Conserved WCPL and CX₄C domains mediate several mating adhesin interactions in *Saccharomyces cerevisiae*

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Running head: Interactions between Aga1p and Fig2p

Key words: Adhesin interactions, yeast mating, Aga1p, Fig2p, WCPL/CX₃C domains
ABSTRACT

Several adhesins are induced by pheromones during mating in *Saccharomyces cerevisiae*, including Aga1p, Aga2p, Sag1p (Agα1p), and Fig2p. These four proteins all participate in or influence a well-studied agglutinin interaction mediated by Aga1p-Aga2p complexes and Sag1p, however they also play redundant and essential roles in mating via an unknown mechanism. Aga1p and Fig2p both contain repeated, conserved WCPL and CX₄C domains. This study was directed toward understanding the mechanism underlying the collective requirement of agglutinins and Fig2p for mating. Apart from the well-known agglutinin interaction between Aga2p and Sag1p, three more pairs of interactions in cells of opposite mating type were revealed by this study, including bilateral heterotypic interactions between Aga1p and Fig2p, and a homotypic interaction between Fig2p and Fig2p. These four pairs of adhesin interactions are collectively required for maximum mating efficiency and normal zygote morphogenesis. GPI-less, epitope-tagged forms of Aga1p and Fig2p can be co-immunoprecipitated from the culture medium of mating cells in a manner dependent on the WCPL and CX₄C domains in the R1 repeat of Aga1p. Using site-directed mutagenesis, the conserved residues in Aga1p that interact with Fig2p were identified. Aga1p is involved in two distinct adhesive functions that are independent of each other, which raises the possibility for combinatorial interactions of this protein with its different adhesion receptors, Sag1 and Fig2p, a property of many higher eucaryotic adhesins.
INTRODUCTION

Adhesion is an important activity for many eukaryotic cell types and is often a property associated with cell differentiation. Cell adhesion interactions are fundamental to many biological processes such as cell-cell and cell-substrate interactions occurring in development of multicellular organisms as well as a variety of contexts important to the life cycles of unicellular organisms. In fungal cells such interactions are known to be both homotypic and heterotypic, allowing the assembly of cells into biofilms and enabling adhesion to abiotic substrates, as well as to other cells during processes such as mating, flocculation and pathogen-host interactions.

Studies in a variety of yeasts have revealed a multitude of adhesins governing different aspects of their life cycles. In the budding yeast *S. cerevisiae*, a number of specific families of adhesins play significant roles in cell-cell interactions during mating (agglutinins) and toward the formation of filaments of vegetative cells during biofilm formation and pseudohyphal growth (flocculins) (DRANGINIS *et al.* 2007; GUO *et al.* 2000; LENGELER *et al.* 2000; LIPKE and KURJAN 1992; STRATFORD 1992; TEUNISSEN and STEENSMA 1995; VERSTREPN *et al.* 2004). For many pathogenic fungi, such as *C. albicans* and *C. glabrata*, formation of biofilms of cells at interfaces such as plastic or host contributes to the antibiotic resistance and virulence of fungal cells and has been demonstrated to depend on multiple different adhesins (CHANDRA *et al.* 2001; DOUGLAS 2003; GARCÍA-SÁNCHEZ *et al.* 2004; GREEN *et al.* 2004; IRAQUI *et al.* 2005; NOBILE *et al.* 2006; NOBILE *et al.* 2008; O’TOOLE *et al.* 2000; REYNOLDS and FINK 2001; VERSTREPN *et al.* 2004). The involvement of adhesins in the attachment of fungal cells to host tissues is therefore considered a critical virulence factor for successful infection in the
pathogenesis of many fungi (Kaur et al. 2005; Sundstrom 1999; Sundstrom 2002; Tunlid et al. 1992; Verstrepen et al. 2004).

Fungal adhesion interactions are mediated by cell surface proteins termed adhesins, which belong to a general class of cell wall proteins typically attached to the cell surface via GPI anchors (Huang et al. 2003; Kapteyn et al. 1999; Orleans 1997; Sundstrom 2002). In S. cerevisiae, the agglutinin genes AGA1, AGA2, SAG1/Agα1, and another mating specific adhesin FIG2 are involved in the mating process (Erdman et al. 1998; Jue and Lipke 2002; Lipke and Kurjan 1992; Sprague and Thorner 1992; Zhang et al. 2002). FLO genes are involved in flocculation and filamentation in response to nutrition signals, as well as invasive growth and biofilm formation (Guo et al. 2000; Lo and Dranginis 1998; Reynolds and Fink 2001; Rupp et al. 1999; Verstrepen et al. 2004, Verstrepen, 2005 #259). In C. albicans, two classes of adhesins have been identified: SAG1-like adhesins (including ALA1, ALS genes), EAP genes, and an unconventional adhesin HWP1, which attaches to epithelial cells by a transglutaminase-mediated mechanism (Li and Palecek 2003; Li and Palecek 2005; Nobile et al. 2008; Sharkey et al. 1999; Staab et al. 1999; Staab et al. 1996; Sundstrom 1999; Sundstrom 2002). In C. glabrata, EPA genes mediate adhesion to mammalian tissues and biofilm formation (Cormack et al. 1999; De Las Penas et al. 2003; Kaur et al. 2005).

Studies of the protein sequences of adhesins have revealed that in addition to signal sequences and GPI addition signals, most contain internally repeated domains. The functions of these domains may be divided into two major categories: domains dedicated to adhesion interactions that are either protein-protein, lectin or
transglutaminase mediated in nature, and domains whose repetition serves to increase
the distance between the C-terminal GPI-associated plasma membrane and cell wall
anchoring domain and the typically N-terminally distributed adhesion mediating domains
(DRANGINIS et al. 2007; FRIEMAN et al. 2002; GUO et al. 2000; HUANG et al. 2003; KAUR
most GPI-anchored adhesins contain both such types of domains, assigning individual
domains to either of these categories may not be done exclusively in all cases, since
recent work suggests that some repeated domains may influence not only overall size,
but also aspects such as the hydrophobicity of adhesin proteins and therefore the cell
surface when expressed in sufficient quantity (DRANGINIS et al. 2007; FIDALGO et al.
2006; ISHIGAMI et al. 2006; VERSTREPN et al. 2004). The repetition of the adhesion
domains within different adhesin proteins is expected to help increase the affinity of
adhesion interactions by providing additional binding sites that may aid in initiating or
maintaining adhesin-receptor interactions (SHEN et al. 2001).

A subset of fungal adhesins in *S. cerevisiae* and *C. albicans*, including Aga1p,
Fig2p, Hwp1p and some flocculins, contain repeated, conserved domains named WCPL
(SHARKEY et al. 1999) and CX₄C domains (HUANG et al. 2003). The WCPL domains are
so named because of the highly conserved YTTW/YCPL residues characteristic of the
C-terminal regions of these domains, and they are present in one to five copies in the
different proteins in which they are found (SHARKEY et al. 1999). The CX₄C domains are
also present in one to multiple copies and their positions appear to be independent of
the WCPL domains, occurring in some cases close to the WCPL region and in other
cases in other locations within the proteins. The highly conserved nature of the WCPL
and CX₄C domains suggest a functional significance of these domains, although no studies have yet been reported addressing the function of these domains.

A number of the adhesins containing WCPL and CX₄C domains function in mating in *S. cerevisiae*, an excellent model system in which to study adhesin functions and interactions. During mating, *MAT*a and *MAT*α yeast cells signal each other and undergo a series of programmed changes to allow their stable contact and cytosolic and nuclear fusion to form zygotes (reviewed in (MARSH and ROSE 1997; SPRAGUE and THORNER 1992)). Mating includes several distinct stages, such as agglutination, cell cycle arrest, mating projection formation, cell fusion, and nuclear fusion. A number of proteins are induced by pheromone from cells of the opposite mating type, including the adhesins Aga1p, Aga2p, Sag1p and Fig2p (CAPPELLARO *et al.* 1994; CAPPELLARO *et al.* 1991; ERDMAN *et al.* 1998; LIPKE and KURJAN 1992; LIPKE *et al.* 1989; ROY *et al.* 1991; SPRAGUE and THORNER 1992). Aga2p and Sag1p are cell type specific in their expression, whereas Aga1p and Fig2p are expressed in both mating types. Aga1p is the anchorage subunit of a-agglutinin, which localizes the binding subunit of a-agglutinin Aga2p on the surface of *MAT*a cells through two disulfide linkages (HUANG *et al.* 2003; SHEN *et al.* 2001). Aga2p binds Sag1p to mediate cell-cell adherence between cells of the opposite mating type and this interaction is critical for mating under conditions that do not promote cell-cell contact (such as in liquid environments). In conditions where cell-cell contact is facilitated (such as on solid substrates), the agglutinins and Fig2p have previously been shown to be collectively required for mating in the Σ1278b strain background via an unknown mechanism (GUO *et al.* 2000).
Aga1p and Fig2p have been relatively well defined at both the gene and protein levels providing good targets for studying the function of the conserved WCPL and CX4C domains. The native, unprocessed Aga1p contains 725 amino acids with a secretion signal at its N-terminus and a GPI anchor addition signal at its C-terminus (ROY et al. 1991). Aga1p contains two repeats termed R1 and R2, separated by multiple copies of a third repeat rich in serine and threonine residues that plays a “spacer” function (HUANG et al. 2003; SHEN et al. 2001). The R1 and R2 domains share 50% homology and each contains a WCPL domain, a CX4C domain, and a CX2C motif, the latter being involved in disulfide linkage of Aga2p (ROY et al. 1991; SHEN et al. 2001). Fig2p shares a similar structure with Aga1p, also bearing N-terminal secretion and C-terminal GPI anchor addition signals (ERDMAN et al. 1998). The native, unprocessed Fig2 protein contains 1609 amino acids with five WCPL domains and nine CX4C domains. Both Aga1p and Fig2p are highly O-glycosylated, and Fig2p is also predicted to receive multiple N-glycosylation modifications; these posttranslational modifications are expected to aid in the adoption of an extended polypeptide structure, conducive to display of the adhesion domains of these proteins (JENTOFT 1990; LU et al. 1995).

In this study we explored the mechanism underlying the collective requirement of agglutinins and Fig2p for mating on solid substrates. We found a novel interaction between Aga1p and Fig2p in cells of the opposite mating type, which is mediated by the conserved WCPL and CX4C domains. An interaction between Fig2p and Fig2p in cells of the opposite mating type also exists, but supports mating function less effectively as compared to the Aga1p interaction with Fig2p. Both of the novel adhesin interactions that we identified were found to play redundant roles to the well-known Aga1p-Aga2p
agglutinin interaction with Sag1/Agα1p in mediating mating and normal zygote morphogenesis on solid substrates. Our findings on the roles of WCPL and CX4C domains are also likely to be relevant to pathogenic fungi whose proteins contain these conserved domains.
Yeast strains, growth conditions, and general methods

The *S. cerevisiae* strains (S288c/BY4743 strain background) used in this study are listed in Table 1. Replacement of the *AGA1* gene with *URA3* was described in (HUANG *et al.* 2003), other single mutants used to construct the following double mutants were obtained from the Yeast Genome Systematic Deletion collection. *MATα aga1::URA3 fig2::KanR, aga1::URA3 aga2::KanR, and aga2::KanR fig2::KanR* strains were constructed by standard mating and sporulation procedures, followed by selecting tetrad progeny on selective media and confirming the genotypes by diagnostic PCRs. *MATα aga1::URA3 fig2::KanR, aga1::URA3 sag1::KanR, and sag1::KanR fig2::KanR* strains were constructed by similar methods. To construct a *MATα aga1 aga2 fig2* and a *MATα aga1 sag1 fig2* triple mutant, the *URA3* marker was used to replace the *AGA1* gene in a *MATα aga2 fig2* or *MATα sag1 fig2* strain. Standard yeast culture medium was prepared as described (SHERMAN *et al.* 1986). All yeast strains were grown at 30°C. Cell densities were measured by optical density as measured at A$_{600}$ with a spectrophotometer (Spectronic Genesys 8).

Construction and integration of plasmids

*AGA1 cloning:* The plasmids used in this study are listed in Table 2. To make an integratable version of the *AGA1* gene, pRS305-AGA1::3HA, the *Sall*-*XbaI* fragment containing the *AGA1* gene was cut from pRS315-AGA1::3HA (described in (HUANG *et al.* 2003)), gel purified, and cloned into the yeast integration vector pRS305 by standard
molecular cloning methods (SAMBROOK et al. 1989). pRS305-AGA1::3HA-R1Δ, pRS305-AGA1::3HA-WCPLΔ, pRS305-AGA1::3HA-CX4Δ, pRS305-AGA1::3HA-CX2Δ, pRS305-AGA1::3HA-R2Δ, and pRS305-AGA1::3HA-GPIΔ were constructed similarly. Deletion of the CX4C domain in the R1 repeat of AGA1 from pRS305-AGA1::3HA-WCPLΔ resulted in pRS305-AGA1::3HA-WCPLΔCX4Δ, following a 2-step cloning procedure (primers P1-P2 and P3-P4) (See Supplementary Table; bases denoted in bold are the heterologous restriction sites used for cloning the fragment). To generate pRS305-AGA1::3HA-WCPLΔCX4Δ-GPIΔ, the SpeI to XbaI fragment in pRS305-AGA1::3HA-WCPLΔCX4Δ was swapped with that in pRS305-AGA1::3HA-GPIΔ (containing a fragment of the AGA1 gene missing the GPI attachment signal). The R1 (residues 305-349/634, based on the native, unprocessed protein, as for the other proteins described in this study) or R2 (residues 370-414/634) repeat in Hwp1p was cloned into pRS305-AGA1::3HA-WCPLΔCX4Δ at a unique PstI site introduced for CX4C deletion (primers P5-P6 or P7-P8). This resulted in pRS305-AGA1::3HA-(HWP1-R1) or pRS305-AGA1::3HA-(HWP1-R2), respectively.

Sixteen site-directed mutants of AGA1 were constructed following the QuickChange™ Site-Directed Mutagenesis Kit instruction manual (Stratagene, La Jolla, Calif.). The amino acid sequence SPA at positions 49-51 was mutated to AAG using primers P9 and P10. Similarly, the CX4C residues at positions 102-107 were changed to AX4A using primers P11 and P12. All the other mutants were constructed as a single amino acid change to alanine. They were as listed below: V53A (primers P13-P14), S54A (primers P15-P16), T55A (primers P17-P18), T65A (primers P19-P20), Y67A (primers P21-P22), T68A (primers P23-P24), T69A (primers P25-P26), W70A (primers
P27-P28), C71A (primers P29-P30), P72A (primers P31-P32), L73A (primers P33-P34), C102A (primers P35-36), C107A (primers P37-P38), and T65S (primers P39-P40).

Mutagenesis in the WCPL domain was carried out on the pRS305-AGA1::3HA-CX₄CΔ construct, whereas mutagenesis in the CX₄C domain was carried out on the pRS305-AGA1::3HA-WCPLΔ construct. All of the AGA1 clones generated were confirmed by sequencing or multiple diagnostic PCR reactions.

**FIG2 cloning:** The FIG2 gene, including 674 bases upstream of the predicted start codon and 500 bases downstream of the termination codon, was PCR amplified from genomic DNA and cloned into pRS306 to produce pRS306-FIG2, following a 2-step cloning strategy (primer P41-P42 and P43-P44). The whole FIG2 fragment was then cloned into pRS305 as a SalI-XbaI fragment, which yielded pRS305-FIG2. To generate a tagged version of FIG2, a 14 amino acid long V5 epitope was PCR amplified from the vector pYD1 (Invitrogen, Carlsbad, Calif.) using primers P45 and P46 and inserted at a unique Nhel site (866/1609) in FIG2, this resulted in pRS305-FIG2::V5. To produce pRS305-FIG2::V5-GPIΔ, the C-terminal 33 amino acids encoding the predicted GPI attachment signal was deleted from pRS305-FIG2::V5 and replaced with a unique EcoRI site by a 2-step cloning strategy (primers P41-P47 and P44-P48). The correct orientation of the V5 insert was confirmed by DNA sequencing.

**Integration:** The plasmids containing the native or modified versions of the AGA1 gene were linearized with HindIII and integrated into aga1 fig2, aga1 aga2 fig2, or aga1 sag1 fig2 strains at the AGA1 locus. Similarly, the plasmids containing the native or modified versions of the FIG2 gene were linearized with BglII and integrated into
desired strains at the FIG2 locus using a high-efficiency transformation method (BURKE et al. 2000). Integrations at the correct loci were confirmed by PCRs.

**Mating assays**

A replica plate patch mating assay was conducted essentially as described (GUO et al. 2000). Briefly, one mating partner was streaked onto a YPAD plate as a patch, and the other mating partner was spread evenly onto another YPAD plate as a lawn. Both strains were grown overnight and replicated by velvet to a plate containing selective medium (SC-Met-Lys). The plate was incubated overnight before observation. Quantitative mating assays were performed via a standard method (GUTHRIE and FINK 1991).

**Measurement of zygote morphology and microscopy**

Samples for observation by microscopy were prepared and observed as described (ZHANG et al. 2002). Briefly, cultures were mated on nitrocellulose filters for four hours, followed by formaldehyde fixation and storage in 1X phosphate-buffered saline (PBS)-1M sorbitol at 4°C. Microscopy was used to observe mating cell morphology in each cross at 100x magnification. Two parameters, relative conjugation bridge length and diameter, were used to describe zygote morphology. Relative conjugation bridge length was defined as the ratio of fusion bridge length to the mean diameter of the parental pair (ERDMAN et al. 1998; ZHANG et al. 2002). Similarly, relative conjugation bridge diameter was defined as the ratio of fusion bridge diameter to the mean diameter of the parental pair. For each cross, 50 zygotes were photographed and
measured by methods as described previously (Zhang et al. 2002). Statistical analyses were conducted with ANOVA using SAS, version 8.0 (SAS Institute, Cary, NC, USA).

In the cross of MATα aga2 fig2 x MATα sag1 fig2, very few zygote-like cells could be observed and those observable were quite abnormal in appearance, thus this cross was excluded from the measurements. To confirm that these abnormal zygote-like cells were actually zygotes, the two mating partners were marked differently before being mixed for mating. Briefly, the mating partners were transformed with either a centromeric plasmid pRS315 (LEU2⁺) or a plasmid, containing a GFP fusion to a Golgi localized protein, (kindly provided by Dr. Veronika Simons). The pRS315-containing mating partner was stained with Calcofluor (Nern and Arkowitz 1999) before mating with the GFP fusion-containing mating partner. This experiment was done bi-directionally with identical results indicating that the zygote-like cells observed corresponded to zygotes formed by the association of two cells of opposite mating type.

**Preparation of protein samples and co-immunoprecipitation**

The 3xHA-tagged, GPI-less AGA1 gene with and without the WCPL and CX₄C domains present in R1 of Aga1p were integrated into a MATα aga1 aga2 fig2 strain (YSE1090), this resulted in strains YSE1090-AGA1::3HA-GPIΔ and YSE1090-AGA1::3HA-WCPLΔCX₄CΔ-GPIΔ, respectively. Similarly, the V5-tagged, GPI-less FIG2 was integrated into a MATα aga1 sag1 fig2 strain (YSE1091), which yielded YSE1091-FIG2::V5-GPIΔ.

The three strains described above were grown separately in YPAD medium overnight to an optical density of A₆₀₀ = 1.5-2.0, followed by mixing 200mls of MATα and
MATα cells at an optical density of A_{600} = 1.5 in 2800ml Fernbach flasks. The mating mixtures were then incubated at 30°C for six hours. Secreted proteins were isolated from the mating mixtures by centrifugation of yeast cells at 12,500rpm twice to remove the cell pellet and the cleared media supernatants concentrated to 600-800μl by Centricon Plus-80 (Millipore). This produced the pre-IP extracts. The pre-IP extracts were brought to the same volume with 1X PBS, and an aliquot (50μl) of each extract was mixed with 2X LDS sample buffer (Invitrogen), boiled for 5 minutes, run on 4-12% tris-acetate gels (Invitrogen, Carlsbad) and Western blot detected with either anti-HA or anti-V5 antibody as described below. The rest of the pre-IP extracts were immunoprecipitated with anti-V5 agarose beads (Sigma, St. Louis) and detected with anti-HA antibodies.

**Western blotting**

All samples were boiled at 95-100°C for 5 min before loading on 4-12% tris-acetate gels (Invitrogen, Carlsbad). Proteins were transferred to polyvinylidene difluoride (PVDF) membrane by semidry blotting and detected with either anti-HA or anti-V5 antibody as indicated, following procedures as described previously ((HUANG et al. 2003)). For Aga1p detection, the antibody used was a mouse monoclonal IgG horseradish peroxidase (HRP)-conjugated anti-HA antibody (Santa Cruz), used at 1:5,000. For Fig2p detection, the primary antibody was a mouse anti-V5 antibody used at 1:5,000 (Invitrogen, Carlsbad), and the secondary antibody was a HRP-conjugated goat anti-mouse IgG (1:1,000) (Pierce). Blots were imaged by X-Ray film.
RESULTS

Four pairs of adhesin interactions mediate mating in S. cerevisiae

The agglutinin interaction between Aga1p-Aga2p and Sag1p is essential for mating in liquid environments but not on solid substrates (LIPKE and KURJAN 1992). In addition to the two agglutinin proteins, Aga1p and Aga2p, MATa cells also express Fig2p, while MATα cells express Sag1p, Aga1p and Fig2p. Therefore three adhesins are present in cells of either mating type: Aga1p, Aga2p and Fig2p in MATa cells; Aga1p, Sag1p and Fig2p in MATα cells. The collective requirement of Aga1p and Fig2p for mating on solid substrates under high cell density conditions (GUO et al. 2000) could reflect an interaction between these proteins. Alternatively, these two proteins may mediate lectin-like activities that underlie this requirement. In order to differentiate these two possibilities, a series of patch mating assays were conducted. To test for an interaction between Aga1p and Fig2p in cells of the opposite mating type, a strain containing either Aga1p or Fig2p as the only adhesin present was mated to a series of mating partners that were either wild type or similarly deficient for different adhesins (Table 3; Fig. 1). When Aga1p was the only adhesin present in one mating partner, the absence of Fig2p in the opposite partner abolished mating ability as judged by the patch mating assay, while the presence of Fig2p as the only adhesin in the opposite mating partner allowed cells to retain wild type patch mating efficiency (Fig. 1A, B). This suggests that Fig2p is both necessary and sufficient for mating under these conditions when Aga1p is the only adhesin present in the opposite mating partner. In contrast, when Fig2p was the only adhesin present in one mating partner, mating was not abolished when Aga1p was missing from the opposite partner, although the mating
efficiency was decreased significantly (Fig. 1C, D). This suggested a possible interaction between Fig2 proteins in cells of the opposite mating type or with other cell wall components that is weaker or mediates mating less efficiently under these conditions. That this interaction occurs between the Fig2 proteins themselves in opposite mating type cells was confirmed by two additional sets of qualitative mating assays. Mating was abolished in crosses where one partner expressed only the Fig2p adhesin while the other partner did not express either Aga1p or Fig2p (Table 3: MATa aga1 x MATα aga1 fig2 and MATα aga1 sag1 x MATa aga1 fig2). Additionally, in experiments conducted to examine requirements for different domains within Fig2p for these interactions, we found that for expressing Fig2p from a centromeric plasmid could rescue the sterility of MATa aga1 aga2 fig2 cells when mated to a wild type partner. Additionally, when such MATa aga1 aga2 fig2 cells expressing Fig2p are mated to MATα cells deleted of all three mating adhesins (MATα aga1 fig2 sag1) and carrying a centromeric plasmid expressing a truncated Fig2p lacking its R1 and R2 domains, this truncated version of Fig2p partially rescued the sterility of the MATα triple mutants (HUANG 2006). Finally, we found there to be either no interaction between Aga1p and Aga1p in cells of the opposite mating type or the interaction is inappropriate to support mating function, because patch matings were abolished when Aga1p was the only adhesin present in MATa and MATα cells (HUANG 2006). These observations based on a semi-quantitative patch mating assay were found to be consistent with quantitative mating assays carried out for all these crosses (below).

These data clearly indicate that aside from the agglutinin interaction of Aga1p-Aga2p complex with Sag1p, three more pairs of adhesin interactions occur during the
mating process of *S. cerevisiae*, namely the two interactions between Aga1p and Fig2p, and a weaker or less productive interaction between Fig2p and Fig2p, in cells of the opposite mating type. While all of these unilateral and bilateral interactions are expected to occur in mating cells, it is the case that one unilateral Aga1p - Fig2p interaction between cells of the opposite mating type is sufficient to enable wild type levels of mating on solid substrates. These interactions are likely to be involved in the adherence of cells of the opposite mating type to each other, as observation of the mating mixtures by microscopy indicated more single cells in crosses missing one or more pairs of the adhesins (data not shown). No obvious cell fusion defects were observed in any of the crosses examined.

The presence of four pairs of adhesin interactions during mating in *S. cerevisiae* leads to the question of how efficiently each of these different interactions mediates mating on solid substrates. To address this question, we quantified the mating efficiencies from crosses where all or none of the interacting pairs were present, as well as matings where there was only one interacting pair present.

Based on quantitative assays of the mating efficiency of the MATα partner, ~60% of haploid cells in our crosses between wild type strains form diploids (Table 4). Although the Aga1p and Fig2p mediated crosses showed a mating efficiency indistinguishable from the wild type cells by patch mating assays, they mediated significantly less efficient matings than the wild type in the quantitative mating assays. This result highlights the fact that patch mating assays, although semi-quantitative, cannot reveal differences in mating efficiencies that are much less than ten-fold in magnitude. We also examined whether it mattered which of the two mating cell types
were expressing specific adhesins of the Aga1p and Fig2p interaction. Surprisingly, the \textit{MATa} Aga1p -\textit{MAT\alpha} Fig2p interaction mediated mating significantly better (39.5\% vs 20.0\%) than the same interaction in which the cell types expressing each protein were reversed (i.e. \textit{MATa} Fig2p-\textit{MAT\alpha} Aga1p mediated mating). Currently, we do not understand why there exists such mating type specificity to this interaction. Roy et al. noted that \textit{AGA1} transcript is expressed at a three-fold higher level in \textit{MATa} than \textit{MAT\alpha} cells (Roy \textit{et al.} 1991). If the Aga1 protein is also expressed at a much higher level in \textit{MATa} than \textit{MAT\alpha} cells, this differential expression of Aga1p in cells of the two mating types might contribute to the directionality of Aga1p and Fig2p interaction.

Consistent with the patch mating assays, the Fig2p-Fig2p pair mediated interaction conferred only a 2.4\% mating efficiency, suggesting a weak affinity or less productive interaction between Fig2 and Fig2p in cells of the opposite mating type (Table 4). In the absence of the other three interacting adhesin pairs, the Aga2p-Sag1p pair conferred a wild type mating efficiency in both patch mating (data not shown) and quantitative mating assays (Table 4), indicating that the agglutinin interaction alone can mediate mating well on solid substrates, in addition to its critical role for mating in liquids. In the absence of all of the four interacting pairs, i.e., when Aga1p was the only adhesin present in both \textit{MATa} and \textit{MAT\alpha} cells, the mating efficiency was extremely low (0.0015\%), corresponding to less than 1/10,000 the efficiency of mating between wild type cells and less than 1/1,000 the efficiency of Fig2p-Fig2p mediated mating. Thus there is either no interaction between Aga1p and Aga1p in cells of the opposite mating type or if the interaction does occur, it is non-productive in supporting mating functions.
The conserved WCPL and CX\textsubscript{4}C domains mediate adhesin interactions

A subset of fungal adhesins, including Aga1p and Fig2p in *S. cerevisiae* and Hwp1p in *C. albicans*, contains highly conserved WCPL and CX\textsubscript{4}C domains whose functions are currently unknown (Sharkey *et al.* 1999). Both Aga1p and Hwp1p contain two sets of conserved domains termed R1 and R2 (Repeats 1 and 2), that each contains one WCPL domain and one CX\textsubscript{4}C domain, whereas Fig2p contains five WCPL domains and nine CX\textsubscript{4}C domains (Fig. 2). Most of the CX\textsubscript{4}C domains in Fig2p are clustered within the C-terminal portion of the protein (Fig. 2A).

Our previous study of Aga1p structure, biogenesis and function indicated its conserved WCPL and CX\textsubscript{4}C domains are not critical for mating under liquid conditions (i.e., agglutination) (Huang *et al.* 2003). We tested whether these domains were required for mating on solid substrates. Truncated Aga1 proteins lacking the R1 or R2 repeat were integrated into a MAT\textsubscript{a} aga1 fig2 strain and mated to a MAT\textsubscript{a} aga1 aga2 strain. The MAT\textsubscript{a} aga1 fig2 strain was essentially sterile, and the wild type Aga1p rescued its mating defect when integrated (Fig. 3A). When the R2 repeat was missing, a wild type mating efficiency was observed, and matings were abolished when the R1 repeat was missing (data not shown), suggesting that R1 is essential for mating on solid substrates. We then deleted the subdomains in the R1 repeat, i.e., the WCPL or CX\textsubscript{4}C domain or the CX\textsubscript{2}C motif, alone or collectively and tested the mating efficiencies the resulting proteins conferred to the sterile MAT\textsubscript{a} aga1 fig2 strain. When the WCPL or CX\textsubscript{4}C domain alone was missing, the mating efficiency was greatly decreased, and essentially no matings were observed when both domains were missing (Fig. 3A; Table 5). Although Aga1p containing one of the WCPL and CX\textsubscript{4}C domains conferred efficient
mating nearly indistinguishable from the wild type in patch mating assays, only a mating efficiency of 1-2% was observed by quantitative mating assays. When a reciprocal cross (i.e., \( \text{MAT}_a \, \text{aga}1 \, \text{fig}2\text{-integrants} \times \text{MAT}_\alpha \, \text{aga}1 \, \text{sag}1 \)) was carried out, similar results were obtained (Fig. 3B). This strongly indicates that the conserved WCPL and CX\(_4\)C domains in the R1 repeat of Aga1p are collectively required for its interaction with Fig2p. Deletion of the CX\(_2\)C motif in Aga1p-R1 did not have any effect on mating efficiency (Fig. 3C, D; Table 5). Furthermore, when the above experiment was conducted using \( \text{MAT}_a \, \text{aga}1 \, \text{aga}2 \, \text{fig}2 \) and \( \text{MAT}_\alpha \, \text{aga}1 \, \text{sag}1 \, \text{fig}2 \) strains (i.e., the wild type or truncated AGA1 gene was integrated into the triple mutants and the mating efficiency was tested), the trend was the same (data not shown).

The CX\(_2\)C motif in the R1 repeat of Aga1p has been found to be critical for mating under liquid conditions (HUANG et al. 2003; SHEN et al. 2001). The role of the WCPL/CX\(_4\)C domains in the R1 repeat of Aga1p in mating on solid substrates led to the question of whether their function is dependent on that of the CX\(_2\)C motif, and vice versa. When both WCPL and CX\(_4\)C domains in Aga1p were gone, a wild type agglutination activity was observed (data not shown), indicating that the function of the CX\(_2\)C motif in agglutination is independent of the WCPL/CX\(_4\)C domains. Similarly, Aga1p missing the CX\(_2\)C motif showed a wild type mating efficiency, both in patch mating assays (Fig. 3C, D) and quantitative mating assays (Table 5). Thus the two different functions (involvement in mating under liquid and solid conditions) of the conserved domains in the R1 repeat of Aga1p (the CX\(_2\)C motif and WCPL/CX\(_4\)C domains) are independent of each other.
Our previous study suggested that the R1 repeat and its subdomains does not affect the biogenesis, stability, or trafficking of Aga1p when the proteins are expressed from a centromeric plasmid pRS315 (HUANG et al. 2003). To confirm that the mating defect mediated by the integrated version of the Aga1 proteins missing R1 and its subdomains is not associated with a defect in Aga1p biogenesis, stability, or trafficking, we performed an immunoblotting experiment on total cell lysates containing these truncated proteins as previously described (HUANG et al. 2003). Similar expression patterns and levels of Aga1 proteins were found for all of the truncated proteins (data not shown). Overall, these data suggest that the WCPL and CX₄C domains in the R1 repeat of Aga1p affect mating through their interaction with the Fig2 protein.

**Three conserved residues are critical for Aga1p and Fig2p interaction**

Since the conserved WCPL and CX₄C domains in Aga1p-R1 are required for its interaction with Fig2p, we carried out a site-directed mutagenesis experiment to determine which residues are critical for this function. As shown in Fig. 2B, 14 residues are highly conserved in the WCPL domain of Aga1p-R1. The SPA residues at positions 49-51 were collectively mutated to AAG, and the CX₄C residues at positions 102-107 were changed to AX₄A. The rest of the conserved residues were changed to alanine individually (Table 6). As described in the Materials and Methods, mutagenesis in the WCPL domain was carried out in an AGA1 construct lacking the CX₄C domain, and vice versa. The resulting mutants were integrated into MATα or MATα aga1 fig2 strains and tested for their abilities to complement the mating defect of the aga1 fig2 strains by both patch mating and quantitative mating assays. As shown in Fig. 4 and Table 6, three
residues, T65, C71 and C102, were found to be critical, mutations in any of them essentially abolished matings. Mutations in four more residues, V53, S54, Y67 and C107, seemed to decrease the mating efficiency slightly in patch mating assays. However, when a quantitative mating assay was carried out, these mutants conferred a similar mating efficiency as their parental constructs on which they were built (i.e., Aga1p-WCPLΔ or Aga1p-CX4CΔ) (Table 5, 6).

The collective requirement of the two cysteine residues in Aga1p, C70 in the WCPL domain and C102 in the CX4C domain, for adhesin function in mating suggests that Aga1p might interact with Fig2p through disulfide linkages. The requirement of T65 suggested two possibilities. Since Ser/Thr residues are potential sites for O-glycosylation, the requirement of T65 could reflect an importance of O-glycosylation at this site. It is also possible that T65 itself is important, and represents a structural constraint for Aga1p functions in mating. To distinguish between these two possibilities, a T65S mutant was constructed and its mating efficiency tested. If O-glycosylation at this residue occurs and is important for mating, and assuming that the T65S mutation behaves similarly with respect to its modification, the T65S mutant should have a wild type mating efficiency. However, the yeast strain containing the T65S mutation showed a similar mating efficiency to that containing a T65A mutation (Fig. 4). While strong conclusions cannot be drawn from these experiments due to the difficulties inherent in the study of O-linked modifications at specific residues within secreted proteins containing a great number of such modified residues, one possible interpretation of these results is that it is less likely that O-glycosylation at this site matters and that a structural role of the T65 residue may be a more likely function.
**Aga1p physically interacts with Fig2p**

The fact that a single set of Aga1p and Fig2p interactions between cells can mediate efficient mating suggests that there may be a physical interaction between these proteins in cells of the opposite mating type. To test whether such an interaction occurs, we constructed GPI-less, epitope-tagged, integrated versions of $AGA1$ (3xHA-tagged) and $FIG2$ (V5-tagged). A GPI-less, integrated, 3xHA-tagged $AGA1$ lacking the WCPL and CX$_4$C domains in its R1 repeat was also constructed. The two different forms of the $AGA1$ gene were integrated into a $MAT_a$ $aga1$ $aga2$ $fig2$ strain (YSE1090) separately, and the tagged $FIG2$ gene was integrated into a $MAT_\alpha$ $aga1$ $sag1$ $fig2$ strain (YSE1091). Overnight cultures of these strains were inoculated into a mating mixture and the GPI-less proteins were secreted into the growth medium. After several hours of exposure to cells of the opposite mating type, we collected the medium and removed the cells by centrifugation to test whether the GPI-less Aga1p or Aga1p-WCPL$_\Delta$CX$_4$C$_\Delta$ interacts with the GPI-less Fig2p in a mating mixture via co-immunoprecipitation. As shown in Fig. 5, the GPI-less Aga1p was pulled down together with Fig2p by anti-V5 agarose beads from a concentrated medium supernatant. This interaction was eliminated when the WCPL and CX$_4$C domains in Aga1p-R1 were absent, demonstrating both the specificity of the interaction and its requirement for the same domains that support functionality of the protein in mating assays. A western blot on the pre-IP extracts indicated that about an equal amount of Aga1 and Fig2 proteins were present in both mating mixtures and that the apparent molecular weights of the wild type and deleted forms of Aga1p were consistent with those observed in our previous studies.
of these proteins (Huang et al. 2003). Therefore Aga1p physically interacts with Fig2p in cells of the opposite mating type, and this interaction requires the WCPL and CX₄C domains in Aga1p-R1. We reproducibly detected two distinct high molecular weight forms of Fig2p in this experiment, the smallest form being at least 250 kD or greater based on migration of molecular weight standards. These forms might result from differential N-glycosylation and/or O-glycosylation of Fig2p.

**The conserved domains in Hwp1p are partially functional in S. cerevisiae**

Hwp1p is a hyphae-specific adhesin in *C. albicans*, the most common yeast pathogen in humans. Hwp1p is critical for virulence, as *C. albicans* *hwp1/hwp1* strains are avirulent (Staab et al. 1999; Sundstrom 1999). As shown in Fig. 2B, Hwp1p contains two sets of WCPL and CX₄C domains, named R1 and R2. Since the WCPL and CX₄C domains are clearly conserved in *S. cerevisiae* and *C. albicans* (identities ~50%, similar residues ~66%), we tested whether the WCPL/CX₄C domains in Hwp1p can functionally replace those in Aga1p. Two chimeric Aga1::Hwp1 proteins were generated, where the WCPL/CX₄C domains in Aga1p-R1 were replaced by the R1 or R2 repeat in Hwp1p. Surprisingly, the chimeras rescued the mating defect of *aga1 fig2* mutants to different degrees when integrated. Based on patch mating assays, the Aga1::Hwp1p-R2 chimera rescued the mating defect of *aga1 fig2* mutants completely, but the Aga1::Hwp1p-R1 chimera only rescued the mating defect of *aga1 fig2* mutants partially (Fig. 6). A quantitative mating assay revealed a two-fold difference (3% vs 1.5%) in mating efficiency conferred by these two chimeras, suggesting that the conserved domains in Hwp1p function in *S. cerevisiae*, albeit relatively inefficiently. The R1 and R2 repeats in Hwp1p share ~80% identity, and 11 out of 45 residues are
different between them (Fig. 2B). Presumably one or more of these different residues contribute to the difference in mating efficiency conferred by the two chimeras.

**Mating cell morphogenesis reflects different adhesin interactions**

All of the four adhesins, Aga1p, Aga2p, Sag1p and Fig2p are highly induced by pheromones and localize predominantly to mating projections, presumably facilitating adhesive interactions and contact-dependent events in mating (ERDMAN et al. 1998; LIPKE and KURJAN 1992). Agglutinins have not been reported to participate in mating cell morphogenesis, but fig2Δ cells form hyperpolarized mating projections and narrower conjugation bridges (ERDMAN et al. 1998; ZHANG et al. 2002). Thus it would be of interest to see whether any or all of the four different pairs of adhesin interactions are important for mating cell morphogenesis. To address this question, zygotes from crosses with only one interacting adhesin pair were observed and compared to wild type zygotes. When Aga1p-Fig2p was the only interacting pair present, zygotes with longer and narrower conjugation bridges were more frequently observed relative to those generated by wild type matings (Fig. 7A, B). A similar trend was observed when Fig2p-Aga1p or Fig2p-Fig2p were the only interacting pairs (Fig. 7C, D). When Aga2p-Sag1p was the only interacting pair present, zygotes exhibited a different morphology: the conjugation bridges seemed to be narrower, but not longer, than the wild type (Fig. 7E). When all of the four interacting pairs were missing, most cells remained single and exhibited abnormal morphology, often elongated and swollen at one end. This suggests that those cells managing to become associated with a mating partner likely had difficulties adhering to and fusing with their partners and in most cases aborted mating.
relatively early in the process. Very occasionally, a few abnormal zygote-like cells could be observed and were confirmed to be zygotes (Materials and Methods). Interestingly, when all of the four interacting pairs were absent, only either very short conjugation bridges or none at all between cells were observed for most of the zygotes (Fig. 7F).

To gain a better understanding of the zygote morphologies arising from different crosses, the relative conjugation bridge length and diameter in fifty zygotes were measured for each cross (Table 7). These measurements confirmed the previous qualitative observations. The conjugation bridges were significantly longer and narrower in the Aga1p-Fig2p, Fig2p-Aga1p, and Fig2p-Fig2p mediated crosses than those in the wild type cross. Consistent with its lowest mating efficiency, the Fig2p-Fig2p pair mediated cross exhibited the most aberrant zygote morphology, with the longest and narrowest conjugation bridges. In the Aga2p-Sag1p mediated cross, the relative conjugation bridge length was similar to that in the wild type, but the relative conjugation bridge diameter was significantly smaller. Therefore different adhesin interactions play redundant roles in zygote morphogenesis and conjugation bridge length and diameter depend on proper cell-cell adhesion. Previous studies showed that the zygote morphogenesis defect in bilateral matings of fig2Δ cells (containing the agglutinin pair) can be rescued by elevated osmolarity (Zhang et al. 2002), therefore it would be interesting to examine whether the morphogenesis defects observed in mating cells bearing other adhesin pairs can also be rescued by elevated osmolarity conditions.
DISCUSSION

In addition to the well-known agglutinin interaction between the Aga1p-Aga2p complex and Sag1p, we identified two more adhesin interactions, Aga1p-Fig2p and Fig2p-Fig2p, which occur during yeast mating. Because both Aga1p and Fig2p are expressed in both cell types and, as we have shown, mediate both heterotypic and homotypic adhesion interactions that are bilateral between the cells, this brings the total number of distinct adhesin interactions between mating cells to four. The extensive number of adhesin interactions that characterize the yeast mating process and the bifunctional nature of the role of Aga1p in binding two different receptor proteins raise several questions regarding the nature of fungal adhesion proteins. Why are there so many different adhesin interactions that are apparently redundant for some functions and not others? How are these proteins related to one another and to other fungal adhesins, and what information concerning the organization and function of these proteins may be understood from their primary sequences and interactions? Finally, might adhesins containing interaction domains for multiple receptors interact with these targets in a combinatorial manner?

Requirements of adhesins in S. cerevisiae mating

Our identification of heterotypic adhesin interactions between Aga1p and Fig2p and a homotypic interaction between Fig2p and Fig2p in both MATa and MATα mating yeast cells explains why the agglutinins and Fig2p were found to be collectively required for efficient mating on solid substrates (Guo et al. 2000). The involvement of multiple adhesin interactions in S. cerevisiae mating suggests that the different pairs of
adhesin interactions are collectively required to establish and maintain stable cell-cell connections during mating so that the process can proceed efficiently. Several possibilities exist as to why this multitude of adhesins operates during mating. One possibility is that together these interactions serve to maximize the speed and efficiency of cell-cell association, polarization and fusion events during mating. Because mating cells must thin their cell walls in the zone of cell fusion, it is important for them to accurately localize such events and likely to also make certain they proceed rapidly to minimize the likelihood of exposure to sudden environmental changes in osmolarity that could lyse cells preparing to fuse (MARSH and ROSE 1997; PHILIPS and HERSKOWITZ 1997; ZHANG et al. 2002). Our results support the notion that adhesins aid in coordinating contact and growth events between mating cells as zygote morphologies in the area of cell-cell contact are affected by the complement of adhesins present on mating cells. A second and not necessarily exclusive reason for the presence of multiple different adhesin interactions on mating cells is that these different adhesin pairs may play different roles under different conditions that mating cells might encounter, such that some pairs of interactions may be dispensable or redundant under certain environmental conditions, but are stronger or play more important roles under other conditions. Some evidence of such different roles already exists, as the Aga1p-Aga2p agglutinin interaction with Sag1p is critical for mating in liquid conditions, but is dispensable for mating on solid media. The interactions between Aga1p and Fig2, and Fig2p with itself are similarly sufficient to allow mating on solid media, but are insufficient in liquid conditions when the interaction between Aga1p-Aga2p and Sag1p is defective. Thus, while our assays point to different effects of the different adhesion
interactions on mating success, for example suggesting the Fig2p-Fig2p interaction to be the least effective in performing the essential function of the adhesins in mating, this interaction or others might prove more crucial under other mating conditions cells encounter in nature as compared to the standardized conditions employed for mating assays in the laboratory.

We found the different adhesin interactions in yeast to be distinct in their abilities to mediate the essential mating function they perform as judged by qualitative and quantitative measures of mating. Further studies are needed to determine whether the differences in mating efficiencies and zygote morphogenesis associated with specific adhesin pairs reflect different molecular interactions of distinct strength or quality between the different adhesins, or are due to cell type differences in adhesin expression levels or effects due to losses of distinct adhesins. Evidence for both types of changes in mating cells due to the loss of adhesins is indicated by our results or has previously been reported. The ability of the Aga1p-Fig2p interaction to allow efficient mating depends on the cell types on which these adhesins are expressed indicates that cell type can influence these interactions (Table 4). Cells lacking Fig2p display reduced viability under mating conditions, incur cell fusion defects at elevated rates and possess altered distribution of zygotic nuclei relative to wild type mating cells (ERDMAN et al. 1998; ZHANG et al. 2002). Cells lacking Fig2p also display elevated levels of agglutination in liquid mating conditions, and it has been proposed that Fig2p might compete with other GPI-anchored proteins expressed in mating cells for receiving GPI anchors, other posttranslational modifications within the secretory pathway or for their postsecretory traffic to the cell wall, or that Fig2p might act as an
indirect “masker” of the active sites of Sag1p (ERDMAN et al. 1998; JUE and LIPKE 2002). In both the S288c and W303 strain backgrounds of budding yeast, the hyperagglutination caused by fig2 mutants is largely α partner specific (ERDMAN et al. 1998; JUE and LIPKE 2002; ZHANG et al. 2002). Our finding that Aga1p interacts directly with Fig2p on cells of the opposite mating type provides additional insight to this phenomenon. Our results show that Aga1p on the MATa cell is a bifunctional molecule that might have the potential to engage either of two distinct adhesin receptors on MATα cells: Sag1p via Aga2p binding and interaction with Fig2p. When Fig2p is missing from the α mating partner, more Aga1p on the MATα mating partner could be released to interact with Sag1p, resulting in higher agglutination activity. Conversely, when Fig2p is missing from the MATα mating partner, there is no influence on agglutination activity, because Fig2p does not interact with Aga1p from the same mating type or Sag1p from the opposite mating type. Nonetheless, fig2 mutants showed the highest agglutination activity in a bilateral mating context (i.e., fig2 x fig2) (ERDMAN et al. 1998; JUE and LIPKE 2002; ZHANG et al. 2002). Perhaps in addition to competing for MATα cell Aga1p receptors, Fig2p does also indirectly mask other agglutinins or compete with them for modifications during biogenesis. Finally, it is also possible that the cell wall structure is slightly different in the bilateral and unilateral mating contexts lacking Fig2p, contributing further to the differential agglutination activities (ZHANG et al. 2002). Interestingly, pmt1 and pmt2 mutants also showed higher agglutination activities in a bilateral mating context than in unilateral matings where Pmt1p or Pmt2p was missing from the MATα cells (HUANG et al. 2003). Additional work
will be needed to distinguish which of these complementary or potentially synergistic effects causes the effects on agglutination due to the absence of Fig2p function.

**Structural organization and evolution of mating cell adhesins**

In considering the different roles of adhesins containing WCPL and CX$_4$C domains in mediating mating cell adhesion interactions, it may be useful to consider more broadly the relationships of the proteins containing these domains both within *S. cerevisiae* and in other species. As noted previously, a subset of fungal adhesins in *S. cerevisiae* and *C. albicans* contain the well-conserved WCPL and CX$_4$C domains (HUANG et al. 2003; SHARKEY et al. 1999). In a further BLAST search against the fungal proteome database, these conserved domains are also found in cell wall proteins from other fungal species, including *C. glabrata* and *Debaryomyces hansenii* (HUANG and ERDMAN). However, functions for these domains remain unknown for any of the proteins in which they occur. We demonstrated these conserved domains mediate adhesion interaction between Aga1p and Fig2p in cells of the opposite mating type. As Fig2p contains multiple copies of both domains, it is likely that the Fig2p-Fig2p interaction is also mediated through interactions among these domains. Among the *S. cerevisiae* proteins possessing WCPL and CX$_4$C domains, all are predicted or documented GPI-anchored proteins whose postsecretory traffic links a significant portion of them to the β-1,6 glucan component of the cell wall. In baker’s yeast three classes of proteins contain these domains, the mating adhesins Aga1p and Fig2p, a protein functioning in cell integrity, Sed1p, and the flocculins Flo1, Flo5, Flo9 and Flo10 (Flo11 does not contain any recognizable copies of either the WCPL or CX$_4$C domains) (HAGEN et al. 2004;
SHARKEY et al. 1999). Comparisons of the primary amino acid sequences of these proteins to one another and to known and predicted proteins encoded by the C. albicans genome indicates that the yeast proteins are most closely related to one another with the flocculins and the mating adhesins sharing the feature of a small insertion of four additional amino acids comprising the sequence SPAY/I within the middle of the WCPL domain. Fig2p, Aga1p and Sed1p, are further related by a presumed ancestral rearrangement removing an N-terminal segment of the WCPL domain, separating the CX4C domains from the WCPL sequences. The FIG2 and AGA1 genes are found in their respective locations within the yeast genome adjacent and in the same orientation relative to genes encoding a pair of predicted serine/threonine protein kinases, YNR047 and KIN82. These kinases are also clearly evolutionarily related homologs and perform overlapping functions as judged by their additive phenotypes associated with resistance or sensitivity to different chemical agents (S. Erdman, unpublished). These features strongly suggest that FIG2 and AGA1 arose from an ancestral single locus, perhaps during the genome duplication event that occurred some 40 MYA in the evolution of the S. cerevisiae genome (WOLFE and SHIELDS 1997). Fig2p has been shown previously to be capable of substituting in function for Flo11p in mediating invasive growth, similar to the majority of the other cell surface flocculins (GUO et al. 2000). It is also interesting that Flo11p has recently been demonstrated to possess homotypic adhesion ability (DOUGLAS et al. 2007), similar to Fig2p, suggesting that this family of proteins may generally behave in this manner. Thus, one likely inference is that the ancestral adhesin gene that gave rise to the present day AGA1 and FIG2 genes was more FIG2-like in its nature and that the features of Aga1p that
mediate interactions with Aga2p and Sag1p are more recent evolutionary developments. Overall the repeated nature of the adhesins and their roles in providing specificity to the mating reaction has likely facilitated their relatively rapid evolution as such proteins have been shown to be strong candidates for natural selection causing their rapid change (CORONADO et al. 2007).

The WCPL and CX₄C domains are repeated in nearly all proteins in which they occur. Repetition of adhesion mediating domains is a common feature of adhesins that may serve to increase the affinity of the adhesion interactions they mediate (LIPKE and KURJAN 1992; SHEN et al. 2001). However, in few cases has the functionality of multiple adhesion domains aside from the most N-terminal repeat of each adhesin been directly assessed. Studies to date have demonstrated distinct adhesion functions associated with the Aga1p-R1 domain, which is a common theme among fungal adhesins, typically bearing an N-terminal adhesion domain (FRIEMAN et al. 2002; LIPKE 1996; SUNDSTROM 2002; VERSTREPNEN et al. 2004). We have been unable to find evidence for any direct adhesion functionality for the R2 domain of Aga1p in either our previous studies of its agglutination activity or in its ability to support the essential mating specific function of the adhesins we describe here (SHEN et al. 2001); G. Huang and S. Erdman, unpubl.). Specifically, we tested whether the R2 domain could mediate the heterotypic interactions of Aga1p with Fig2p. The R1 and R2 regions in Aga1p each contain a conserved WCPL domain, a CX₄C domain, and a CX₂C motif (HUANG et al. 2003; ROY et al. 1991). R1 is more toward the N-terminus of Aga1p and therefore more exposed at cell surface. We also tested whether differences in the abilities of these two repeats to mediate mating are simply a consequence of their positions or are more likely domain
sequence/structure effects. An Aga1p derivative with the positions of R1 and R2 swapped was constructed and integrated into a MATa aga1 fig2 strain. This construct showed no agglutination activity in liquid culture and mated poorly with strains lacking Fig2p or Sag1p on solid substrates (HUANG 2006). This suggests that R2 cannot functionally replace R1 even when located closer to the surface or external to the cell wall. Another potential conclusion from this experiment is that the R1 repeat in this Aga1p derivative, when placed toward the inner side of the cell wall, is apparently non-functional in this location, although we cannot rule out that it may be so because it competes with the R2 repeat in binding Fig2p or Aga2p, but somehow functions non-productively after binding these proteins in this context. These studies do not address the question of whether R2 plays a low-affinity, ancillary role in different adhesion interactions that are below the thresholds of detection for the assays we have employed. Overall, these results suggest that the R2 repeat in Aga1p may now be evolving to function as a spacer to facilitate the extension of Aga1p molecule and therefore the exposure of the R1 repeat closer to the cell surface (HUANG et al. 2003). Such a conclusion is consistent with the observation that the sequence of the R2 WCPL domain is divergent and contains several replacements in the conserved SPA residues present in the insertion carried by all three other repeats collectively present in Aga1 and Fig2 proteins, although our mutagenesis of these residues failed to uncover strong effects on adhesion activities (Figure 2, this study).

In contrast to our results regarding the functionality of repeats in its evolutionarily related partner Aga1p, our initial studies of Fig2p requirements for interaction with Aga1p have found some evidence for adhesion activities associated with C terminally
located repeats of Fig2p. A construct deleted of the two N-terminal most WCPL domain repeats of Fig2p remained capable of interaction with the wild type Aga1 and Fig2 proteins, but was compromised for interaction with Aga1p lacking either WCPL or CX_4C domains of R1 or in bilateral homotypic interactions mediated solely by these N-terminally mutated Fig2 proteins (HUANG 2006). These data point to adhesin activities associated with the C terminal repeats of Fig2p. The structure of Fig2p is more complicated than that of Aga1p, with its nine CX_4C repeats being located predominantly near the C terminal end of the protein and its WCPL domains located nearer the N terminus, an organization unique to Fig2p among all WCPL/ CX_4C repeat containing adhesins. Additional studies will be required to fully understand the nature of the interactions of these two domains with one another in mediating adhesin interactions.

Aspects common to mating adhesion systems of many unicellular eukaryotes

Our identification of additional homotypic and heterotypic adhesion interactions between S. cerevisiae mating partners also highlights a general feature of mating cell adhesion characteristic of many different unicellular organisms. During the early stages of S. cerevisiae mating, a weaker interaction occurs between cells of the opposite mating type, which can be disrupted by various mechanical agitations (FEHRENBACHER et al. 1978). Agglutinin binding assays also revealed both a weaker and a stronger interaction in the mating process (LIPKE et al. 1987; TERRANCE and LIPKE 1981). Based on these observations and the repeated structures of the agglutinin proteins Aga1p and Sag1p, Lipke and Kurjan proposed that binding of an agglutinin to its cognate adhesion receptor is initiated with a weak interaction, followed by conversion to a tighter
interaction (LIPKE and KURJAN 1992). In light of the additional presence of three more pairs of adhesin interactions in the mating process of *S. cerevisiae* as defined by this study, it seems reasonable to propose that one function of the Aga1p-Fig2p and Fig2p-Fig2p interactions is to help initiate a weak adherence between cells of both the same and opposite mating types before mating projection formation, partner induced signaling and extensive engagement of Aga1p-Aga2p and Sag1p agglutinin interactions serve to pair cells of the opposite mating type as mating partners. All of the three agglutinins and Fig2p are highly induced by pheromones and mainly distributed to the tips of the mating projections (ERDMAN et al. 1998; LIPKE and KURJAN 1992), and these interactions further concentrate the localized secretion of these proteins, facilitating their interactions. However, given their pheromone regulation, the interactions between Aga1p-Fig2p, Fig2p-Aga1p, and Fig2p-Fig2p probably continue to occur during or after mating projection formation, perhaps conferring another level of adherence or context of interaction between cells of the opposite mating type. Evidence that this is the case can be seen from our results demonstrating the distinct effects of the different adhesin pair functions on zygote morphogenesis. Collectively these four pairs of interactions might also serve to confer a stronger adherence between mating cells that could be still more resistant to disruption once established.

The presence of two distinct phases of adherence and at least two parallel adhesin systems operative during *S. cerevisiae* mating, suggests a two-stage adhesion model mediated by Aga1p-Aga2p and Sag1p interactions that may be preceded, followed, or complemented by Aga1p and Fig2p, and Fig2p and Fig2p interactions. Such a two-stage model of adhesion is also attractive as such interactions have been
reported or predicted to characterize mating systems of other unicellular eucaryotes and, as will be discussed below, fungal pathogen-host interactions. In the mating process of the green alga *Chlamydomonas*, two stage adhesin interactions have also been reported, where an initial contact is mediated through peripheral adhesin molecules and later on, a more intimate contact mediated through specific adhesion of membranes (Lipke 1996). In the mating processes of other unicellular eukaryotes such as the protist *Tetrahymena thermophila*, adhesion also proceeds by multiple contact-mediated interactions (Brown et al. 1993). *C. albicans* has recently been found to have a sexual life cycle and Hwp1, a critical virulence factor in *C. albicans*, is also found to be pheromone-regulated and specifically expressed on the conjugation tubes formed by MAT\textit{a} cells (Bennett and Johnson 2003; Bennett et al. 2003; Daniels et al. 2003; Soll 2003). Hwp1 contains two WCPL/CX\textsubscript{4}C domains in addition to its adhesion repeats responsible for transglutamylation-mediated interactions with host tissues (Sharkey et al. 1999; Staab et al. 1999). Deletion of Hwp1p and another pheromone-regulated, WCPL/CX\textsubscript{4}C domain-containing cell wall protein, Rbt1p, from both mating partners has also been reported to reduce mating efficiency (Bennett et al. 2003). It is not clear whether Hwp1 interacts with Rbt1p or other adhesins during the mating process in *C. albicans*. Rbt1p is also involved in the virulence of *C. albicans* (Braun et al. 2000), but it is not known whether it has a function in adherence. If there is an interaction between Hwp1 and Rbt1 or another *C. albicans* WCPL/CX\textsubscript{4}C domain containing protein, it seems likely that the conserved WCPL/CX\textsubscript{4}C domains may participate in this process. It is also interesting to note that complementary and heterotypic interactions between Hwp1 and certain Als adhesins (which do not contain
recognizable WCPL/CX₄C domains) have recently been documented to play an important role in biofilm formation (Nobile et al. 2008). These adhesin interactions might also occur during C. albicans mating, or these biofilm forming interactions may have evolved from an ancestral interaction or function of these proteins in mating as suggested by the authors. Our studies of the Aga1p-Fig2p and Fig2p-Fig2p mediated adhesion interactions suggest that multiple different adhesin interactions, some homotypic and others heterotypic, might also characterize the C. albicans mating process.

**Combinatorial aspects of adhesin function in fungal systems**

Our finding that Aga1p is a bifunctional adhesin capable of interacting with two different adhesion receptors, Sag1p and/or Fig2p, through distinct domains of its structure, suggests the possibility that Aga1p may interact in a combinatorial way with its adhesion partner proteins. Such interactions might be either temporally or spatially separate, or simultaneous if the two interactions that Aga1p participates in are not mutually exclusive of one another on the same polypeptide.

Aga1p represents the first example of a fungal adhesin capable of interacting with more than one type of adhesion receptor, a feature common to proteins mediating cell adhesion in higher eucaryotes. In C. albicans the known adhesins Eap1 and Hwp1 both contain predicted WCPL/CX₄C domains within their sequences. Hwp1 has a well defined adhesion domain mediating its transglutamylation interaction with host epithelial cells and two WCPL/CX₄C domains. By analogy to Aga1p, these domains could be dedicated toward functions of Hwp1 associated with mating, or they might
conceivably participate in concert with the N terminal repeats responsible for transglutamylolation reactions to mediate combinatorial interactions of Hwp1 with different adhesin target proteins during establishment of infection and transitions to mating conditions during the life cycle of *C. albicans*. A variety of adhesion related activities have been reported for Eap1 and these have been mapped to different domains of the protein, however the roles of WCPL related repeats in these activities have not been investigated (Li and Palecek 2008). Eap1 may therefore represent another example of a multi-functional adhesin, however it remains somewhat unclear whether substrates other than human cells that it has been shown to interact with represent native targets of the protein. A two-stage model of adhesion mediated by two different classes of adhesins has previously been proposed for adherence of *C. albicans* to its host tissues: an initial weaker interaction mediated mainly by the conventional adhesins such as Als proteins followed by a stronger interaction mediated by the unconventional adhesin Hwp1p (Sundstrom 1999). It will be interesting to determine whether WCPL/CX₄C mediated interactions also play roles in these processes or are dedicated to mating specific functions. Given that the fungal adhesin, Aga1p, and most likely Hwp1, present in two different fungal species possess the potential to interact with multiple adhesion receptors, it will be of interest to determine what other fungal adhesion events in different species are mediated by similarly multifunctional adhesins.
ACKNOWLEDGEMENTS

This work was supported by funding from the Ruth Myers Scholars Fund and Renee Schine Crown Honors Program of Syracuse University to S.D., and a grant from NIH/NIDCR DE14443-01 to S.E. The authors are grateful to J. Belote, R. Hallberg, W. Powell, C. Boddy and two anonymous reviewers for their valuable comments on this work.

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HAGEN, I., M. ECKER, A. LAGORCE, J. FRANCOIS, S. SESTAK et al., 2004 Sed1p and Srl1p are required to compensate for cell wall instability in Saccharomyces cerevisiae mutants defective in multiple GPI-anchored mannoproteins. Mol. Microbiol. 52: 1413-1425.


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ZHANG, M., D. BENNETT and S. E. ERDMAN, 2002 Maintenance of mating cell integrity requires the adhesin Fig2p. Eukaryotic Cell 1: 811-822.
Figure Legends

FIGURE 1. Aga1p interacts with Fig2p in cells of the opposite mating type. MATα or MATα cells containing Aga1p or Fig2p as the sole adhesin were grown as a lawn and mated to the indicated mating partners in the form of patches. The mating mixtures were directly replica plated to a selective medium (Sc-Met-Lys) plate without pre-incubation on a YPAD plate. Strain growth occurs due to the formation of diploids by mating in this assay and is a semi-quantitative indicator of mating efficiency. (A) A MATα aga2 fig2 lawn containing Aga1p as the only adhesin was mated to the indicated MATα mating partners with or without Fig2p. (B) A MATα sag1 fig2 lawn containing Aga1p as the only adhesin was mated to the indicated MATα mating partners with or without Fig2p. (C) A MATα aga1 aga2 lawn containing Fig2p as the only adhesin was mated to the indicated MATα mating partners with or without Aga1p. (D) A MATα aga1 sag1 lawn containing Fig2p as the only adhesin was mated to the indicated MATα mating partners with or without Aga1p.

FIGURE 2. ScAga1p, ScFig2p, and CaHwp1p contain highly conserved WCPL and CX₄C domains. (A) Schematics of Aga1p and Fig2p. Both Aga1p and Fig2p precursors contain a secretion signal (white boxes) at their N-termini and a GPI attachment signal (cross-hatched boxes) at their C-termini. The R1 and R2 repeats in Aga1p include amino acids 45-149/725 and 387-493/725, respectively. For each repeat, the WCPL domains (white-dotted boxes), CX₄C domains (black hatched boxes), and CX₂C motif (white boxes) are shown. The 3HA epitope tag carboxy terminal to the R2 repeat is shown as a black bar. The region between R1 and R2 contains 19 copies of an imperfect heptapeptide sequence, TSTSS/PSS (solid gray bar). The five WCPL domains and nine CX₄C domains in Fig2p are represented by similar patterns as those in Aga1p. The V5 tag is inserted at a unique Nhel site (866/1609), shown as a black bar. For both proteins, the N-terminus is to the left. Some elements are not drawn to scale. (B) The alignment of the WCPL and CX₄C domains in ScAga1p, ScFig2p, and CaHwp1p. The R1 and R2 repeats in Hwp1p include amino acids 305-349/634 and 370-414/634, respectively. The CX₂C domains in Aga1p and Fig2p were not shown since they are C-terminal to the WCPL domains in Aga1p and scattered in the
C-terminal portion of Fig2p as depicted in (A). A consensus sequence is shown below, with the conserved WCPL and CX₄C residues highlighted in bold. The conserved residues that were mutated in Aga1p (see Table 6) are underlined.

**FIGURE 3.** The conserved WCPL and CX₄C domains in Aga1p-R1 are collectively required for the interaction between Aga1p and Fig2p. MATα or MATα cells containing Fig2p as the sole adhesin were mated to the indicated mating partners as described in the legend to Fig. 1. (A) A MATα aga1 aga2 lawn was mated to a MATα aga1 fig2 strain integrated with wild type Aga1p or truncated Aga1 proteins lacking one or two of the conserved WCPL and CX₄C domains in R1. (B) A MATα aga1 sag1 lawn was mated to a MATα aga1 fig2 strain integrated with the wild type or truncated Aga1 proteins as described in A. (C) A MATα aga1 aga2 lawn was mated to a MATα aga1 fig2 strain integrated with the wild type Aga1p or Aga1p lacking the CX₂C motif in R1. (D) A MATα aga1 sag1 lawn was mated to a MATα aga1 fig2 strain integrated with the wild type Aga1p or Aga1p lacking the CX₂C motif in R1.

**FIGURE 4.** Patch mating efficiency of selected site-directed mutants of Aga1p. A MATα aga1 aga2 lawn was mated to the indicated MATα mating partners. In the α mating partners, wild type Aga1 or Aga1 containing either a WCPL or a CX₄C domain in R1 and a site-directed mutation in the domain present was integrated into an aga1 fig2 strain. Crosses were also conducted in the other direction and similar results were obtained.

**FIGURE 5.** GPI-less Aga1p and Fig2p physically interact with each other. The concentrated culture medium from mating cells was immunoprecipitated (IP) with anti-V5 agarose beads and immunoblotted (IB) with anti-HA antibody. The before IP extract was obtained and detected with either anti-HA or anti-V5 antibody as indicated. The secreted forms of Aga1p and Fig2p were both estimated to be equal to or greater in size than 210 kD and 250 kD, respectively. Lane 1: YSE1090-AGA1::3HA-GPIΔ x YSE1091-FIG2::V5-GPIΔ; Lane 2: YSE1090-AGA1::3HA-WCPLΔCX₄CΔ-GPIΔ x YSE1091-FIG2::V5-GPIΔ.
FIGURE 6. The R1, R2 repeats in Hwp1p can function in Aga1p. A MATa aga1 aga2 lawn was mated to a MATα aga1 fig2 strain containing the wild type Aga1p or a chimeric Aga1::Hwp1 protein. Similar results were obtained when crosses were conducted in the other direction.

FIGURE 7. Zygote morphology in crosses with different adhesin interactions. Mating mixtures were obtained and collected as described in the Materials and Methods. Two representative zygotes were shown for each cross. (A) Wt x Wt; (B) aga2 fig2 x aga1 sag1; (C) aga1 aga2 x sag1 fig2; (D) aga1 aga2 x aga1 sag1; (E) fig2 x aga1 fig2; (F) aga2 fig2 x sag1 fig2.
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<sup>a</sup> All strains are derived from the Yeast Genome Deletion collection (WINZELER et al. 1999) unless otherwise indicated.
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TABLE 3. Mating of strains lacking one or two of their three mating adhesins

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The left column represents the \( \text{MAT}^a \) mating partner, and the top row represents the \( \text{MAT}^\alpha \) mating partner. The mating efficiency was based on patch mating assays. “+” means no difference from the wild type cross, “-” means no or very few matings, “±” means a mating efficiency between “+” and “-”.
**TABLE 4. The four pairs of adhesin interactions mediate matings to different degrees**

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<sup>a</sup> For each protein pair, the first protein listed is present in MAT<sup>a</sup> cells, and the second one is present in MAT<sup>α</sup> cells.

<sup>b</sup> The mating efficiency of the MAT<sup>α</sup> mating partners was quantified. The data shown were the mean ± standard error of four independent assays. The letters after the mating percentages indicate statistical significance, with the same letter indicating no significant differences at α=0.05 level.
TABLE 5. Mating efficiency conferred by Fig2p and different Aga1 proteins

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<td>2.17±0.44 c</td>
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<tr>
<td>Fig2p-(Aga1p-CX4CΔ)</td>
<td>0.84±0.19 c</td>
</tr>
<tr>
<td>Fig2p-(Aga1p-WCPLΔCX3CΔ)</td>
<td>0.00069±0.00038 c</td>
</tr>
<tr>
<td>Fig2p-(Aga1p-CX5CΔ)</td>
<td>20.65±1.35 b</td>
</tr>
</tbody>
</table>

A quantitative mating assay was performed on crosses between a MATa \( aga1 \) aga2 strain and the MAT\( \alpha \) mating partners as shown in Fig. 3A. In all of the crosses except the wild type, the only adhesin interaction present is that between Fig2p and different forms of Aga1p (wild type or truncated proteins lacking different domains). The letters after the mating percentages indicate statistical significance, with the same letter indicating no significant differences at \( \alpha=0.05 \) level.
TABLE 6. Mating efficiencies of the 16 site-directed Aga1 mutants

<table>
<thead>
<tr>
<th>Amino acid mutated&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mating efficiency&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+ (48.72±2.66)</td>
</tr>
<tr>
<td>SPA(49-51)AAG</td>
<td>+</td>
</tr>
<tr>
<td>V53A</td>
<td>± (0.94±0.09)</td>
</tr>
<tr>
<td>S54A</td>
<td>± (1.04±0.16)</td>
</tr>
<tr>
<td>T55A</td>
<td>+</td>
</tr>
<tr>
<td>T65A</td>
<td>-</td>
</tr>
<tr>
<td>T65S</td>
<td>-</td>
</tr>
<tr>
<td>Y67A</td>
<td>± (1.02±0.18)</td>
</tr>
<tr>
<td>T68A</td>
<td>+</td>
</tr>
<tr>
<td>T69A</td>
<td>+</td>
</tr>
<tr>
<td>W70A</td>
<td>+</td>
</tr>
<tr>
<td>C71A</td>
<td>-</td>
</tr>
<tr>
<td>P72A</td>
<td>+</td>
</tr>
<tr>
<td>L73A</td>
<td>+</td>
</tr>
<tr>
<td>C102A</td>
<td>- (0.0138±0.00043)</td>
</tr>
<tr>
<td>C107A</td>
<td>± (3.18±0.25)</td>
</tr>
<tr>
<td>CX&lt;sub&gt;4&lt;/sub&gt;C(102-107)AX&lt;sub&gt;4&lt;/sub&gt;A</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> The position indicated represents the location in the native, non-processed protein.

<sup>b</sup> The mating assays were conducted on crosses between a MAT<sup>a</sup> aga1 aga2 strain and a MAT<sup>α</sup> aga1 <i>fig2</i> strain integrated with Aga1p bearing different mutations. The mating efficiency was based on patch mating assays. “+” means no difference from WT, “-” means no or very few matings, “±” means slightly reduced matings relative to WT. For some mutants, data from quantitative mating assays (mean ± standard error of three independent assays) is also shown.
TABLE 7. Relative conjugation bridge dimensions in crosses with different adhesin interactions

<table>
<thead>
<tr>
<th>Interacting pairs</th>
<th>Length</th>
<th>Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt-Wt</td>
<td>1.33±0.016 a</td>
<td>0.65±0.014 a</td>
</tr>
<tr>
<td>Aga1p-Fig2p</td>
<td>1.38±0.019 ab</td>
<td>0.49±0.010 b</td>
</tr>
<tr>
<td>Fig2p-Aga1p</td>
<td>1.43±0.022 b</td>
<td>0.52±0.009 b</td>
</tr>
<tr>
<td>Fig2p-Fig2p</td>
<td>1.46±0.021 b</td>
<td>0.46±0.013 c</td>
</tr>
<tr>
<td>Aga2p-Sag1p</td>
<td>1.34±0.022 a</td>
<td>0.50±0.008 b</td>
</tr>
</tbody>
</table>

* The data shown are the mean ± standard error of measurements of 50 representative zygotes for each cross. The letters after the mating percentages indicate statistical significance, with the same letter indicating no significant differences at α=0.05 level.
FIGURE 1

A

MATα
aga2 fig2

WT  fig2  aga1 sag1

B

MATα
sag1 fig2

WT  fig2  aga1 aga2

C

MATα
aga1 aga2

WT  aga1  sag1 fig2

D

MATα
aga1 sag1

WT  aga1  aga2 fig2
Figure 2

A

Aga1p

B

Fig2p

Hwp1-R1 T T E H D T V V T V T S C S N S V C T E S E - - - - V T T G V I V I T S K D T I Y T T Y C P L
Hwp1-R2 T S E Q S T T V I T V T S C S S E S S C T E S E - - - - V T T G V V V V T S E E V Y T T T F C P L
Aga1-R1 T T T V S P A L V S T S T I V Q A G T T T L Y T T W C P L
Aga1-R2 S T T I P S F S M S T Y F T T V S G V T T M Y T T W C P Y
Fig2-R1 T S T T S P A Y V S T A T K T V D G V I T E Y V T W C P L
Fig2-R2 T S T T S P A Y V S T A T K T V D G V I T E Y V T W C P L
Fig2-R3 T S G K Q T L V L S T V T T T V N G A A T E Y T T C P A
Fig2-R4 S S T P S Q Y S L S T A T T T I N G I K T V Y T T W C P L
Fig2-R5 S Q T S I Q Y T L S T A T T T I S G L K T V Y T T W C P L

Highly Conserved Residues
T - T - - - T T V - T V T S C - - - - C T - - - - - - V S T - - - - - - - - - - T - Y T T W C P L
FIGURE 3
**FIGURE 4**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>aga1 fig2</th>
<th>AGA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MATa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aga1 aga2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-V53A</td>
<td>-S54A</td>
<td>-T65A</td>
<td></td>
</tr>
<tr>
<td>-Y67A</td>
<td>-C71A</td>
<td>-C102A</td>
<td></td>
</tr>
<tr>
<td>-C107A</td>
<td>-CX&lt;sub&gt;4&lt;/sub&gt;C(102-107)AX&lt;sub&gt;4&lt;/sub&gt;A</td>
<td>-T65S</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 5

IP: α-V5
IB: α-HA

α-HA
Pre-IP extract

α-V5
FIGURE 6