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Sexual Agglutination in Budding Yeasts: Structure, Function, and Regulation of Adhesion Glycoproteins

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INTRODUCTION

Cell adhesion proteins mediate many cellular interactions, including mating reactions. The sexual agglutinins are cell adhesion proteins that mediate direct cell-cell contact during mating in budding yeasts (for previous reviews, see references 6a, 14, 17, 52, 109a, and 112). Until recently, most of the work on the agglutinins involved biochemical analysis. In the past few years, a molecular genetic approach has been initiated for *Saccharomyces cerevisiae* that is greatly facilitating the study of these highly glycosylated cell wall proteins. The yeast system provides an opportunity to study glycoprotein-glycoprotein interactions by using a combination of genetic, molecular, and biochemical approaches. In this review, we will describe results from both the biochemical and the molecular genetic approaches and discuss some of the intriguing questions that have arisen from recent studies.

The initial observation that led to the discovery of yeast sexual agglutinins was that particular strains of the yeast *Hansenula wingei* aggregate upon mixing but are not self-aggregable (8, 100). Further experiments with several species indicated that coaggregating strains are haploid and of opposite mating type and that the agglutination process involves specific cell surface glycoproteins expressed by each of the haploid mating types of each species (100, 101). The complementary agglutinins of each species interact with one another to promote stable cell-cell contact, which is an early step in the mating process (59, 89). Aggregates of thousands of cells are formed, and pairs of cells of opposite mating type fuse within these aggregates to form diploid zygotes (18, 37, 78). The ability of the complementary agglutinins to interact with one another indicates that the binding domain of each agglutinin (i.e., the domain that interacts with the complementary agglutinin) is accessible on the exterior surface of the cell wall. Sexual agglutination is

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TABLE 1. Nomenclature and properties of yeast agglutinins

Species	Mating type	Agglutinin name	Agglutinates target cells?	No. of binding sites per molecule	No. of molecules per cell	K_a (M^{-1})
α-Agglutinin analogs						
S. cerevisiae	α	α-Agglutinin, α-agglutination substance	No	1	5×10^{4a}	1×10^{9b}
S. kluyveri	17	17-Agglutinin	No	1	5×10^5	3×10^8
	α	α-Agglutinin				
H. wingei	21	21-Factor, 21-agglutinin	No	1	3×10^6	10^5–10^7
P. amethionina	**a**	**a**-Agglutinin	No	1	ND[c]	ND
a-Agglutinin analogs						
S. cerevisiae	**a**	**a**-Agglutinin, **a**-agglutination substance	No	1	3×10^{4a}	1×10^{9b}
S. kluyveri	16	16-Agglutinin	No	1	ND	3×10^8
	a	**a**-Agglutinin				
H. wingei	5	5-Factor, 5-agglutinin	Yes	5–8	ND	10^5–10^7
P. amethionina	α	α-Agglutinin	Yes	ND	ND	ND

[a] Sites per cell after induction by pheromone.
[b] Weaker sites also present.
[c] ND, not determined.

cation independent and is distinguishable from the flocculation or self-aggregation that occurs with some yeast strains, which involves a calcium-dependent mechanism and has different biochemical and genetic components (45, 57).

The budding yeasts in which the sexual agglutinins have been studied share several characteristics. Haploid cells exist as either of two mating types which can fuse to form a diploid. Each of the haploid mating types expresses a set of proteins specific to that mating type, including the sexual agglutinins, peptide pheromones, and pheromone receptors (18, 78, 112). The pheromones have been most extensively analyzed in *S. cerevisiae*, but homologs to the α-factor peptide secreted by *S. cerevisiae* α cells have been identified in *Saccharomyces kluyveri* (secreted by cells of the 17 or α mating type) and *H. wingei* (secreted by cells of the 21 mating type). One of the responses to pheromone is induction of agglutinability. The agglutinins promote close interactions between cells of opposite mating type and may increase the efficiency of mating by facilitating response to pheromone.

OVERVIEW OF AGGLUTININ FEATURES

The biochemistry of the sexual agglutinins has been studied most extensively in four species of budding yeast: *H. wingei*, *Pichia amethionina*, *S. kluyveri*, and *S. cerevisiae*. Combining the analyses from these yeasts, a consistent pattern emerges (9) (Table 1; Fig. 1). In each species, cells of one mating type (analogous to *S. cerevisiae* α cells) express a heat-labile glycoprotein consisting of a single polypeptide. The opposite mating type (analogous to *S. cerevisiae* **a** cells) expresses a heat-insensitive, highly glycosylated protein composed of a cell surface anchorage subunit and one or more small subunits attached by disulfide linkage. Treatment of intact cells with reducing agents eliminates agglutinability by releasing the small subunits, which contain the domains for binding to the opposite agglutinin present on target cells.

Historically, the nomenclature for the mating types of the different yeast species has no consistent basis and is potentially confusing (Table 1). We will refer to the agglutinins that share properties with the *S. cerevisiae* α- and **a**-agglutinins as α- and **a**-agglutinin analogs, respectively. The agglutinins from some yeast species have in some cases been called factors rather than agglutinins. To prevent confusion with the pheromones (α-factor and **a**-factor), we will use the term agglutinin (e.g., *H. wingei* 21-agglutinin rather than 21-factor).

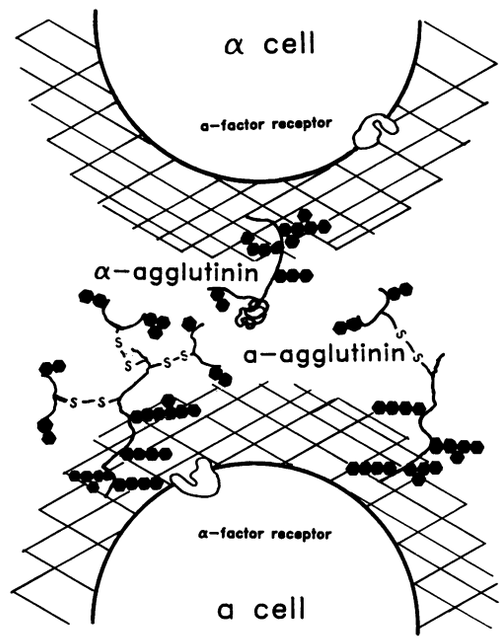

FIG. 1. Model for sexual agglutinins in budding yeasts. Plasma membranes are shown as thick lines; cell walls are cross-hatched; and hexagons represent agglutinin carbohydrate moieties. α-Agglutinin analogs are composed of a single polypeptide with N- and possibly O-linked carbohydrate. The **a**-agglutinin analogs consist of a highly O-glycosylated core subunit, which mediates cell surface anchorage, and one or more binding subunits. *H. wingei* and *P. amethionina* **a**-agglutinin analogs are multivalent (i.e., contain multiple binding subunits per core molecule [left]), and *Saccharomyces cerevisiae* and *Saccharomyces kluyveri* **a**-agglutinins are monovalent (i.e., contain a single binding subunit per core molecule [right]).

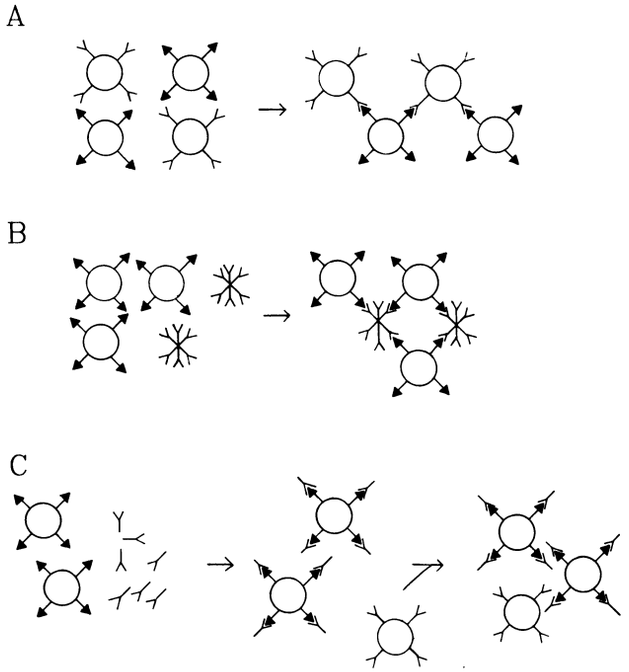

FIG. 2. Agglutination assays. (A) Cells of opposite mating types interact to form macroscopic aggregates. (B) Multivalent **a**-agglutinin analogs bind to multiple target cells, resulting in aggregates that are assayed as in panel A. (C) Monovalent agglutinins mask target sites and therefore inhibit agglutination.

Assays

Assays for agglutinability of intact cells involve visual inspection or spectrophotometric measurement of the degree of clumping in mixtures of cells of opposite mating type (Fig. 2A) (9, 14, 89, 117). Assays for solubilized or purified agglutinins are based on their ability to bind specifically and reversibly to cells of the opposite mating type (target cells). Specific binding of ^{125}I-labeled agglutinin to target cells can be assessed directly (67, 88). Unlabeled multivalent agglutinins, which contain multiple binding sites per molecule, can be assayed by their ability to agglutinate target cells in a manner similar to agglutinating antibodies (Fig. 2B). Monovalent agglutinins, which contain a single binding site per molecule, cannot agglutinate target cells. Instead, they are assayed by their ability to occupy binding sites on the target cells, inhibiting agglutination (14, 75, 88) (Fig. 2C).

Species Specificity

Agglutinins from one yeast species do not interact efficiently with the agglutinin of the other class from other species. Species specificity has been tested both by assaying agglutination of cells of different species and by using binding assays with purified agglutinins. Burke et al. (9) showed that *H. wingei* type 5 cells formed only very small aggregates when mixed with *P. amethionina* **a** cells and showed no significant aggregation with *S. kluyveri* 17 cells (contrary to a previous observation by Taylor [83]). None of the purified agglutinins showed reactions with cells of the other species. ^{125}I-labeled *S. kluyveri* 16-agglutinin binding subunit bound specifically to *S. kluyveri* type 17 cells, but not to *H. wingei* type 21 cells or *P. amethionina* **a** cells (67). Yamaguchi et al. tested agglutination by pairs of strains of both mating types of the four yeast species discussed above as well as several other species (107). No cross-agglutination between species was seen. Similar results were reported in a study of eight species of budding yeasts from the genera *Saccharomyces*, *Saccharomycodes*, and *Pichia* (117). These findings indicate that the biochemical similarities of the analogous agglutinins from different species do not include common binding determinants and do not promote agglutinin interactions between the species.

The detailed biochemical analyses that led to this overview of agglutinin structure are described in the next sections. The reported sizes of the agglutinins vary greatly, since different isolation techniques yield differences in carbohydrate content and degree of proteolysis. After describing the biochemistry, we discuss the molecular genetics and regulation of the agglutinins and end with some thoughts on the role of the agglutinins in mating.

BIOCHEMISTRY OF α-AGGLUTININ ANALOGS

The α-agglutinin analogs are cell surface glycoproteins containing 30 to 50% carbohydrate (Tables 1 and 2). Much of the carbohydrate is N linked, because it is removed by endo-β-*N*-acetylglucosaminidase H (endo H). This N-linked carbohydrate is not required for activity. The activity of these agglutinins is heat labile but is unaffected by reducing agents. Biochemical and genetic analyses imply that the α-agglutinins are monovalent (possess a single binding site) and are each composed of a single polypeptide.

H. wingei 21-Agglutinin

H. wingei 21-agglutinin was first isolated by Crandall and Brock following trypsinization of type 21 cells and affinity purification on type 5 cells (14). The binding activity of purified 21-agglutinin is destroyed by alkali or prolonged heating above 60°C. The tryptic fragment sediments at 2.9S (about 30,000 to 40,000 Da) and contains 35% carbohydrate. Burke et al. repeated this purification and isolated a form of molecular weight 27,000 (9). This material contained 5% carbohydrate, all released by treatment with endo H, indicating that it is N linked. A larger form was solubilized by treatment of type 21 cells with Zymolyase, a lytic enzyme mixture containing (β1–3)-glucanase and proteases (9, 118). Both forms of 21-agglutinin are acidic (pI = 3.8; 25 to 30% Asx and Glx) and contain a high proportion of hydroxyamino acids (32%). It was suggested that the larger form released by Zymolyase treatment contains a portion of the protein involved in cell wall anchorage that is not present in the smaller form released by trypsin. The low carbohydrate content of the 27,000-Da form, which possesses binding activity, suggests that the binding domain contains little carbohydrate.

P. amethionina a-Agglutinin

In *P. amethionina* the mating-type nomenclature is reversed relative to *S. cerevisiae* (56), so the agglutinin from **a** cells is analogous to *H. wingei* 21-agglutinin and *S. kluyveri* and *S. cerevisiae* α-agglutinins. A large mannoprotein is released by Zymolyase treatment and a smaller (27,000-Da) active protein is released by subtilisin digestion. The **a**-agglutinin activity is labile to heat but insensitive to reducing agents. The 27,000-Da species contains 22% Asx and Glx and 32% Ser and Thr residues (Table 2) (56). **a**-Agglutinin did

TABLE 2. Composition of yeast agglutinins

Amino acid	Amino acid composition (mol%) of:									
	α-Agglutinin analogs					a-Agglutinin analogs				
						Core or core plus binding subunits			Binding subunits	
	H. wingei 21-agglutinin (10)[a]	*P. amethionina* a-agglutinin (56)[b]	*S. kluyveri* 17-agglutinin (67)[c]	*S. cerevisiae* α-agglutinin (48, 104)[d]	*S. cerevisiae* α-agglutinin (48)[e]	*H. wingei* 5-agglutinin (115)[f]	*P. amethionina* α-agglutinin (56)[g]	*S. cerevisiae* a-agglutinin core (69)[h]	*H. wingei* 5-agglutinin (115)[i]	*S. cerevisiae* a-agglutinin (11)[j]
Asp				7.3	4.5			1.1		–[n]
Asp/Asn[k]	15.9	11.6	8.8			2.9	6.2		10.1	
Asn				8.5	6.5			1.1		4.3
Glu				4.5	4.5			3.2		5.8
Glu/Gln[k]	12.7	10.3	8.3			7.2	6.3		7.1	
Gln				3.9	3.1			2.3		5.8
His	0.8	2.3	TR[l]	1.2	1.2	0.2	1.1	1.2	0.1	1.4
Arg	0.9	0.6	1.3	1.5	1.2	1.2	0.3	0.3	3.8	–
Lys	1.7	1.7	6.3	2.7	2.3	0.4	1.9	1.4	0.1	4.3
Ser	8.3	26.4	10.4	11.5	15.7	52.4	30.8	33.6	17.4	14.5
Thr	12.9	6.4	8.3	10.9	13.5	8.9	20.2	21.5	26.8	15.9
Cys	ND[m]	ND	0.5	1.8	1.1	0.5	ND	1.5	3.5	2.9
Met	–	ND	0.9	1.5	1.1	–	ND	1.5	–	1.4
Pro	4.3	4.2	4.2	2.4	3.1	2.0	ND	4.3	2.2	7.2
Gly	7.9	15.7	13.3	5.8	4.8	1.7	6.4	1.5	2.7	5.8
Ala	6.1	12.0	5.9	6.7	6.2	5.2	7.1	4.5	7.0	2.9
Val	7.7	2.3	7.1	3.9	4.9	6.5	8.5	5.9	8.3	5.8
Ile	6.3	1.4	5.9	5.2	6.0	3.5	3.3	4.7	3.7	5.8
Leu	7.5	1.9	6.2	7.6	6.9	3.6	3.2	5.8	5.9	5.8
Tyr	4.3	1.7	3.6	5.5	4.2	1.1	0.8	2.2	0.8	5.8
Phe	3.1	1.6	9.2	6.4	5.4	0.7	1.1	1.8	0.1	4.3
Trp	ND	ND	ND	1.5	0.9	ND	ND	0.4	ND	–

[a] Agglutinin purified from trypsin digests of type 21 cells. MW 27,000; 5% carbohydrate.
[b] Agglutinin purified from subtilisin digests of **a** cells. MW 27,000; carbohydrate not determined.
[c] Agglutinin purified from Zymolyase digests of type 17 cells. MW 60,000 determined by SDS-gel electrophoresis, which may be inaccurate for glycoproteins; <20% carbohydrate.
[d] Composition of amino acid residues 20 to 351 of the α-agglutinin gene *AGα1*. The corresponding glycoprotein secreted by cells harboring a truncation construct has an MW of 43,000 (SDS-gel electrophoresis); 14% carbohydrate (104). This fragment contains the binding domain of α-agglutinin and may correspond closely to the α-agglutinin fragments released by proteolysis of other species.
[e] Composition of amino acid residues 1 to 650 of the *AGα1* gene. The corresponding glycoprotein purified from α cell extracts has an MW of 160,000 and contains 50% carbohydrate.
[f] Agglutinin purified from subtilisin digests of type 5 cells. MW 10^6; 85% carbohydrate.
[g] Agglutinin purified from Zymolyase digests of α cells. MW 10^6; 80% carbohydrate.
[h] Composition of amino acid residues 1 to 725 of the *AGA1* gene. The glycoprotein purified from **a** cell extracts has an MW of >200,000 and contains 90% carbohydrate.
[i] Subunit obtained from β-mercaptoethanol treatment of 5-agglutinin. MW 12,500; 30% carbohydrate.
[j] Composition of amino acid residues 19 to 87 of the *AGA2* gene. The subunit released from **a** cells with dithiothreitol has an MW of 18,000 (by SDS-gel electrophoresis) with 61% carbohydrate.
[k] Asp/Asn and Glu/Gln are shown as one value where the residues were not distinguished.
[l] TR, trace.
[m] ND, not determined.
[n] –, not detected.

not agglutinate α cells but could inhibit the agglutinability of α cells, indicating that it is monovalent.

S. kluyveri 17- or α-Agglutinin

The agglutinin from *S. kluyveri* 17 cells has been isolated in several forms following Zymolyase digestion of intact cells or cell walls. Purification was achieved by affinity chromatography on columns containing the binding fragment from the opposite mating type (41, 57, 99). Agglutinin activity is destroyed by incubation with trypsin or pronase or by incubation at 60°C for 20 min. Use of partially purified β-glucanase allows the isolation of 17-agglutinin of molecular weight >200,000 (99) or >400,000 (41). The >400,000-Da form includes 90% carbohydrate. Treatment with endo H indicates that much of the carbohydrate is N linked, but the resulting product retains carbohydrate, suggesting that there may also be O-linked carbohydrate. The endo H-treated agglutinin is active, and Lasky and Ballou (41) estimated that the intact protein has a mass of 90,000 Da.

A major active form of 60,000 Da and a minor form of 40,000 Da were obtained following limited proteolysis of the >200,000-Da glycoprotein. The protein portion of the active 60,000-Da fragment has a mass of 32,000 Da, consisting of 19% Ser and Thr and 17% Asx and Glx residues (Table 2) (67). Because carbohydrate is not removed from the 60,000-Da fragment by endo H treatment, Weinstock and Ballou (99) proposed that this domain contains only O-linked carbohydrate and that the larger glycoproteins include a domain that is highly N glycosylated and is involved in anchorage to the cell wall. These results suggest that N-linked carbohydrate is not required for agglutinin activity, but they do not provide information concerning a requirement for putative O-linked carbohydrate.

S. cerevisiae α-Agglutinin

S. cerevisiae α-agglutinin has been isolated in various forms ranging from 140,000 Da to a heterogenous mixture of 250,000 to 400,000 Da (31, 73, 75, 88, 109). Endo H treatment of a 160,000-Da form isolated from broken cells yields species of 160,000, 105,000, 72,000, 68,000, 50,000, 48,000, and 46,000 Da, all of which are active (88). Peptide analysis indicates that these forms are proteolytic products of a single protein (44). Endo F treatment of a 250,000- to 400,000-Da form released from cells by Zymolyase (β-glucanase) yields active forms of 72,000, 68,000, 57,000, 54,000, and 51,000 Da, all of which have the same N-terminal sequence (31). A 96,000-Da active glycopeptide isolated by trypsinization of α cells followed by treatment with endo F contains 19% Ser and Thr and 23% Glx and Asx residues (75). N-linked carbohydrate contents in the range of 30 to 50% have been reported, and N-linked carbohydrate is not required for activity (31, 75, 88). These results are consistent with the model proposed for the *S. kluyveri* 17-agglutinin, with a C-terminal highly glycosylated cell wall anchorage domain and a protease-releasable binding domain.

BIOCHEMISTRY OF a-AGGLUTININ ANALOGS

The *S. cerevisiae* **a**-agglutinin and its analogs from other yeasts are characterized by heat stability, susceptibility to reagents that reduce disulfide bonds, and carbohydrate contents of 80 to 95%, most or all of which is O linked. These agglutinins are disulfide-linked oligomers; a large, very highly glycosylated subunit mediates cell surface attachment (the core subunit), and small, less highly glycosylated subunits bind to the α-agglutinin analogs (the binding subunits). The *H. wingei* and *P. amethionina* **a**-agglutinin analogs are multivalent (i.e., they consist of multiple binding subunits attached to the core), whereas *S. kluyveri* and *S. cerevisiae* **a**-agglutinins are monovalent (i.e., they consist of a single binding subunit attached to the core). A single observation implying multivalence of *S. cerevisiae* **a**-agglutinin (25) has not been repeated.

H. wingei 5-Agglutinin

H. wingei 5-agglutinin was the first yeast agglutinin to be isolated, and it remains the best characterized biochemically. It has been isolated from β-glucanase-treated cell supernatants (83) or following limited proteolysis of whole cells (84). This released 5-agglutinin is a heterogeneous mannoprotein complex with a molecular weight of about 10^6, composed of 85% carbohydrate, 10% protein, and 5% phosphate (Table 2) (115). The protein portion consists of over 60% Ser and Thr residues, most or all of which have carbohydrate attached. Most of the carbohydrate is released by β-elimination, indicating that it is O linked (5, 115). Activity is eliminated by either pronase or α-mannanase, suggesting that both protein and carbohydrate are necessary for activity.

The 5-agglutinin preparations agglutinate cells of the opposite mating type (called type 21 cells). Treatment with a reducing agent eliminates this activity and generates two products. One product is a large (molecular weight between 500,000 and 1,000,000) inactive subunit that contains most of the protein and carbohydrate, does not differ much in size from the original 5-agglutinin, and has a somewhat higher proportion of Ser and Thr residues (71%). The second product is a small (12,000-Da) subunit that is not active in agglutination of type 21 cells but retains the ability to bind specifically to type 21 cells (86). The amino acid composition of the binding subunit differs from that of the original 5-agglutinin, including a lower proportion (40%) of Ser and Thr residues (Table 2). These results led to the model of a large core glycopeptide disulfide bonded to binding subunits; it is estimated that about six binding subunits are attached to each core. Each 5-agglutinin binding subunit (12,500 Da) is estimated to contain 28 amino acids and 60 mannose units (115) and binds weakly to 21-factor ($K_a = 10^4$ to 10^7 M^{-1} [85, 86]). The presence of multiple binding subunits greatly increases the strength of binding ($K_a = 10^{10}$ to 10^{14} M^{-1}).

The carbohydrate present on 5-agglutinin is composed of branched chains of 10 to 15 sugar units attached to Ser and Thr residues (115). In contrast, the O-linked carbohydrate of bulk yeast cell wall mannan consists of short linear oligosaccharides (dimer to tetramer [5, 114]). The observation that the activity of **a**-agglutinin and its analogs is destroyed by β-elimination or glycosidases and by some proteases suggests that both carbohydrate and protein are essential for binding. No information is available as to whether peptide or oligosaccharide or both are responsible for the specificity of the binding reaction.

P. amethionina α-Agglutinin

The agglutinin from *P. amethionina* α cells shows properties similar to the *H. wingei* 5-agglutinin (56). The *P. amethionina* α-agglutinin released from cells by Zymolyase (β-glucanase) treatment has a molecular weight of 1×10^6 and consists of 80% mannose, 12% protein, and 5% phosphate by weight. The overall proportions of acidic, hydroxy, and hydrophobic amino acids are similar to those of the *H. wingei* 5-agglutinin (Table 2), including over 50% Ser and Thr residues, most of which are glycosylated. The α-agglutinin is able to agglutinate **a** cells, indicating that it is multivalent. Reducing agents eliminate this agglutination activity, but the released monovalent binding subunit retains the ability to inhibit the agglutination of **a** cells by α cells (56). The agglutinability of α cells is also eliminated by reducing agents, presumably owing to release of the α-agglutinin binding subunit.

S. kluyveri 16- or a-Agglutinin

The *S. kluyveri* 16-agglutinin released by spheroplasting has a molecular weight of 5×10^5 and consists of over 95% carbohydrate (67). This agglutinin does not agglutinate type 17 cells but can inhibit their agglutinability, indicating that it is monovalent. The agglutinability of type 16 cells is eliminated by reducing agents, and an active binding fragment can be obtained by treatment of the purified 16-agglutinin or whole type 16 cells by reducing agents. The binding subunit from a wild-type strain has a molecular weight of 35,000. Binding activity is heat stable but sensitive to some proteases, α-mannosidases, and mild β-elimination, suggesting that the 16-agglutinin binding determinant includes both carbohydrate and peptide components (67).

S. cerevisiae a-Agglutinin

Different isolation techniques have yielded **a**-agglutinin species of different sizes. A >200,000-Da form was isolated from broken cell extracts (97), a 10^6-Da form was isolated by spheroplasting, and 23,000- and 130,000-Da forms were isolated from broken cell extracts from autoclaved cells (28,

73, 108). All of the forms have similar binding properties, and forms of higher molecular mass have higher carbohydrate contents with the largest forms containing >90% carbohydrate. The activity of the 130,000-Da species is labile to several proteases. Both the 130,000 and 23,000-Da forms were reported to be inactivated by reducing agent. This sensitivity of the smaller form is inconsistent with subsequent results (65, 91) and with the structure proposed for analogous agglutinins, i.e., an active binding fragment released from a core subunit by reducing agents. The very high concentration of reducing agent used in these experiments is sufficient to result in denaturation of proteins; the loss of activity is therefore likely to be an artifact of the experimental protocol. Other results are consistent with a structure similar to the **a**-agglutinin analogs in other yeasts (see below).

An O-glycosylated protein of 22,000 Da with 29% carbohydrate is released by dithiothreitol treatment of **a** cells that have been induced with the pheromone α-factor, but is not released from unexposed cells (65). Immunofluorescence studies with antibodies isolated against this protein indicate that it localizes to the cell surface of **a** cells only after exposure to pheromone. This glycoprotein inhibits agglutination of α cells, indicating that it is the binding subunit of **a**-agglutinin. The gene encoding this peptide, *AGA2*, has been sequenced (11) and shows an amino acid composition that is neutral and rich in Ser and Thr residues (Table 2). The activity of the binding subunit is stable to reduction and alkylation of the single cysteine residue (65, 75, 98). Treatment of **a**-agglutinin with periodate under conditions that are specific for destruction of carbohydrate or treatment with proteases eliminates activity, suggesting that both carbohydrate and peptide determinants are required for activity. Treatment of intact pheromone-induced **a** cells with reducing agents also eliminates agglutinability. Together, these results are consistent with the pattern seen in the other yeast species, that the **a**-agglutinin consists of a binding subunit attached to a core subunit by disulfide linkage.

AGGLUTININ BINDING

Intermolecular complexes of agglutinins from opposite mating types have been observed in *H. wingei*, *S. kluyveri*, and *S. cerevisiae* (14, 16, 28, 67), indicating that the agglutinins interact directly with each other, i.e., that the agglutinins are involved in complementary interactions. The nature and properties of the interactive bonds in complexes of purified agglutinins are similar to those of cell-cell bonds formed between intact cells (9, 89, 116). The binding domains appear to interact in a 1:1 ratio; i.e., each **a**-agglutinin binding domain interacts with a single molecule of α-agglutinin (16, 67). Analysis of the area of cell-cell contact implies that cells interact through binding of the agglutinins to each other at multiple points on the cell surface of both cells (66). The ability of *H. wingei* 5-agglutinin to agglutinate type 21 cells indicates that at least two binding subunits of a single agglutinin molecule can bind to target cells (86) to facilitate aggregate formation. For strains with monovalent agglutinins, each cell must interact with several other cells via multiple agglutinin sites to result in aggregation (66).

Characterized association constants for agglutinin interactions are in the range of 10^8 to 10^{10} M^{-1}. For multivalent agglutinins a weaker association constant of the binding fragment (e.g., 10^4 to 10^7 M^{-1} for *H. wingei* 5-agglutinin) is compensated for by the existence of multiple intermolecular bonds per agglutinin molecule (86). The number of binding sites per cell varies between species and between strains within a species, depends on growth conditions, and is increased by exposure to pheromone (see below). In *S. cerevisiae*, basal expression varies from undetectable to 10^4 molecules per cell for **a**-agglutinin and from about 10^3 to 10^4 molecules per cell for α-agglutinin. After exposure to pheromone, both mating types express 2×10^4 to 5×10^4 molecules per cell (90, 98, 106). *H. wingei* type 21 cells and *S. kluyveri* α cells show a basal expression of 5×10^5 sites per cell (Table 1) (67, 85). The strength of the agglutination reaction is more closely correlated with site number than with binding constant. *S. cerevisiae*, which is the weakest agglutinator of the four well-characterized yeasts, has a relatively large association constant, but the fewest binding sites per cell. Induction by pheromone increases agglutinability by increasing the number of binding sites with no change in association constant (90).

In *S. cerevisiae* both agglutinins show complex binding characteristics with cells of the opposite mating type (46, 47). Binding of ^{125}I-labeled α-agglutinin shows both a weak interaction ($<10^8$ M^{-1}) and a stronger interaction (10^9 M^{-1}). This dual affinity is mirrored in assays of **a**-agglutinin binding to α cells (46). Formation of tight bonds is cold sensitive, as is interaction of the intact cells (47, 89). Both weak and tight interactions show similar pH dependence, and the same agglutinin molecules can interact in either the weak or tight mode. These data suggest either that binding of agglutinin to the opposite agglutinin occurs at heterogeneous sites or that an initial weak interaction is followed by a cold-sensitive conversion to a tighter interaction. Given the similar binding behavior of the two agglutinins, the second hypothesis seems more likely. Recently, Cappellaro et al. have shown that His_{292} of α-agglutinin is essential for binding, and they have proposed that the agglutinins interact through complementary charged amino acid residues (11).

Role of Carbohydrate

Carbohydrates were first implicated as binding determinants in cell adhesion systems when Roseman suggested that they have the diversity, specificity, and plasticity required for cellular interactions in developing systems (68). Such interactions have been modeled on lectin-sugar interactions, and the hypothesis was confirmed by the discovery of cell adhesion proteins with lectinlike domains and carbohydrate ligands (7).

Mutants of *H. wingei* type 5 cells (76), *S. kluyveri* type 16 cells (67), and *S. cerevisiae* **a** cells (5) in which certain carbohydrate moieties of cell wall mannoproteins, including the agglutinins, are lacking have been isolated. Specifically, mutants of *H. wingei* and *S. kluyveri* lacking mannosylphosphate residues and mutants of *S. cerevisiae* with defects in formation of mannosyl α1–2, mannosyl α1–3, and mannosyl α1-phosphate linkages have been tested. All of these mutants show normal agglutination indicating that at least these features of the carbohydrate are not necessary for activity (5, 67, 76, 88). Despite the implication of carbohydrate as playing a role in **a**-agglutinin function, no glycosides have been reported to inhibit agglutination in any of the yeasts (25, 44). A role for carbohydrate in binding specificity therefore remains uncertain.

Release of N-linked carbohydrate from α-agglutinin analogs does not affect activity, and the *S. kluyveri* 17-agglutinin binding domain has relatively little carbohydrate, suggesting that the specificity is determined by protein for these agglutinins (67). Some carbohydrate remains after endo H treat-

ment; it is unclear whether the remaining carbohydrate is O linked or unreleased N linked and whether it is necessary for binding activity.

MOLECULAR GENETICS OF *S. CEREVISIAE* AGGLUTININS

A molecular genetic approach to the study of the agglutinins in *S. cerevisiae* has been undertaken recently. The isolation of agglutinin structural genes has provided new information concerning agglutinin structure and is facilitating the analysis of agglutinin function. Two approaches have been used to obtain agglutinin structural genes. In collaboration, investigators in our laboratories have used a genetic approach: we isolated mutants defective in agglutination, determined which mutants affect agglutination in only one mating type (and therefore are candidates for structural gene mutations), and cloned the genes by complementation (48, 69). Investigators in W. Tanner's laboratory have used a biochemical approach: they purified the agglutinins, obtained amino acid sequence information, synthesized degenerate oligonucleotides corresponding to these sequences, and used the oligonucleotides to clone agglutinin structural genes (11, 31).

Isolation of Putative Agglutinin Structural Gene Mutants of *S. cerevisiae*

Agglutinin structural gene mutants might be predicted to have **a**-specific or α-specific mating defects; however, such mutants were not identified in screens for sterile mutants. Sterile mutants that do show severe agglutination defects have mutations in the mating-type locus or are nonspecific sterile mutants (i.e., show defects in both **a** and α cells) that are defective in components of the pheromone response pathway (18, 30, 78, 92). To isolate mutants specifically defective in agglutination, we developed an enrichment for nonagglutinable cells among a population of agglutinable cells. We used this enrichment to obtain agglutination-defective mutants after ethyl methanesulfonate mutagenesis of both **a** and α strains (48, 69). These mutants show variable levels of pheromone production and mating. Mutants showing pheromone production similar to the wild type (eliminating many pleiotropic mutants) were crossed to wild-type strains of the opposite mating type (completely sterile mutants were not analyzed further), and the diploids were sporulated and tetrads were analyzed. The majority of the mutants show nonspecific agglutination defects and low mating frequencies; they may represent nonspecific sterile mutants involved in the pheromone response pathway. The remaining mutants (five isolated in the α strain and nine isolated in the **a** strain) show α- or **a**-specific agglutination defects and levels of mating similar to those of the wild type in qualitative assays. The lack of a clearly observable mating defect explains the lack of **a**- and α-specific agglutination-defective mutants among the sterile mutants and is discussed further below.

Complementation and dominance tests involved construction of **a**/**a** diploids by using the *HO* gene to switch mating types for the **a**-specific mutants and construction of α/α diploids by protoplast fusion for the α-specific mutants (48, 69). All of the agglutination-specific mutants are recessive. The α-specific mutants fall into a single complementation group, consistent with the biochemical results indicating that α-agglutinin contains a single peptide. An α-specific agglutination-defective mutant, *agα1*, had been identified earlier and shown to be linked to *CEN10* (80, 81). Mapping indicated that our single complementation group corresponds to *AGα1* (48). Several of the **a**-specific mutants are defective in pheromone response; this pleiotropic effect indicates that they are not candidates for **a**-agglutinin structural genes. Five of the six remaining mutants fall into a complementation group called *AGA1*, which is immediately adjacent to the *PET494* gene on chromosome XIV (54, 69). A single mutation complements all the *aga1* mutants and therefore identifies a second complementation group, called *AGA2*, which has not been mapped.

S. cerevisiae α-Agglutinin Gene

The *AGα1* gene was cloned by complementation of *agα1* mutants (48) and by identification of DNA fragments complementary to oligonucleotide probes derived from the α-agglutinin amino acid sequence (31). Sequencing indicates that the *AGα1* gene could encode a protein of 70,500 Da (31, 48). Several lines of evidence indicate that *AGα1* encodes α-agglutinin. An anti-α-agglutinin antibody recognizes an *Escherichia coli* fusion protein containing amino acids 128 to 356 of *AGα1*. This antibody inhibits agglutination when preincubated with α cells, and the *AGα1* fusion protein prevents this inhibition. Antibody against the fusion protein itself also inhibits agglutination (48). Finally, the peptide sequence of the N terminus of the α-agglutinin fragments is found within the *AGα1* open reading frame (31).

Examination of the *AGα1* open reading frame reveals several interesting features (Fig. 3). The N and C termini are hydrophobic and are likely to be involved in cell surface localization (see below). There are 12 potential N-glycosylation sites, most of which are in the C-terminal half of the open reading frame. Ser and Thr, which are potential O-glycosylation sites, are 29% of the residues and are also clustered in the C-terminal half (Fig. 3). We hypothesized that the C-terminal half of the protein is highly glycosylated and involved in cell wall anchorage. We also speculated that the acidic region between residues 200 and 300 corresponds to the binding domain, which would contain little or no carbohydrate. This pattern is consistent with the model based on biochemical results for the *S. kluyveri* α-agglutinin.

α-Agglutinin Binding Domain

Several lines of evidence suggested that acidic residues are involved in binding activity of the α-agglutinin analogs. The active fragments of *H. wingei* and *P. amethionina* α-agglutinin analogs are acidic (Table 2). Binding has characteristics of an ionic interaction (89), because it is perturbed by high salt but not by nonionic detergents. The pH optima for binding in *H. wingei* and *S. cerevisiae* show that titrable groups with pI in the range of pH 4.5 and 6.5 are important (85, 89), and Asp and Glu residues are often titrated in this range. We therefore proposed that the acidic region between amino acids 200 and 300 of *AGα1* made up at least a portion of the binding site (Fig. 3). Several results provide evidence for a role of this domain in binding. Antibodies that recognize and block the binding domain should inhibit agglutination; therefore inhibition of agglutination by antibodies directed against a fusion protein containing amino acids 128 to 356 of *AGα1* indicates that the binding domain is within this region (104, 105). This fusion peptide is also able to prevent the inhibition of agglutination by antibody against intact α-agglutinin, indicating that the fusion peptide neutralizes antibodies directed against the α-agglutinin active site (49).

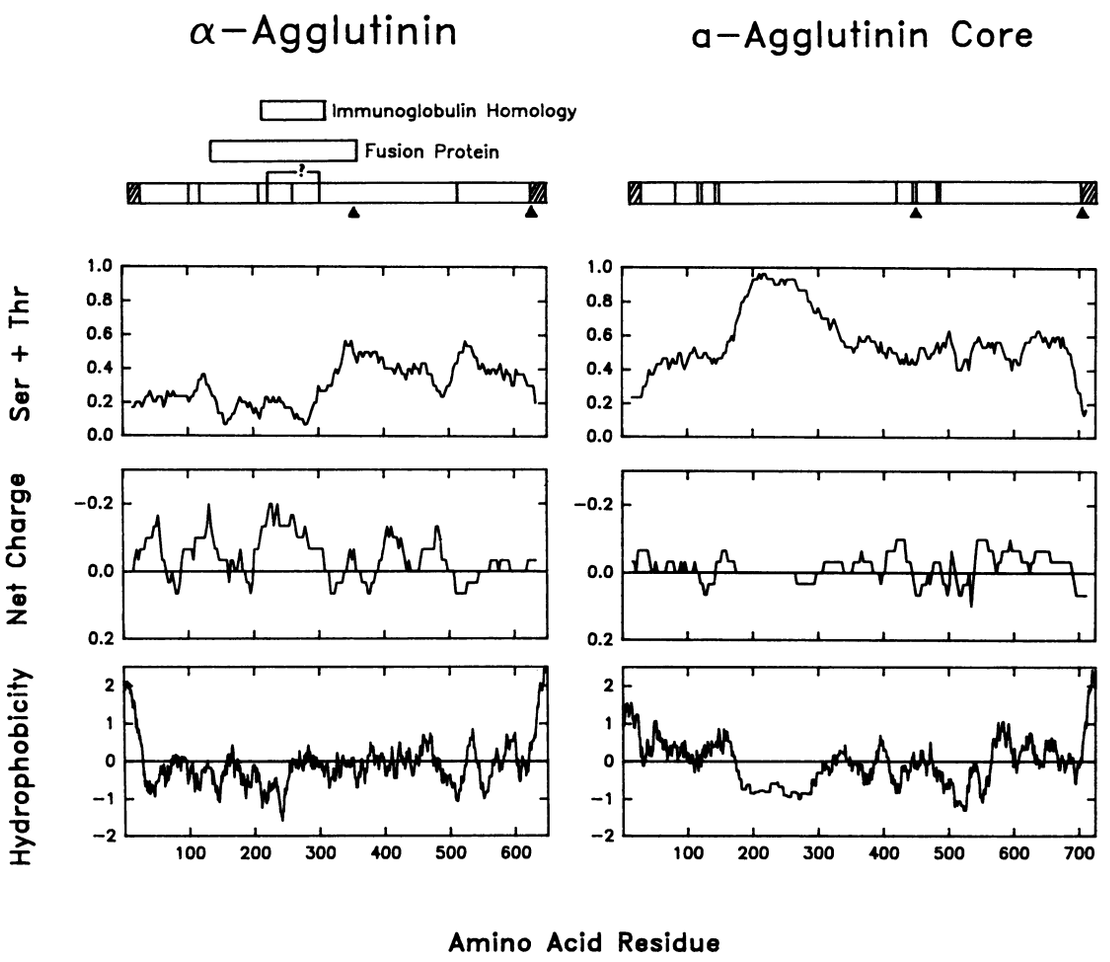

FIG. 3. Profiles of agglutinin structural gene coding sequences. At the top, the open reading frames of *AGα1* and *AGA1* are shown as rectangles, hydrophobic regions are shown by diagonal lines, and Cys residues are shown as vertical lines. In α-agglutinin, the region of the binding domain that elicits blocking antibodies and the region of immunoglobulin homology are shown. The putative immunoglobulin fold disulfide is also shown (marked by ?). Solid triangles denote truncations that secrete active agglutinins. The profiles aligned below each map show frequency of Ser + Thr, net charge, and Kyte-Doolittle analysis (40), each with a window of 20 amino acids. The profiles were prepared by using the University of Wisconsin Genetics Computer Group package.

All of the active proteolytic products of endo F-treated α-agglutinin isolated by Hauser and Tanner (31) had the same N-terminal sequence, which starts at amino acid 20 of the *AGα1* coding sequence, indicating that the binding domain is within the N-terminal two-thirds of the protein. Finally, a truncation mutant that expresses only the first 351 amino acids of *AGα1* produces an α-agglutinin fragment that inhibits agglutination and that binds to a cells with an affinity similar to that of wild-type agglutinin (Fig. 3) (104, 105). A truncation at amino acids 278, within the acidic region, is inactive. The essential histidine residue identified by Capellaro et al. is at position 292. On the basis of these results, the C-terminus of the binding domain is between amino acids 292 and 351, consistent with our speculation.

This proposed binding domain shows a feature found in adhesion proteins in higher eukaryotes. Many proteins involved in cell surface recognition phenomena contain regions similar to domains originally found in immunoglobulins, called the immunoglobulin fold (1). These domains contain two β sheets, each composed of several β strands. Cys residues at conserved positions in the two sheets stabilize the structure. Proteins with similarity to immunoglobulin fold domains have been divided into V-type and C-type proteins, which are similar to domains of immunoglobulin variable and constant regions, respectively (102). Conserved residues are found within the β strands that make up the β sheets, whereas sequences between strands are poorly conserved both with respect to sequence and length (102).

The *AGα1* sequence between amino acid residues 200 and 320 shows features similar to the immunoglobulin V-type fold, in both the position of predicted β strands and the presence of conserved amino acid residues. The degree of similarity to the consensus sequence is comparable to immunoglobulin fold domains of CD4, CD8, Thy1, Po, and the CAM family, all of which are cell adhesion proteins (102). The presence of an immunoglobulin fold structure indicates that this cell recognition motif dates to a time more ancient than the split between animals and fungi. Ligands of the animal cell adhesion proteins can be identical proteins (e.g., CAMs), other members of the immunoglobulin superfamily (e.g., T-cell receptor binding to major histocompatibility

complex antigens), or unrelated proteins (antibody binding to antigens). It will be interesting to determine whether the a-agglutinin binding subunit shows similarity to the immunoglobulin fold consensus.

S. cerevisiae a-Agglutinin Genes

Molecular genetic analysis implies that *AGA1* encodes the a-agglutinin core subunit (69). The *AGA1* open reading frame could encode a protein of 725 amino acids. The N and C termini are hydrophobic, consistent with a signal sequence that targets the protein through the secretory pathway and a C-terminal sequence involved in cell surface localization (see below). Overall, the open reading frame consists of about 50% Ser and Thr residues and does not contain potential sites for N glycosylation, consistent with a protein with a high proportion of O-linked carbohydrate and no detectable N-linked carbohydrate, as has been shown for the a-agglutinin analogs. The amino acid composition is similar to the amino acid compositions determined for the a-agglutinin analogs from *H. wingei* and *P. amethionina* (Table 2). No region of the *AGA1* open reading frame has an amino acid composition similar to that of the binding subunit (Table 2) (69, 98), indicating that *AGA1* does not encode the a-agglutinin binding subunit. The *aga1* mutants secrete active a-agglutinin binding subunit into the growth medium, suggesting that they are defective in cell wall anchorage of the binding subunit. Together, the secretion of a-agglutinin binding subunit by *aga1* mutants and the structural features of the *AGA1* gene seem most consistent with the conclusion that *AGA1* encodes the a-agglutinin core, i.e., the subunit involved in cell wall anchorage of the a-agglutinin binding subunit.

An interesting feature of the *AGA1* coding sequence is the presence of two homologous regions separated by a region of about 250 amino acids (Fig. 3). These repeats each contain 5 of the total 11 Cys residues present in *AGA1*. Cys-rich regions of unknown function are also found in some animal cell adhesion proteins (70). The remaining Cys residue is within the hydrophobic C-terminal region that we propose to be involved in cell surface localization and attachment (see below). It is therefore likely that the disulfide linkage to the single Cys residue of the binding subunit is to one of these two repeats. Given the multivalent nature of the analogous agglutinins from *H. wingei* and *P. amethionina*, we have speculated that the core genes from these yeasts contain several such repeats (69). The region between the two repeats contains a 100-residue region of about 90% Ser and Thr residues, which may be very highly O glycosylated. Mendonca-Previato et al. suggested that the high degree of glycosylation of the core subunit of a-agglutinin analogs would result in a low degree of secondary structure (56). The repeats may represent compact, structured domains necessary for attachment of the binding subunit within a relatively unstructured glycoprotein.

The *AGA2* gene may encode the a-agglutinin binding subunit. The *aga2* mutant has a mating phenotype similar to the *agα1* and *aga1* mutants (described below), but produces no active a-agglutinin, consistent with a structural gene defect in the binding subunit (69). Cappellaro et al. have determined the N-terminal sequence of the purified a-agglutinin binding subunit and have used oligonucleotides to identify the a-agglutinin binding subunit gene *AGA2*. It has not yet been determined whether this gene complements the *aga2* mutant identified by Roy et al. (69) or whether it corresponds to a different gene (82).

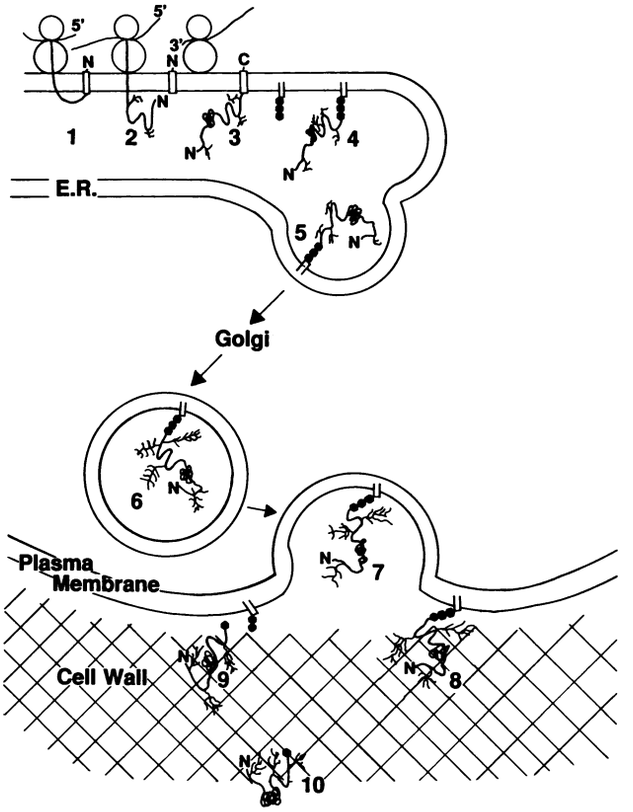

FIG. 4. Model for agglutinin localization. Boxes in the primary translation product indicate hydrophobic signal sequences. We propose that the agglutinins are translocated into the endoplasmic reticulum (E.R.), where the secretion signal sequence is cleaved and O-linked and core N-linked glycosylation occur. The C-terminal hydrophobic sequence is cleaved, and a GPI anchor is attached. The agglutinin would then be transported through the secretory pathway attached to the anchor. Further glycosylation occurs in the Golgi apparatus (93, 94), but is not shown. Because the mature agglutinin is likely to be covalently attached to the cell wall matrix rather than integrated into the plasma membrane, we propose that the agglutinin is transferred from the GPI anchor to the cell wall glycan. The mature agglutinin remains anchored to the cell wall with the binding domain exposed at the cell surface.

CELL SURFACE LOCALIZATION AND ANCHORAGE OF AGGLUTININS AND SPECULATIONS ON CELL WALL STRUCTURE

Experiments involving *S. cerevisiae* mutants defective in secretion have shown that localization of both a- and α-agglutinin to the cell surface occurs through the secretory pathway (93, 94). Consistent with this finding, both *AGα1* and *AGA1* have hydrophobic N-terminal sequences with features similar to signal sequences (31, 48, 69, 96). Mature α-agglutinin begins at amino acid 20 of the *AGα1* open reading frame, indicating that a 19-amino-acid signal sequence is removed during processing (31).

AGA1 and *AGα1* also contain C-terminal hydrophobic sequences that lack the basic residues characteristic of transmembrane domains (48, 69). These hydrophobic domains are reminiscent of sequences present at the C termini of precursors to eukaryotic cell surface proteins that are transported and linked to cell membrane by glycosyl phosphatidylinositol (GPI) anchors (19, 49). GPI anchors are

associated with cell surface proteins of diverse function in both unicellular and multicellular eukaryotes. Such proteins are synthesized as precursors with hydrophobic C-terminal sequences (Fig. 4). In an early step after transport into the endoplasmic reticulum, this sequence is cleaved and the remaining C-terminal amino acid becomes amide-linked to an ethanolamine residue, which is in turn linked through a phosphodiester bond to the C-6 position of a mannose residue in a complex glycan. The reducing terminus of the glycan is in turn linked to the inositol moiety of a phosphoinositide, resulting in attachment to the cell membrane.

The C-terminal half of α-agglutinin is not long enough to allow cell membrane anchorage of the mature form and simultaneous exposure of the binding domain at the surface of the cell wall, which is 1,000 to 2,000 Å (100 to 200 nm) thick (5, 66). In addition, agglutinins are released from cells by treatment with β-glucanase, which would not occur if they remained attached to the membrane by a GPI anchor. α-Agglutinin is therefore likely to be bound to the cell wall. We proposed that α-agglutinin is transported to the cell surface by a GPI anchor and then released from the membrane and transferred into the matrix of the cell wall (Fig. 4) (48). Transfer would involve release of a portion of the GPI moiety by *trans*-glycosylation or some other reaction (Fig. 4). Unlike the sugars in N- and O-linked oligosaccharides, the reducing ends of the sugars in a GPI anchor are oriented away from the peptide; therefore a *trans*-glycosylation reaction is possible. The finding that *AGA1* also has a hydrophobic C terminus leads us to extend this proposal to the cell wall anchorage of **a**-agglutinin as well as α-agglutinin (69).

The effects of truncation mutations indicate that the C-terminal hydrophobic sequences are involved in cell surface anchorage. Secretion of a truncated *AGα1* product of 350 amino acids that is active in binding to **a** cells (described above) indicates that cell wall anchorage involves the C-terminal 300 amino acids. Truncations that remove just the hydrophobic C terminus or replace this sequence with a random hydrophilic sequence also result in secretion of active α-agglutinin, indicating that the hydrophobic C terminus itself is involved in cell surface anchorage (104, 105). Truncations of the *AGA1* gene give similar results; active **a**-agglutinin binding subunit is secreted into the medium (69). Experiments in progress will test whether a GPI anchor is involved in this cell surface attachment.

Various other lines of evidence are consistent with this proposed role of a GPI anchor in cell wall attachment of the agglutinins. GPI-anchored proteins have been identified in *S. cerevisiae* (12, 13, 64). The components of a glycosidic linkage similar to linkages in GPI anchors are present in yeast cell walls (5, 55, 79). Mannose 6-phosphate is a common residue in yeast mannans and is often found in phosphodiester linkages (5). Mutations that prevent the formation of GPI tails are lethal, although N- and O-glycosylation are also affected in the known mutants (64a).

The *S. cerevisiae* cell wall is composed of fibrous polysaccharides (β-glucans) and proteoglycans (mannoprotein) (5, 24). Cross-links are necessary for the integrity of the composite structure. It is possible that cross-links are formed when a portion of bulk cell wall mannoprotein becomes incorporated into the cell wall by a mechanism involving linkage to a GPI anchor and transfer to the cell wall in a process similar to that proposed for the agglutinins. Elucidation of the nature of these cross-links is necessary to determine whether their structure is consistent with GPI anchor-mediated linkages (24, 55). In support of this model, it is known that inositol is required for cell wall biosynthesis and cell expansion (2, 29, 71), and phosphoinositols and variable amounts of lipid are also found in the cell wall (5). Some cell wall proteins are clearly not anchored to the cell wall by a GPI-mediated linkage; several proteins in addition to the **a**-agglutinin binding subunit are released from cells by treatment with reducing agents, indicating that covalent attachment is mediated solely through disulfide bonds (38, 65).

REGULATION OF EXPRESSION

Agglutinin expression in *S. cerevisiae* is subject to a variety of types of regulation. Agglutinin expression is limited to haploids, with one haploid mating type expressing one of the agglutinins and the opposite mating type expressing the complementary agglutinin. Exceptions to this pattern are the expression of a 5-agglutinin-like activity upon growth of *H. wingei* diploids in the presence of 0.1 to 2 mM vanadium salts and of a 21-agglutinin-like activity upon growth in the presence of EDTA (15). The physiological relevance of these findings is not clear. Agglutinins are also induced by exposure to pheromone, and levels of expression vary depending on temperature, carbon source, and aeration.

Mating-Type Regulation

α-Agglutinin is expressed by α cells but not by **a** or **a**/α cells, and **a**-agglutinin is expressed by **a** cells but not by α or **a**/α cells (18, 112). The analysis of agglutinin expression in mutants altered at the mating-type locus (*MAT*) suggested that the agglutinins are regulated by a mechanism similar to the regulation of other α- and **a**-specific products.

The *MAT*α locus encodes two proteins (32). *MATα1* is a positive regulator of α-specific genes, thus allowing their expression in α cells. *MATα2* is a negative regulator of **a**-specific genes, thus preventing their expression in α cells. In **a** cells, the **a**-specific genes are expressed constitutively. In **a**/α cells, the *MATα2* protein combines with a product of the *MAT***a** locus, *MAT***a***1*, to repress haploid-specific genes. Haploid-specific genes are expressed constitutively in cells of either the **a** or α mating type.

Mutations in the *MATα1* locus result in defects in agglutinability and α-agglutinin production (77, 92), suggesting that α-agglutinin expression is positively regulated by *MATα1*. For other α-specific genes, this regulation has been shown to be at the RNA level. Experiments with the *AGα1* gene confirmed this hypothesis (48). Northern (RNA) analysis showed that an *AGα1* transcript is present in α but not **a** or **a**/α cells. A sequence with homology to the consensus sequence for positive regulation by *MATα1* is found upstream of the *AGα1* coding region. Regulation of *AGα1* is therefore similar to that of other α-specific genes.

Mutations in *MATα2* result in expression of both α- and **a**-specific products: *MATα1* is present and induces expression of the α-specific genes and *MATα2* is absent, allowing expression of the **a**-specific genes (32). Some *matα2* mutants are nonagglutinable under standard growth and assay conditions (92), but express either α- or **a**-agglutinin when growth or assay conditions are changed; therefore the nonagglutinability observed under standard conditions was suggested to result from formation of inactive complexes of **a**-agglutinin with α-agglutinin, resulting in no free agglutinin of either type on the cell surface. Other *matα2* mutants are agglutinable with either **a** or α tester cells (77). These observations suggest that both α- and **a**-agglutinin are expressed by *matα2*

mutants and therefore that *MATα2* negatively regulates **a**-agglutinin.

Surprisingly, an *AGA1* transcript is expressed in both **a** and α cells at low levels (69). Consistent with this haploid-specific expression pattern, a sequence similar to the consensus sequence for haploid-specific expression, but not one for *MATα2*-mediated repression, is present upstream of the *AGA1* coding sequence. This result suggests that the **a**-agglutinin core subunit is expressed in both **a** and α cells. We have not seen any phenotype associated with *aga1* mutations in α cells. Because the **a**-agglutinin binding subunit is necessary for **a**-agglutinin activity, **a**-specific expression of the **a**-agglutinin binding fragment gene would be sufficient for **a**-specific expression of active **a**-agglutinin. In contrast, the **a**-agglutinin binding subunit gene *AGA2* is expressed only in **a** cells (11). This specificity ensures that active **a**-agglutinin is expressed only by **a** cells despite the nonspecific expression of the gene for the core subunit (69).

Induction by Pheromones

Initiation of sexual agglutination occurs within minutes of mixing in most *H. wingei* (14) and *P. amethionina* (56) strains but may take 20 to 30 min in *Saccharomyces* strains owing to the need for pheromone induction of agglutinin expression (52). Significant strain variability is seen in the basal level of agglutinin expression in *S. cerevisiae* and *H. wingei*; some strains are constitutively agglutinable, whereas others show no detectable basal agglutinin expression (10, 14, 52, 112). The sex pheromones induce agglutinin expression in their target cells in inducible strains and increase agglutinin levels in most constitutively agglutinable strains (6, 74, 89, 113, 117). The constitutively expressed and pheromone-induced agglutinins are biochemically indistinguishable (89), and both forms of α-agglutinin are encoded by a single gene (48, 106). Most α strains are constitutively agglutinable and show low levels of induction by pheromone, and most **a** strains agglutinate only after treatment with pheromone. This mating-type-dependent pattern of agglutinin expression resulted in isolation of mutants defective in pheromone response among potential **a**-specific agglutination-defective mutants, because expression of only basal levels of agglutinins has little effect in α cells but results in a severe agglutination defect in **a** cells (69).

Pheromones are known to induce increased agglutinability in *S. kluyveri* and *H. wingei*, as well as in *S. cerevisiae* (26, 27, 110). In *S. cerevisiae*, induction by pheromone is rapid and requires pheromone concentrations in the range of 3×10^{-11} to 1×10^{-9} M (3, 4, 59, 72). The pheromone concentration and exposure times are similar for agglutinin induction and for pheromonal induction of cell cycle arrest (59). Agglutinability of G_1 and G_2 cells is induced at a similar rate, indicating that cells are competent to respond throughout the cell cycle (90).

Northern analyses with the cloned agglutinin structural genes indicate that pheromone induction occurs at the RNA level. *AGα1* RNA levels in α cells increase at least 20-fold upon exposure to **a**-factor (48). This increase in RNA level is higher than the increase in the cell surface α-agglutinin level (2- to 7-fold) or in α-agglutinability (1.2- to 2.5-fold) (106). The explanation for this discrepancy between mRNA, protein, and agglutinability induction levels may be due to the stability of the constitutively expressed protein, which can provide a substantial pool of previously synthesized agglutinin even when constitutive transcription levels are low (37, 62, 87, 91, 104). Sequences homologous to the consensus sequence for pheromone induction are found upstream of the *AGα1* coding sequence.

AGA1 mRNA is induced in both mating types by exposure to pheromone from the opposite mating type (69). Several sequences with close matches to the consensus sequence for pheromone induction are found upstream of the *AGA1* coding sequence. Northern analysis also indicates that the **a**-agglutinin binding subunit gene *AGA2* is induced in **a** cells by exposure to α-factor (11). Only 57 base pairs of upstream sequence have been determined; any regulatory consensus motifs must lie farther upstream (11, 82).

Regulation by Growth Conditions

The level of constitutive agglutinin expression varies depending on the strain background as well as environmental conditions. Most strains lose constitutive agglutinability upon growth at temperatures above 30°C (21, 63, 91, 106). The carbon source used also affects agglutinin expression; growth on nonfermentable carbon sources lowers constitutive expression of the agglutinin (112). The temperature and carbon source dependence of agglutinin expression may depend on genes involved in general metabolic regulation. For example, *SNF5*, which was identified as being involved in glucose regulation of invertase expression, also regulates the constitutive expression levels of both **a**-specific (*BAR1*) and α-specific (*MFα1*) mRNAs (42) and therefore may also regulate the constitutive level of agglutinin expression.

Mutations Affecting Agglutinin Expression

A number of mutants that show altered agglutinin expression have been identified. Pleiotropic regulators of glucose metabolism (e.g., *SNF5*) may affect agglutinin function (42). Alleles of *tup1* (also called *umr7*, *flk1*, and *cyc9*) cause sterility in *MATα* cells by mimicking *matα2* mutants in their inappropriate expression of **a**-specific genes (43, 103). Such mutants express both agglutinins on their cell surface, resulting in a nonagglutinable phenotype due to formation of inactive complexes of the agglutinins (45, 77). *TUP1* has been proposed to be involved in glucose repression and cell surface phenomena, but the mechanism by which *tup1* mutations result in **a**-agglutinin expression in α cells is unclear. The original sterile mutants show reduced agglutinin expression and have now been shown to identify components of the pheromone response pathway (18, 30, 39, 78, 95). The decreased basal expression of pheromone-inducible gene products, including the agglutinin structural genes, can account for the agglutination defects observed in these mutants. Such pleiotropic mutants do not provide information on specific regulation of the agglutinins.

Mutants with altered basal expression of α-agglutinin have been reported. *SAG1* and *SAG2* were identified as being necessary for constitutive sexual agglutinability at 36°C in *MATα* cells (21). The *sag1* mutation was originally reported to be tightly linked to *MATα* (21), but was later reported to be an allele of *AGα1*, which is not linked to *MAT* (20). The *sag2* mutation was shown to be a temperature-sensitive allele of *MATα1* (22). Two mutations that led to loss of constitutive agglutinability at 36°C were also identified; the *cag1* mutation is linked to *MAT*, and the *cag2* mutation is unlinked to *MAT* (23). Neither of the mutants has been studied further. A recessive mutation, *saa1*, caused loss of constitutive agglutinability in cells of both mating types grown at 28°C. The mutation was unlinked to *MAT* or any tested markers including *sag1*. Several dominant mutations

causing loss of constitutive agglutinability in both mating types were also identified (111). One of these genes was tightly linked to *MAT*. Three other genes, *IND1*, *IND2*, and *IND3*, showed complicated segregation patterns. Whether any of these genes identify specific regulators of agglutinin expression has not been determined (60, 61).

ROLE OF AGGLUTININS IN MATING AND PHEROMONE RESPONSE

Nonagglutinable strains of *H. wingei* and *S. cerevisiae* that show normal pheromone response are able to mate (14, 48, 69, 81). This result explains the lack of identification of agglutinin structural gene mutants among the sterile mutants in *S. cerevisiae*. After obtaining agglutinin structural gene mutants, we investigated their mating properties in more detail. Mutations in *AGα1* (α-agglutinin structural gene), *AGA1* (a-agglutinin core subunit structural gene), and *AGA2* (putative a-agglutinin binding fragment structural gene) all result in very mild effects on mating efficiency (two- to fourfold decrease) when assayed on solid medium. When assayed in liquid medium, however, the mutants showed mating frequencies three to seven orders of magnitude lower than those of the isogenic wild-type strains (48, 69). This unusual mating phenotype indicates that the agglutinins are essential for mating under conditions that do not promote cell-cell contact (liquid medium) but are not essential under conditions that promote cell-cell contact (solid medium). For two cells of opposite mating type to fuse, they must be in direct contact. On solid medium with a high concentration of cells (the conditions under which standard quantitative mating assays are done), there is a high probability that a cell of one mating type will have a cell of the opposite mating type in direct contact. In liquid medium, however, cells of opposite mating type that randomly contact one another must remain in contact in order to mate. The agglutinins provide a mechanism for this prolonged contact to occur.

The apposition of cells resulting from the complementary agglutinin interaction may also facilitate efficient pheromone response and hence efficient mating. Jackson and Hartwell have shown that a cell can choose between mating partners and will mate preferentially with the cell that produces the higher level of pheromone (34, 35). This choice may be dependent on pheromone concentrations much higher than those necessary to induce increased expression of genes (3, 4, 34, 35). Induced a-agglutinin and, to a lesser extent, α-agglutinin have been shown to localize to the region of the cell called the shmoo tip, which is the region of cellular elongation that is induced by prolonged exposure to pheromone (98, 104). The *FUS1* cell fusion protein is also localized to this area (95), as is newly synthesized acid phosphatase (18). Since secretion appears to be directed to the shmoo tip, pheromones and pheromone receptors synthesized after pheromone induction are likely to be concentrated in this region (18), where fusion eventually occurs (35). An interaction of agglutinins at the shmoo tip will therefore provide a situation in which the concentrations of pheromone and pheromone receptors are highest at the region at which the agglutinins provide cell-cell adhesion. Such an effect might not be very significant under conditions of high cell density and limited diffusion of the pheromones, such as the mating assays on solid medium, where pheromone concentrations surrounding the cells are high. In liquid medium, diffusion of pheromone should result in lower pheromone concentrations. The agglutinins, by facilitating close apposition of secretory membrane and pheromone receptor at the shmoo tip, may increase the frequency of mating by raising the local pheromone concentration substantially, or possibly by providing a gradient of pheromone concentrations. This mechanism may be analogous to the function of synapses in the nervous system of higher animals (suggested by Fred Cross); in synapses, adhesive proteins hold transmitter receptors in close juxtaposition to the portion of the presynaptic cell that secretes the transmitter, thus reducing the amount of transmitter that must be secreted in order to effect the postsynaptic response (33). As in synapses, there are mechanisms for inactivation of excess pheromone. Pheromone inactivation should prevent gratuitous stimulation of adjacent cells (36, 50, 51, 53, 58).

PERSPECTIVE

When we initiated the molecular genetic approach to the study of yeast agglutinins, there was no evidence to indicate whether the molecular mechanisms of interaction between adhesion proteins were similar in yeasts and higher eukaryotes. The finding that the putative α-agglutinin binding domain shows similarity to the immunoglobulin fold structures of animal cell adhesion proteins suggests that these mechanisms may be evolutionarily highly conserved. The analysis of yeast cell adhesion protein interactions by a combination of biochemistry and molecular genetics should greatly enhance our understanding of cell-cell adhesion in this system.

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