Delineation of Functional Regions within the Subunits of the Saccharomyces cerevisiae Cell Adhesion Molecule a-Agglutinin*

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The Saccharomyces cerevisiae agglutinins are cell surface mannoproteins that mediate cell-cell adhesion of haploid cells during mating. The a- and a-agglutinins, expressed by MATα cells, respectively, interact directly with 1:1 stoichiometry. Exposure to the pheromone expressed by the opposite mating type induces agglutinin expression and thus facilitates the agglutination reaction (1).

a-Agglutinin is a single polypeptide that contains both N- and O-linked carbohydrate. The a-agglutinin gene AGA1 (2, 3) encodes a 650-residue precursor with an N-terminal signal sequence that directs a-agglutinin to the secretory pathway and a C-terminal hydrophobic sequence that results in attachment to a glycosyl phosphatidylinositol (GPI) anchor (4, 5). Attachment of many cell wall proteins, including the agglutinins, involves transfer from a plasma membrane-linked GPI anchor to cell wall glycan by a transglycosylation reaction (5, 6). The N-terminal half of Aga1p contains the binding site for a-agglutinin and is a globular region rich in antiparallel β-sheets. Homology models predict that there are three immunoglobulin-like folds within this region (7–11). Residues involved in ligand binding were identified through site-directed mutagenesis of the third Ig domain (12). Homologs of a-agglutinin mediate adhesion of the pathogenic yeast Candida albicans to epithelia (13).

*a-Agglutinin is the product of the genes AGA1 and AGA2. The 725-amino acid Aga1p precursor includes hydrophobic N and C termini implicated in transfer into the secretory pathway and GPI anchor attachment, respectively. The remainder of Aga1p has 55% serine and threonine residues, consistent with the high level of O-glycosylation reported in analogs from other yeasts (1, 14). The ten cysteine residues in mature Aga1p are clustered in two Cys-rich repeats containing five cysteine residues each (Fig. 1). aga1 mutations allow secretion of Aga2p into the growth medium, consistent with the role of Aga1p in cell wall attachment of Aga2p (15, 16). Aga1p is expressed by both MATα and MATα cells in a pheromone-responsive manner (15–17). It is required for efficient mating in liquid cultures and in cells with a deletion of the homolog FIG2. The deletion can be complemented by overexpression of related genes FIG2, FLO11, or FLO10 (18).

Aga2p is a small glycoprotein expressed only by MATα cells. It consists of 69 residues after cleavage of an 18-residue signal sequence, and at least 10 of the 21 serine and threonine residues are O-glycosylated (19). Cys residues at positions 7 and 50 of mature Aga2p (corresponding to positions 25 and 68 of the open reading frame) anchor it to the cell surface through Aga1p; both cysteine residues must be mutated to lose cellular agglutinability (20). Aga2p is necessary and sufficient for binding to α-agglutinin, and binding is tight and practically irreversible over the time course of a mating reaction (21, 44). Synthetic or proteolytic peptides containing the ten C-terminal residues of Aga2p are sufficient to bind α-agglutinin, but at concentrations 4- to 5-fold higher than the full-length peptide (20), indicating that this region has a major binding determinant and that carbohydrate or
structural features of the full-length protein contribute to binding.

We purified and characterized Aga2p alone and in complexes with fragments of Aga1p to characterize its activity and structure. We have also characterized the effects of a number of mutations in AGA2. These studies have identified regions and specific residues in each subunit important in subunit interactions. The results have also clarified the role of specific Aga2p residues in interaction with the receptor α-agglutinin.

EXPERIMENTAL PROCEDURES

Strains—Escherichia coli strains TG1 and DH5α were used for plasmid analysis and subcloning. Supercompetent E. coli strain XL2 Blue (Stratagene), was used to clone inverse PCR products.

The α-agglutinin constructs were expressed in S. cerevisiae strains isogenic to W303-1A (MATα ade2-1 can1-100 trp1-1 ura3-1 his3-11,15 leu2-3,112). The biochemical analysis of AGA2 was conducted in YEpPGK, as YEp has been used extensively to request from the corresponding author. (24). All primer sequences and construction details are available upon request from the author.

Plasmids—Agglutinin constructs were expressed in S. cerevisiae from YEᵖGk, a URA3-containing expression vector that contains the PGK promoter and terminator sequences (22); pRS316, a URA3-containing centromeric plasmid (23); or pRS424, a TRP1-containing YEp plasmid (24). All primer sequences and construction details are available upon request from the corresponding author.

N- and C-terminal Histidine-tagged versions of Aga2p were constructed in pGEM-AGA2 as template with standard PCR and restriction techniques (16). These constructs were sequenced to confirm the correct insertion. An AGA2 clone containing its homologous promoter was created by PCR amplification of about 1200 bp of the AGA2 upstream sequences from W303-1A genomic DNA. The product was subcloned into pRS316 to make pRS316-AGA2-us. An intact AGA2 gene was reconstructed by subcloning the EcoRI-Hin1 AGA2 fragment from YEPGPKAGA2 into pRS316-AGA2-us. The EcoRI-Hin1 fragments from various AGA2 mutants were also subcloned into pRS316-AGA2-us to construct mutant genes expressed from the AGA2 promoter on a CEN plasmid.

AGA1 DNA was amplified from genomic DNA by PCR (Expand Long Template PCR system, Roche Molecular Biochemicals, Basel, Switzerland). The product was cloned into pGEM-T and made pGEM-AGA1 (Promega, Madison, WI) and was subsequently used as template for polymerase chain reactions in the construction of other plasmids. pRS424-AGA1 (containing the entire open reading frame of AGA1) and pRS424-AGA1(1-664) (which lacks the GPI anchor sequence) were constructed following amplification of pGEM-AGA1. To construct YEᵖGk-AGA1(1-149), which encodes the 22-residue signal sequence and the first 127 amino acid residues of the mature Aga1p, inserts were amplified using pGEM-AGA1 as template and subcloned into YEᵖGk. The YEᵖGk-AGA1(1-149) Xho1-Sal1 fragment, which contains AGA1(1-149) and the PGK promoter and terminator, was subcloned into pRS424 to create pRS424-AGA1(1-149). All other truncation and deletion plasmids shown in Fig. 1B, as well as a C-terminal HA-tagged version of pRS424-AGA1(1-149) were constructed by PCR using pGEM-AGA1 as template with appropriate primers and subcloning the product into pRS424-AGA1(1-149).

Random Mutagenesis of AGA2—Random mutagenesis followed by gap repair reconstruction was performed as described in Muhlrad et al. (25). Random PCR mutagenesis was carried out on YEᵖGk-AGA2, varying ratios of MgCl2:MnCl and limiting amounts of each nucleotide (25). The PCR products were ethanol-precipitated and isolated from an agarose gel with the QIAquick gel extraction kit (Qiagen Inc., Valencia, CA).

Site-directed Mutagenesis—C-terminal AGA2 mutants were generated using YEᵖGk-AGA2 DNA as template in polymerase chain reactions (PCR) using an upstream oligonucleotide containing an EcoRI site and downstream oligonucleotides with the desired mutation and a BamHI site. The resulting mutant AGA2 EcoRI-BamHI fragments were subcloned into YEᵖGk. Additional mutations were made by the QuikChange site-directed mutagenesis method (Stratagene, La Jolla, CA). The mutant aga2 genes were sequenced to confirm that no extraneous mutations were present.

Internal regions of AGA2 for which mutations obtained in the random mutagenesis affected agglutination were subjected to further mutagenesis using a double-PCR method (26). A primary PCR reaction with pGK-AGA2 as a template used appropriate primers to create a fragment of ~200 bp. This fragment was purified and used as an upstream mega-primer in a second PCR reaction; the products were cloned into YEᵖGk and sequenced.

Production and Purification of α-Agglutinin Polypeptides—α-Agglutinin polypeptides expressed from PGK plasmids were produced in W303 diploid or W303-1B aga1::LEU2 haploid cells. Cells were grown to density of 2–2.5 × 10⁷/ml in synthetic medium before harvest and collection of the extruded protein in the culture supernatants. The supernatants were buffered to pH 5.8 with sodium acetate and concentrated ~10-fold through a MilliPore filter with a 30,000-Da cutoff.

Aga2p expressed from pPGK-AGA2 was dialyzed overnight against 20 mM sodium phosphate buffer, pH 5.5, 1 mM EDTA, 0.01% NaN3, then either lyophilized, resuspended in the same buffer with 10% (v/v) glycerol, or centrifuged, and applied to a Bio-Gel P-60 column. The eluted fractions were examined by gel electrophoresis, and stained with Comassie Blue or periodic acid Schiff reagent (PAS) (27). (Comassie Blue-stained Aga2p was not stably fixed in the gels, and bands disappeared after 24 h at room temperature). Extended storage or lyophilization of purified Aga2p generated in a ladder of bands of apparent molecular size 30 kDa, 60 kDa, and larger upon SDS-gel electrophoresis. These apparent multimers were reduced to 30 kDa following treatment with dithiothreitol.

For His-tagged versions of α-agglutinin, the concentrated culture supernatant was dialyzed overnight against 20 mM sodium phosphate buffer, pH 5.5, 500 mM NaCl, and then either lyophilized or applied to a His-Trap column (Amersham Pharmacia Biotech and Upjohn, Inc., Downers Grove, IL), washed in buffer A, and eluted with buffer A containing 500 mM imidazole. The eluate was dialyzed against buffer B (10 mM sodium phosphate, pH 6.7, 0.01% NaN3). In some cases, the material was further purified by gel filtration on Bio-Gel P-60.

Protein Analysis—Polyacrylamide gel electrophoresis of proteins was according to the method of Laemmli (28). Protein concentrations were determined by A₂₆₀ using an extinction coefficient of 1 per mg/ml for impure solutions, and for pure polypeptides, the extinction coefficients were calculated from the amino acid composition (29). Pyrogallolase was used according to manufacturer's directions (Roche Molecular Biochemicals, Indianapolis, IN). Partial deglycosylation was effected with jack bean α-mannosidase (100 μg/ml, used according to manufacturer's directions; Sigma Chemical Co., St. Louis, MO). Peptide sequences were generated on an ABI gas phase sequencer in the Hunter College Sequence and Synthesis Facility. CD spectra were obtained on a JASCO J710 spectropolarimeter.

For immunoblots and dot blots, the proteins were transferred to polyvinylidene difluoride or nitrocellulose paper under standard conditions. The primary antibodies were anti-HA (Roche Molecular Biochemicals), anti-His-Tag (Amersham Pharmacia Biotech), and polyclonal antibodies to Aga2p (provided by W. Tanner (30) or produced for this study); these polyclonals were multiply adsorbed with W303-1B and W2180-1B cells. An anti-rabbit antibody conjugated to horseradish peroxidase (Sigma) was used as a secondary antibody, developed with 4-chloro-1-naphthol (Sigma) or ECL reagent (Amersham Pharmacia Biotech, Arlington Heights, IL).

Agglutinin Activity Assays—Agglutinins were assayed by published procedures (14, 31). Wild type and mutant aga2 genes expressed from the AGA2 promoter on CEN plasmids were also tested for the ability to complement the aga2::LEU2 agglutination defect by cellular agglutination assays (14).

RESULTS

Our results demonstrate that Aga2p is unstable and most of the purified subunit is inactive. Aga2p can be stabilized in an active form by a small fragment of Aga1p, and the two subunits interact through a pair of disulfide bonds and at least one other region of Aga2p. Biochemical and molecular analyses imply that the C-terminal-mannose-residing region of Aga2p is critical for binding to its receptor α-agglutinin.

Biochemical Properties of Aga2p—Aga2p expressed from plasmids had properties similar to that released from cell surface (14, 19, 20). For both Aga2p and Aga2p with His₉ immediately following the secretion sequence (His₉-Aga2p), the yield averaged 1–2 × 10⁴ units/liter of culture, and the purified protein showed a single diffuse band on gel electrophoresis when stained with Comassie Blue or the glycoconjugate stain.
constructs were coexpressed under the PGK AGA1, and Cys-rich regions are lines/ml, three independent experiments. The criteria for activity levels were: Aga2p, and the activity of culture supernatants was assayed in at least GPI anchor signal are units. The hydrophobic N-terminal signal sequences and C-terminal clized N-terminal pyroglutamate. yielded the expected peptide sequence of residues 2–8 after predicted size of the polypeptide is under 7 kDa, and the gly-

149), as well as His6-Aga2p monomer and dimer (Fig. 2

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FIG. 1. Features of a-agglutinin. A, diagram of a-agglutinin sub-units. The hydrophobic N-terminal signal sequences and C-terminal GPI anchor signal are solid black. Cys residues are marked as vertical lines, and Cys-rich regions are cross-hatched. B, features and activity of AGA1 constructs. Internal deletions in AGA1 are indicated as lines. All constructs were coexpressed under the PGK promoter along with His6-Aga2p, and the activity of culture supernatants was assayed in at least three independent experiments. The criteria for activity levels were: +, mean activity < 25 units/ml, maximum activity < 50 units/ml; ++, 70 units/ml < mean activity < 100 units/ml; ++++, mean activity > 150 units/ml.

PAS. The $M_{r}^{app}$ was 30 kDa (Fig. 2A, lane 3), although the predicted size of the polypeptide is under 7 kDa, and the glycopeptide is probably less than 20 kDa (14, 19). Purified Aga2p yielded the expected peptide sequence of residues 2–8 after glycoprotein was treated with pyroglutaminase to remove cyzized N-terminal pyroglutamate.

The specific activity of purified Aga2p or His6-Aga2p ranged up to $1.4 \times 10^{12}$ units/mol, comparable to most previous reports (14, 19, 20). However, we found that the activity of both Aga2p and His6-Aga2p decreased during storage except at $-70^\circ$ C. Activity was not restored or preserved by glycerol, dithiothreitol, EDTA, divalent metal ions, or peroxide oxidation of Cys residues. CD spectroscopy of the purified Aga2p or His6-Aga2p yielded spectra typical of denatured peptides, with only aperiodic structures (data not shown). These results implied that much of the isolated Aga2p was in an inactive and denatured form.

Co-expression of Aga2p with Aga1p—Disulfide linkage of Aga2p to Aga1p involves two disulfide bonds (19). The ten Cys residues in Aga1p are present in two repeats with five Cys residues each (Fig. 1A). We constructed short versions of Aga1p containing a single Cys repeat to determine whether such fragments might support secretion of higher levels of Aga2p activity. Aga1p-(1–149) contains the signal sequence, a Ser/ Thr-rich region, and the first Cys-rich region. Strains expressing this form of Aga1p alone did not secrete measurable a-agglutinin activity. However, when this construct was coexpressed with His6-Aga2p, secreted activity was 10-fold higher than when His6-Aga2p was expressed alone. Nickel-affinity chromatography of the supernatant yielded a complex of His6-Aga2p with a larger component, presumably Aga1p-(1–149), as well as His6-Aga2p monomer and dimer (Fig. 2B, lane 5), which were removed by gel filtration (lanes 6–10). The purified complex stained with PAS, indicating glycosylation (Fig. 2C), and upon reduction with DTT yielded two bands, one with the size of His6-Aga2p near 30 kDa, and a band of apparent size 105–110 kDa. The upper band was not stained with Coomassie Blue (Fig. 2C). The 30-kDa subunit was also stained by antibody to His6 or to Aga2p (data not shown). When His6-Aga2p was co-expressed with Aga1p-(1–149)-HA, the larger subunit was stained with antibody to the HA epitope (data not shown). Therefore, co-expression of His6-Aga2p with Aga1p-(1–149) resulted in secretion of an active complex of these two proteins.

Properties of the His6-Aga2p-Aga1p-(1–149) Complex—The specific activity of the His6-Aga2p-Aga1p-(1–149) complex was $(6 \pm 1) \times 10^{12}$ units/mol (S.E., six independent determinations on two preparations), which was 43-fold higher than the activity of Aga2p or His6-Aga2p alone and similar to the specific activity of purified α-agglutinin. The far-UV CD spectrum of the His6-Aga2p-Aga1p-(1–149) complex showed a positive rise centered at 204 nm and negative peaks at 208, 212, and 217 nm (Fig. 3). A slight negative shoulder is visible at 222 nm. These positions and intensities resulted in an estimate based on the program SELCON that the structure included 13% α-helix, 27% β-sheet, 24% turn, and 35% aperiodic structures (32, 33).

The His6-Aga2p component of the complex had smaller apparent size than the corresponding subunit when expressed alone (Fig. 2A, lane 5 versus lane 3). This result implied that the subunit in the complex might contain less carbohydrate than when expressed alone. When His6-Aga2p or the His6-Aga2p-Aga1p-(1–149) complex was digested with jack bean α-mannosidase, the apparent size was reduced (Fig. 2A). Aga2p expressed alone was reduced to about 19 kDa (lane 2), whereas Aga2p released from the complex generated heterogeneous material with apparent size ranging from the dye front up to 25 kDa (lane 4). Within the smear there were visible bands at 15 kDa (poorly visible in the photo), 23 kDa, and 25 kDa. Deglycosylated Aga2p isolated alone or in complex with Aga1p-(1–149) remained stainable by PAS reagent (data not shown).

Role of Aga1p in Stabilization of a-Agglutinin—In addition to high specific activity and measurable secondary structure, the purified His6-Aga2p-Aga1p-(1–149) complex was stable for several months at $-20^\circ$ C. To determine whether disulfide bonding of the subunits stabilized an active form of α-agglutinin, the complex was treated with DTT for various times, diluted, and assayed (Fig. 4). The activity decreased with a halftime of about 20 min; the unreduced complex was much more stable under these conditions.

Characterization of the Stabilizing Region of Aga1p—To determine the minimal region of Aga1p needed to stabilize Aga2p, we constructed deletions within the AGA1 gene and co-expressed the various forms of Aga1p with His6-Aga2p. Aga1p-(1–136) terminates after the intact Cys-rich region and allowed secretion of activity equivalent to Aga1p-(1–149) (Fig. 1B). In contrast, Aga1p-(1–132) lacks two Cys residues and supported activity only at a level equivalent to that of Aga2p alone. Therefore, it is likely that Cys133, Cys136, or both are supported activity only at a level equivalent to Aga1p-(1–149). In contrast, Aga1p-(1–132) lacks two Cys residues and supported activity only at a level equivalent to that of Aga2p alone. Therefore, it is likely that Cys133, Cys136, or both are important for maintenance of activity of Aga2p.

To determine the N terminus of the domain required for stabilization of Aga2p, we constructed internal deletions that fused Aga1p residues 1–29, including the signal sequence, in-frame to various positions. Expression of Aga1p-(1–29,71–136), which consists only of the signal sequence and the intact cysteine-rich region, stabilized Aga2p activity, as did Aga1p-(1–29,106–136), which lacks the first Cys and 34 other residues but retains four Cys residues. More extensive deletions, to give Aga1p-(1–29,111–136) and Aga1p-(1–29,112–136), which re-
contain three and two cysteine residues, respectively, still stabilized activity at levels about 25% of the constructs with four Cys residues (Fig. 1B). In conclusion, activity of a-agglutinin was maximal when the binding subunit Aga2p was coexpressed with fragments of Aga1p that contained the last four Cys residues of the first Cys-rich repeat. The last two Cys residues, at positions 133 and 136 are particularly important.

The complex containing Aga1p-(1–29,111–136) with His6-Aga2p was purified to yield material with specific activity of $1.2 \times 10^{12}$ units/mol, 8.6-fold greater than Aga2p alone and about 5-fold less that the complex with Aga1p-(1–149). The far-UV CD spectrum was consistent with a structure contain-
Table I

| Mutant name | Mutation | Percent of wild type secreted activity | Complementation of aga2::LEU2 |
|-------------|----------|---------------------------------------|-----------------------------|
| Vector      |          |                                       |                             |
| Wild type   | 58FEYYS61| 100                                   |                             |
| 1–31        | S        | 319                                   |                             |
| 1–50        | .G       | 113                                   |                             |
| h13         | .C       | 200                                   |                             |
| 58–2        | .P       | 200                                   |                             |
| 58–9        | .F       | 400                                   |                             |
| 2–4         | .H       | 95                                    |                             |
| 59–1        | .S       | 238                                   |                             |
| 59–3        | .T       | 283                                   |                             |
| 59–6        | .L       | 400                                   |                             |
| 3–61        | .P       | 225                                   |                             |
| INS         | 55YAAAY59| 131                                   |                             |
| DEL         | Δ55FEYYS60| 93                                   |                             |

*Activity in growth supernatant from strains harboring overexpression constructs. Three independent transformants were tested in duplicate.

Agglutinability of MATa aga2::LEU2 cells harboring CEN plasmids with wild type or mutated AGA2 genes under the homologous promoter. Mutants where assays are not shown were not tested.

Agglutinability of MATa aga2::LEU2 cells harboring high copy plasmids with wild type or mutated AGA2 genes under the homologous promoter. Assays not listed showed complementation of the agglutination defect.

Mutations were generated by random mutagenesis.

Role of Cys Residues in Aga2p—Cappellaro et al. (20) showed that both Cys residues of Aga2p needed to be mutated to lose the ability to complement an aga2 mutation, indicating that only a single disulfide bond was sufficient for cellular agglutination activity (20). However, such cellular agglutination assays are less sensitive in determining loss of activity than the assays of secreted Aga2p (see below). To test whether disulfide bonding to Aga1p through one or both Cys residues is important for maintaining the active conformation of Aga2p, we tested single and double Cys-to-Ser mutations of Aga2p for stabilization of activity by co-expression of Aga1p-(1–149). Dot blots with anti-Aga2p indicated that all supernatants contained similar amounts of Aga2p. With Aga2p expressed alone, the single mutations (C25S and C68S) did not affect activity, and the double mutant showed a mild decrease in activity (Fig. 5). In this experiment co-expression of Aga1p-(1–149) with wild type Aga2p resulted in a 4-fold increase in activity. The Aga2p C25S, C68S double mutant should not be disulfide-linked to Aga1p, and as expected, co-expression of Aga1p-(1–149) did not affect activity. Interestingly, co-expression of Aga1p-(1–149) also did not increase the activity of either Aga2p single mutant. These results suggest that both disulfide bonds are necessary for the Aga1p interaction to stabilize Aga2p in its active conformation.

Mutational Analysis of Aga2p Determinants for α-Agglutinin Binding—We used mutagenesis to identify regions of Aga2p important for activity. The mutants were tested using several assays. First, assays of culture supernatants from cells overexpressing Aga2p were used to determine gross activity. Second, assays of supernatants from cells coexpressing Aga2p mutants and Aga1p-(1–149) were used to determine whether the mutated forms of Aga2p were stabilized by Aga1p. Finally, we tested the ability of mutant forms of aga2 genes expressed on CEN or high copy plasmids from the AGA2 promoter for the ability to complement the aga2::LEU2 mutant using cellular agglutination assays.

The affinity of agglutinins or other adhesion proteins can be estimated from assays using different concentrations of the proteins. Although protein concentrations in bulk solution can be easily estimated, the actual cell surface concentrations are significantly higher, so cellular assays will show lower affinity interactions observable at higher agglutinin concentrations (34). We can estimate the agglutinin concentration within 0.5 μm of the cell surface, corresponding to a spherical shell with 1.7-μm inner radius (the cell diameter) and 2.2-μm outer radius. For a spherical cell of radius 1.7 μm, the volume is 23 × 10⁻¹⁸ liters. For the 2.2-μm sphere the volume is 45 × 10⁻¹⁸ liters. On average there are 1–5 × 10⁴ molecules of agglutinin within this spherical shell (19, 21, 35); therefore the concentra-
Peptides containing the C-terminal ten amino acids of Aga2p bind to α-agglutinin (Ref. 20 and data not shown), indicating that this region comprises part or all of the α-agglutinin binding site. Therefore this region of Aga2p was explored by site-specific mutagenesis to identify residues important for activity (Table II). In the first series of mutants, pairs of residues were mutated to alanine. The most C-terminal double mutations, VF(86–87)A and QY(84–85)A showed undetectable Aga2p activity when assayed alone or when coexpressed with Aga1p (1–149). The agglutination defects became less severe for more N-terminal mutants, with GS(78–79)A showing wild type activity. When these mutants were re-assayed in the more sensitive cellular agglutination assays for complementation of aga2:LEU2, most showed some activity. The exception was VF(86–87)A, which did not show detectable activity. Immuno blotting of the supernatants showed that all mutant forms were secreted at levels of at least 25% of wild type level.

Single mutations in this region were generated by site-specific and random mutagenesis. These mutants showed a similar trend; mutations in residues closer to the C terminus had greater defects (Table II). Mutations that resulted in secretion of inactive Aga2p altered the two most C-terminal residues (F87A, F87L, and V86G). The severity of the defect varied with the particular amino acid substitution, with F87Y, V86A, and V86I showing 20- to 50-fold decreases in activity when expressed alone, but each showed activity close to wild type when coexpressed with Aga1p (1–149). In addition, the Y85A mutant showed a 20-fold decrease in activity, and was stabilized significantly by coexpression with Aga1p (1–149). Other mutations near the C terminus, Q84A, T83A, I81A, and P80A, also reduced activity about 2- to 4-fold, and had close to wild type activity. When these mutants were re-assayed in the more specific mutagenesis to identify residues important for activity (Table II). In the first series of mutants, pairs of residues were mutated to alanine. The most C-terminal double mutations, VF(86–87)A and QY(84–85)A showed undetectable Aga2p activity when assayed alone or when coexpressed with Aga1p (1–149). The agglutination defects became less severe for more N-terminal mutants, with GS(78–79)A showing wild type activity. When these mutants were re-assayed in the more sensitive cellular agglutination assays for complementation of aga2:LEU2, most showed some activity. The exception was VF(86–87)A, which did not show detectable activity. Immuno blotting of the supernatants showed that all mutant forms were secreted at levels of at least 25% of wild type level.

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activity when coexpressed with Aga1p(1–149). Again, all mutant proteins were secreted at levels at least 25% that of wild type. No single mutation inactivated Aga2p completely; all mutants complemented the aga2::LEU2 mutant under conditions that measured binding affinities in the millimolar range. Only the C-terminal F87A mutant showed a detectable reduction in complementation of aga2::LEU2. These results indicate that the most C-terminal residues of Aga2p are critical for its binding activity. In accordance, a His tag at the C terminus of Aga2p eliminated activity, indicating that activity is dependent on a free C terminus (data not shown).

**Other Regions of Aga2p May Contribute to Ligand Binding**—Many protein-protein interactions involve multiple noncontiguous regions of protein (12). AGA2 was therefore randomly mutagenized by PCR, and the products were co-transformed with a gapped AGA2 plasmid to obtain intact genes by homologous recombination. Those plasmids mediating altered a-agglutinin activity were isolated, retested to confirm the phenotype, and sequenced to identify the mutation(s).

Of the missense mutants, N48I showed almost a 3-fold reduction in secreted Aga2p activity that was only partially complemented by Aga1p(1–149). Alanine scanning mutagenesis of the surrounding residues identified several mutants that also showed mild decreases in secreted Aga2p activity (Table III), but all of these mutants could complement the cellular agglutination defect in aga2::LEU2 cells (not shown). The reduced activity of some of these mutants suggests that this domain may contribute to a-agglutinin binding.

**DISCUSSION**

*S. cerevisiae* a-agglutinin has been extensively characterized and shares features with mammalian adhesion proteins, including immunoglobulin fold domains. In contrast, structure and function of the complementary adhesion protein a-agglutinin remains poorly understood. It is composed of two subunits with well-defined functions: one for α-agglutinin binding and one for cell surface anchorage (1). Although *C. albicans* homologs of α-agglutinin are known (13), no homologs of the a-agglutinin binding subunit Aga2p have been reported. The results of this paper define molecular roles for individual residues and regions in the binding subunit and show a previously unrecognized role for the anchorage subunit in maintenance of the active conformation of the binding subunit. These findings are summarized in Fig. 7.

**Roles of the Anchorage Subunit Aga1p in Aga2p Activity**—Purification of Aga2p alone led to an unstable protein with low specific activity. Co-expression of fragments of Aga1p along with Aga2p potentiated α-agglutinin activity, increasing secreted activity 10- to 20-fold (Fig. 1B), and increasing molar specific activity 43-fold. In addition, disruption of the Aga1p-Aga2p complex decreased α-agglutinin activity (Fig. 4). CD spectroscopy identified well-defined secondary structure for Aga2p-Aga1p complexes, whereas isolated Aga2p did not show well-defined structure. This Aga1p-mediated secondary structure derived, at least in part, from the Aga2p component, because the total α-helical, β-sheet, and β-turn content (53% of the residues) was greater than the fraction of residues contributed by Aga1p to the complex (30%) for the His<sub>6</sub>-Aga2p-Aga1p(1–29,111–136) complex. This result was consistent with the high fraction of structured residues in the larger His<sub>6</sub>-Aga2p-Aga1p(1–149) complex as well, with the difference in secondary structure content implying that the extra residues in Aga1p contributed additional α-helix and β-turn regions. These results indicate that Aga1p stabilizes the structure of the binding determinants in Aga2p.

The Aga2p-Aga1p interaction involves disulfide linkages to the two Cys residues in mature Aga2p, Cys<sup>25</sup> and Cys<sup>68</sup>. Cappellaro et al. (20) showed that both Cys residues must be mutated to lose cellular agglutinability, indicating that both are involved in the disulfide linkage. There have been no previous reports of which of the ten Aga1p Cys residues are involved. These residues are grouped into two repeats with five Cys residues each. Only a small part of Aga1p, including the N-terminal Cys repeat was necessary to stabilize α-agglutinin, and the Aga1p(1–149)-Aga2p complex had the highest specific activity so far reported for any form of the agglutinin. Deletion of sequences from the N-terminal through the second Cys residue (Cys<sup>106</sup>) resulted in Aga1p fragments that supported high activity. However, the C terminus of the repeat, including the fourth and/or fifth Cys residues, was essential for increased activity. Even a short fragment with the third, fourth, and fifth Cys residues (amino acids 111–136 fused to the signal sequence) supported increased secretion of a-agglutinin activity and an 8-fold increase in specific activity of the purified complex relative to Aga2p alone.

The a-agglutinin analogs from *Pichia amethionina* and *Hansenula wingei* contain multiple binding subunits disulfide-linked to a single anchorage subunit (1). Therefore, the Aga1p homologs from these yeasts may contain multiple Cys-rich clusters, with each cluster involved in disulfide linkage to a single Aga2p homolog. Similar clusters are found on many fungal cell wall proteins and are common in mammalian extracellular matrix proteins (36–38).

**Regions of Aga2p**—Cappellaro et al. (19) have reported that a C-terminal decapeptide of Aga2p has a-agglutinin activity about 20% that of intact Aga2p. Our results further define the binding region: Mutations of this C-terminal region reduced Aga2p activity, with the last two residues, Val<sup>86</sup> and Phe<sup>87</sup>,...
showing the most important role. No other region of Aga2p that is critical for activity was identified in the random mutagenesis, but mutations in a central region (residues 48–50) decreased activity, suggesting that this region may also participate in binding.

Although Aga2p alone and the C-terminal decapeptide can bind α-agglutinin, a highly glycosylated small peptide such as Aga2p may not have enough hydrophobic character to form a stable structure on its own. The ability of the Aga1p Cys-rich domain to stabilize Aga2p in an active structure suggests the interaction between the two proteins constrains Aga2p to a functional conformation. Because mutations of either Aga2p Cys residue involved in disulfide linkage eliminated this stabilization, both disulfide bonds appear to be critical for this constraint.

A complementary but unexpected phenotype was observed with mutations of residues between 56 and 61. These mutations increased secretion of fully active Aga2p, but the mutated proteins were not stabilized by co-expression of the Aga1p fragment. In addition, these mutations reduced the ability to complement an aga2 mutation in the cellular agglutinability assay. These results suggest that this region may be important for interaction of Aga2p with Aga1p; mutations in this region would therefore prevent cell surface anchorage of Aga2p.

The central region of Aga2p (residues 45–72) is 37% identical and 81% similar to sequences in several Nod genes from soy (Fig. 6) (39, 40). Also within this region of Aga2p, a short sequence motif shows similarity to von Willebrand Factor structure, and Dr. D. J. Tipper for peptide sequencing and syntheses.

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