprecipitated from the aqueous phase (data not shown). The retention of the inositol label and loss of the palmitate label are consistent with the known mechanism of PI-PLC release of GPI-anchored proteins from the membrane, which occurs by cleavage of the anchor between the diacylglycerol and the inositol phosphate (14). Therefore, these results confirm a covalent attachment of an inositol-containing phospholipid to the 140-kDa membrane protein.

GPI addition is associated with membrane anchorage of

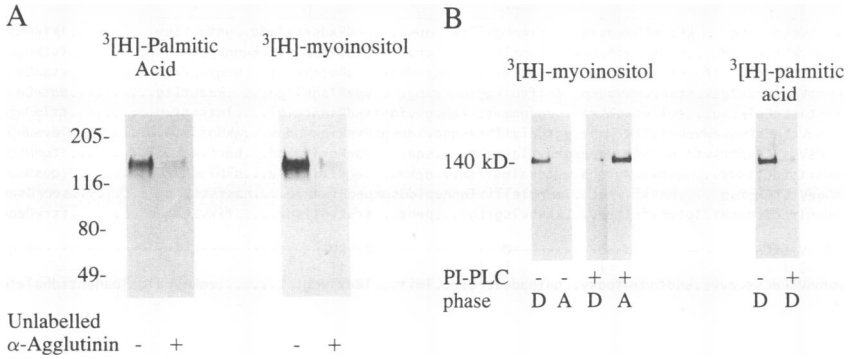

FIG. 4. Incorporation of [^{3}H]inositol and [^{3}H]palmitic acid into membrane-bound α-agglutinin and PI-PLC-mediated release. Extracts of labeled cells were immunoprecipitated with (+) or without (−) preincubation of the antiserum with purified unlabeled α-agglutinin, separated by electrophoresis, and detected by fluorography. (B) Triton X-114 phase partitioning of labeled α-agglutinin without digestion (−) or after digestion with PI-PLC. A, aqueous phase; D, detergent phase. Sizes are indicated in kilodaltons.

α-agglutinin. We speculated that the loss of cell surface anchorage of the $AG\alpha1_{621\cdot}$ and $AG\alpha1_{635\cdot}$ mutants resulted from a defect in attachment of the GPI anchor to the protein. Intracellular forms of α-agglutinin were therefore analyzed in these mutants. In extracts from wild-type cells, the majority of the 140-kDa protein was in the detergent phase, whereas in extracts of the $AG\alpha1_{621\cdot}$ strain, most of the protein was in the aqueous phase (Fig. 5). The small amount of detergent-soluble material seen in the *agα1-3*[pAGα1$_{621\cdot}$] strain was also present in the *agα1-3*[YEp352] strain, indicating that some protein is expressed from the *agα1-3* allele. In $AG\alpha1_{635\cdot}$ extracts, about 10% of the α-agglutinin was membrane associated (Fig. 5), which is similar to the amount that remained associated with the cell surface (Table 1). In $AG\alpha1_{635\cdot}$, approximately half of the C-terminal sequence was replaced by a more hydrophilic sequence; however, the remaining hydrophobic sequences may allow inefficient attachment of a GPI anchor. Alternatively, some other mechanism, such as carbohydrate interactions with the cell wall, may allow residual cell surface attachment of α-agglutinin in $AG\alpha1_{635\cdot}$. Taken together, the phenotypes of the $AG\alpha1_{635\cdot}$ and $AG\alpha1_{621\cdot}$ mutants indicate that removal of the hydrophobic C-terminus eliminates membrane association of the 140-kDa form of α-agglutinin. The C-terminal hydrophobic sequence of *AGα1* therefore appears to be essential for membrane anchorage as well as cell wall anchorage of α-agglutinin.

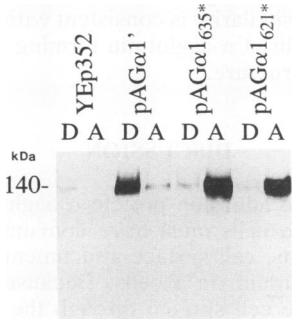

FIG. 5. Analysis of membrane anchorage in *AGα1* mutants. Strain Lα21 (*MATα agα1-3*) was transformed with the indicated plasmids. The cells were grown, treated with a-factor, and extracted with Triton X-114. Proteins in the detergent phase (D) and aqueous phase (A) were precipitated and analyzed by immunoblotting.

Glycosylation of α-agglutinin. There are 12 potential N-linked glycosylation sites in the *AGα1* sequence, five of which are in the first 350-amino-acid residues. All of the truncated proteins and the wild-type α-agglutinins appear as unresolved high-molecular-weight glycopeptides on SDS-polyacrylamide gels (references 16 and 40 and data not shown). Following treatment with endo H, discrete glycopeptides can be visualized (Fig. 2B), indicating that at least one site within the N-terminal half of *AGα1* is N glycosylated.

Some carbohydrate is retained after treatment of α-agglutinin with endo H. This carbohydrate is likely to be O linked (16, 40), although the possibility of endo H-resistant N-linked carbohydrate has not been eliminated (44). Other than the hydrophobic C terminus, the C-terminal half of *AGα1* is about 50% serine and threonine, providing a large number of potential sites for O-linked glycosylation. When the observed molecular weights of endo H-treated peptides were compared with the predicted peptide molecular weights of the truncations, the difference between predicted and observed molecular weights increased as more of the C-terminal half of α-agglutinin was included in the truncations (Table 2). Although the sizes of the glycoproteins determined by gel electrophoresis may be inaccurate, these results suggest that the Ser- and Thr-rich C-terminal half of α-agglutinin contains substantial amounts of endo H-resistant carbohydrate, perhaps as O-linked saccharides. This result is consistent with the observation that endo H-treated α-agglutinin binds to concanavalin A-Sepharose (40).

The binding domain is within the N-terminal half of α-agglutinin. All of the truncated proteins retaining at least 350 N-terminal amino acids of *AGα1* show comparable secreted activities (Table 1). The specific activity of the $AG\alpha1_{350}$ truncation protein was 40,000 to 50,000 U/mg, twofold higher than that of the native protein. Because half of the protein was retained in this truncation, the activity per molecule remained the same as that of the native protein. This result implies that the complete binding domain of α-agglutinin is within the first 350 amino acids of α-agglutinin.

In contrast to the results for $AG\alpha1_{350}$, supernatants from the strain expressing the $ag\alpha1_{278\cdot}$ construct, which lacks an additional 72 amino acids, did not show detectable activity. Only small amounts of immunoreactive ag$\alpha1_{278\cdot}$ peptide were detected in the supernatant in comparison with the other truncations. The ag$\alpha1_{278\cdot}$ peptide was also not de-

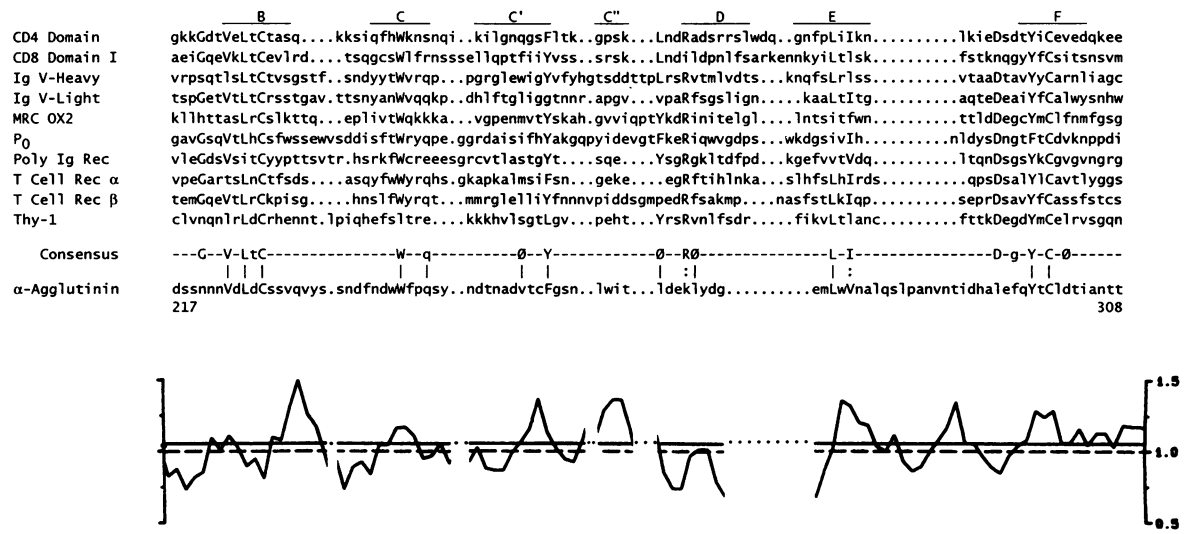

FIG. 6. α-Agglutinin region similar to immunoglobulin-fold V-type domains. The region of AGα1 between amino acids 217 to 308 is compared with sequences of mammalian proteins that are members of the V-type immunoglobulin superfamily (46). The individual β strands of the immunoglobulin fold are overlined and named above the alignment. The A and G strands, which have no consensus residues, are not shown. The AGα1 sequence was aligned to the consensus sequence by the Genetics Computer Group program BESTFIT with a gap penalty of 0.5 and a length penalty of 0. The consensus sequence includes specific amino acid classes (as defined by the Genetics Computer Group program CONSENSUS [9]) present in at least 7 of the 10 aligned domains (capital letters) or at least 5 of the 10 domains (lowercase letters) and hydrophobic residues (ϕ) present in at least 7 of the 10 domains; the program counts very similar amino acids as matches to the consensus residue. The Chou-Fasman β-sheet potential (6) of the α-agglutinin sequence is shown beneath the sequence. Ig, immunoglobulin; MRC OX2, rat T-cell antigen MRC OX-2; Rec, receptor.

tected in cellular extracts, indicating that the small quantity of product present in supernatant was not due to intracellular retention of the peptide. To determine whether the secreted $AG\alpha1_{278*}$ protein retained activity, the supernatant was concentrated 50-fold and reassayed. No activity was detectable from the equivalent of at least 2.5-fold the concentration of immunoreactive peptide detectable by bioassay for the $AG\alpha1_{350}$ truncation (Table 1). Note that fragments of α-agglutinin may lack some epitopes, resulting in reduced immunoreactivity in comparison with wild-type protein; therefore, immunoblots may underestimate the amount of protein, resulting in an overestimate of the specific activity of the fragments (48). Our estimate of specific activity of the $ag\alpha1_{278*}$ peptide therefore represents a maximum estimate. The inactivity of the $ag\alpha1_{278*}$ truncation localizes the C-terminal boundary of the α-agglutinin binding domain to amino acid residues between 279 and 350.

A segment of the binding domain has similarity to immunoglobulin folds. On the basis of the observation that the binding domains of α-agglutinin analogs from other species of budding yeast are acidic, we previously proposed that the most acidic region of *AGα1*, approximately between amino acids 200 to 300, corresponds to the binding domain. The delineation of the C-terminal boundary of the binding domain to between residues 279 and 350 correlates well with this speculation. This acidic region also shows properties similar to those of the immunoglobulin superfamily of mammalian adhesion proteins (Fig. 6). These proteins contain a conserved structure, called the immunoglobulin fold, which consists of two β sheets each composed of several β strands (46). Immunoglobulin folds can be characterized as either variable (V) type, which contain nine β strands, or constant type, which contain seven β strands; the two types also differ in their consensus sequences of conserved residues. Chou-Fasman analysis of the α-agglutinin sequence identified regions between amino acids 217 to 308 with strong β-sheet potential that resemble the V-type immunoglobulin fold (6). There are maxima in the predicted β-strand potentials of this region of *AGα1* at positions corresponding to each β-strand in the immunoglobulin fold. In comparison with the 19 conserved residues of the V-type consensus sequence, this region of α-agglutinin shows 12 exact matches and two conservative substitutions (Fig. 6). To determine whether the alignment match was significant, a residue consensus profile of the variable-region sequences was compiled by using the University of Wisconsin Genetics Computer Group package (9, 46), and the *AGα1* sequence was searched for the best matches. The region between residues 217 and 308 had an alignment score 6.6 standard deviations above the mean score for a set of random sequences with the same amino acid composition ($P < 10^{-7}$). All gaps and insertions in the BESTFIT alignment to the consensus sequence occurred in regions that are exterior loops in the V-type fold. This similarity is consistent with this segment of the binding domain of α-agglutinin forming a V-type immunoglobulin fold structure.

DISCUSSION

The cell surface adhesion protein α-agglutinin expressed by *S. cerevisiae* α cells must have domains involved in at least two functions, cell surface attachment and binding to the target **a**-agglutinin on **a** cells. Because α-agglutinin is transported to the cell surface through the secretory pathway (42, 43), we have been able to analyze secreted forms of truncated α-agglutinin to delineate functional domains of the α-agglutinin structural gene *AGα1*. The C-terminal domain is necessary for cell surface anchorage mediated by a GPI anchor. The N-terminal half of α-agglutinin contains the

binding site for **a**-agglutinin. This binding domain shows striking similarity to the immunoglobulin fold structures present in many mammalian adhesion proteins.

The C-terminal half of α-agglutinin is highly glycosylated and involved in cell surface anchorage. We previously proposed that the hydrophobic C terminus of *AGα1* represents a signal sequence for the attachment of a GPI anchor (14, 26, 28). Precursors to GPI-anchored proteins contain hydrophobic C termini that are cleaved shortly after transport into the endoplasmic reticulum (10). The GPI anchor is linked to the new C-terminal amino acid of the mature protein.

The resemblance of the C terminus of *AGα1* to GPI anchor signal sequences led us to investigate its role in cell surface attachment. Truncations removing all or half of the C-terminal hydrophobic sequence resulted in loss of cell surface attachment (Table 1). Biochemical results show that a form of α-agglutinin is attached to a GPI anchor, thus resulting in membrane attachment (Fig. 3 and 4). *AGA1*, the structural gene for the cell surface attachment subunit of **a**-agglutinin, also has a hydrophobic C terminus, suggesting that a GPI anchor may also be involved in cell surface attachment of **a**-agglutinin (35). GPI-anchored proteins have been identified in yeasts (7, 8, 34), indicating that the machinery for such a mechanism is present. Whereas previously identified GPI-anchored proteins are attached to the plasma membrane (14), the mature agglutinins are likely to be bound to the cell wall matrix rather than the membrane (16, 25). We have therefore proposed that the GPI anchor provides a mechanism to transport the agglutinins to the plasma membrane, after which they would be transferred to the cell wall by a novel mechanism (26). The carbohydrate present on the C-terminal half of α-agglutinin (Table 2) may further stabilize the cell wall interaction by participating in the hydrogen bonding network that stabilizes the cell wall (2, 4, 52). The highly glycosylated C-terminal half is also likely to result in an extended rod-like conformation (19), thus facilitating exposure of the N-terminal binding domain on the surface of the cell wall.

The N-terminal half of α-agglutinin contains the binding domain and shows similarity to immunoglobulin folds. Before further characterization of the mechanism of adhesion mediated by α-agglutinin, the precise domain involved in binding to the target **a**-agglutinin must be determined. Previous results have provided some information on the location of the binding domain. Proteolytic fragments of α-agglutinin lacking the C-terminal third are active (16, 27, 48), indicating that this C-terminal region is not required for binding. A fusion protein containing amino acids 128 to 356 of *AGα1* sequesters anti-α-agglutinin antibodies that inhibit its activity (28) and elicits antibodies that block binding of α-agglutinin to its ligand **a**-agglutinin (48), indicating that residues involved in binding activity are in this region. A His residue at amino acid 292 is essential for α-agglutinin activity (5; amino acid 273 by the nomenclature of this reference). The binding domains of the α-agglutinin analogs from *Hansenula wingei* and *Pichia amethionina* are acidic, and the binding activity of α-agglutinin is pH dependent, with maximal activity at pH 5.0 to 5.5 (26, 40). From these results, we proposed that the most acidic region of α-agglutinin, between residues 200 and 300, corresponds to the binding domain (28).

The C-terminal truncations of *AGα1* allow the further delineation of the α-agglutinin-binding domain. The $AG\alpha1_{350}$ peptide showed activity similar to that of the full-length protein, indicating that the complete binding domain is within the N-terminal 350 amino acids. The inactivity of the $ag\alpha1_{278*}$ peptide suggests that the C-terminal boundary of the binding domain is between residues 278 and 350, although the possibility remains that the $ag\alpha1_{278*}$ peptide is inactive as a result of processing or structural abnormalities.

The region of *AGα1* between residues 217 and 308 shows strong similarity to V-type folds with respect to both conservation of consensus amino acid residues and Chou-Fasman prediction of β-strand positions (6). The degree of similarity to the consensus sequence is comparable to that of immunoglobulin fold domains of CD4, CD8, Thy1, Po, and the CAM family, all of which are cell adhesion proteins (Fig. 6) (46). The loop regions between the strands are of variable length and composition, as found in mammalian immunoglobulin fold domains.

The active $AG\alpha1_{350}$ construction retains the entire predicted immunoglobulin fold domain, whereas the inactive $ag\alpha1_{278*}$ truncation does not. The latter peptide lacks residues in the proposed D, E, F, and G strands of the α-agglutinin V-like domain (Fig. 6), including the second conserved Cys residue. The low levels of $ag\alpha1_{278*}$ peptide found in cell supernatants may result from proteolytic degradation due to loss of a portion of a compact, protease-resistant domain. Deletion of a portion of a highly structured fold domain may therefore account both for the low level of $ag\alpha1_{278*}$ protein and for its lack of α-agglutinin activity. The His residue essential for α-agglutinin activity (5) falls between the E and F strands of the domain. Two nonessential His residues (5) are outside of this proposed immunoglobulin-like domain.

Chou-Fasman β-strand potentials and analyses of the *AGα1* sequence reveal two more possible immunoglobulin domains in the N-terminal half of α-agglutinin, at residues 27 to 104 and 104 to 186. Although the majority of conserved residues or conservative substitutions are present in these regions, neither region has a BESTFIT alignment to the immunoglobulin consensus sequence with a score significantly above the mean for random sequences. With one known exception, members of the immunoglobulin superfamily have multiple immunoglobulin fold domains within a single polypeptide chain, and multiple domains often participate in ligand binding (46). For example, in ICAM-1, an immunoglobulin-related cell adhesion protein, amino acid residues in each of the first two immunoglobulin fold domains interact with the ligand LFA1 (37). The third immunoglobulin fold domain appears to be essential for binding of rhinovirus (36). In immunoglobulins, variable domains from the light and heavy chains interact to form antigen-binding sites. We are currently testing for the presence of one or more immunoglobulin fold structures in α-agglutinin.

Immunoglobulin-like binding domains are a common feature of many cell adhesion molecules of animals (12, 46). The structure of the immunoglobulin fold is based on a sequence of antiparallel β strands arranged in a characteristic order in three dimensions to form a "barrel." At least two prokaryotic proteins have similar β-barrel structures in which the order of β strands is the same as in the immunoglobulin fold (18, 20). These results suggest that immunoglobulin-like β-barrel structure is older than the evolutionary split between the eukaryotes and the prokaryotes (22). On the basis of the alignment of α-agglutinin with immunoglobulin superfamily proteins, we suggest that this β-barrel structure was used as a basis for cell-cell recognition prior to the divergence of fungi and animals.

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