CONFORMATIONAL SWITCHING MEDIATES ATTACHMENT OF THE YEAST CANDIDA ALBICANS TO MAMMALIAN PROTEINS

by

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Abstract

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Advisor: Professor Peter N. Lipke

Candida albicans maintains both commensal and pathogenic states in humans. Both states are dependent on cell-surface-expressed adhesins including those of the Als family. Heterologous expression of Als5p at the surface of *Saccharomyces cerevisiae* results in Als5p-mediated adhesion to various ligands, followed by formation of multicellular aggregates. Following adhesion of one region of the cell to fibronectin-coated beads, the entire surface of the cells became competent to mediate cell-cell aggregation. Aggregates formed in the presence of metabolic inhibitors or signal transduction inhibitors, but were reduced in the presence of 8–anilino-1-naphthalene–sulfonic acid (ANS) or Congo Red (CR), perturbants that inhibit protein structural transitions. These perturbants also inhibited aggregation of *C. albicans.* An increase in ANS fluorescence, which accompanied Als-dependent cellular adhesion, indicated an increase in cell surface hydrophobicity. In addition, *C. albicans* and Als5p-

expressing *S. cerevisiae* showed an aggregation-induced birefringence indicative of order on the cell surface. The increase in birefringence did not occur in the presence of aggregation disruptants ANS or CR. These results suggest a model for Als5p-mediated aggregation in which an adhesion-triggered change in the conformation of Als5p propagates around the cell surface, forming ordered aggregation-competent regions.

The *ALS* Tandem repeat (TR) region has not been well characterized in Als proteins. *ALS5* TR deletion fragments were constructed in order to determine the structural characteristics and to identify the regions in Als5p required for adhesion to fibronectin. Cellular adhesion assays showed the Immunoglobulin-like (IG) domain mediated adherence to fibronectin. This result was consistent with previous studies for Als1p. The Threonine conserved (TC) region was important for secretion of cell surface anchored or soluble protein. SDS-PAGE and dot blot analyses showed the TR region is *O*-glycoslylated. Circular Dichroism (CD) spectroscopy indicated that the TR region increases the overall amount of alpha-helix structures 6-fold. Deletion of the TR region reduced binding to fibronectin in bioassays using soluble Als5p constructs. A reduction in cell-cell aggregation was observed in assays using cell-surface TR deletion constructs. Our findings demonstrate a structural and functional role for the TR region.

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Chapter I

General Introduction

I. The importance of studying fungal pathogens

i. Medical Significance of C. albicans and the C. albicans life cycle

Fungal infections cause significant morbidity and mortality and are increasingly common, especially among immunocompromised patients. The diploid sexual fungus *Candida albicans* is the most common fungal opportunistic pathogen in humans, and can exist in a wide range of associations from a superficial commensal state to systemic diseases in its human or animal host. As a commensal organism *C. albicans* can exist in small numbers on mucosal surfaces for extensive periods of time. However, *C. albicans* becomes more invasive and infectious when certain aspects of the host's environment changes such as, a breakdown in the anatomic integrity of mucosal surfaces, decreases in the resident microbial flora or some aspect of the host immunity becomes compromised (Calderone, 2002). Typical cases of candidiasis in humans include oral and vaginal mucosal infections. Studies have shown that approximately 75% of women may experience a case of vaginal candidiasis (Sobel et al., 1998). Disseminated *Candida* infections are a significant cause of morbidity and mortality in immunocompromised patients (e.g., AIDS cancer chemotherapy, organ or bone marrow transplantation) and patients harboring indwelling medical devices (catheters, dental implants, heart valves and artificial joints) (Arnow, Quimosing, & Beach, 1993; Calderone, 2002; Klepser, Lewis, & Pfaller, 1998; Ueta, Tanida, Yoneda, Yamamoto, & Osaki, 2001). It is estimated that 70-96% of AIDS victims have significant oropharharyngeal candidiasis (Calderone, 2002). In addition, *Candida albicans* is the fourth most common nosocomial infectious

agent overall (Calderone, 2002). Antifungal treatments against candidiasis are compromised by several factors such as a limited number of active fungicidal agents and the development of drug resistance (Masia Canuto & Gutierrez Rodero, 2002). An interesting feature of the *C. albicans* genome is the occurrence of numerous genetic alterations as a means of generating diversity, whereby this adaptive strategy may, in part, facilitate drug resistance (Calderone, 2002). Several mechanisms proposed to account for resistance include: 1. alterations in the cell membrane composition, particularly sterol synthesis (Calderone, 2002; Lupetti, Danesi, Campa, Del Tacca, & Kelly, 2002); 2. decreasing the affinity of the drug for the particular cellular target (Calderone, 2002; Lupetti, Danesi, Campa, Del Tacca, & Kelly, 2002) ; 3. upregulation of the expression of membrane bound drug efflux pumps (Calderone, 2002; Lupetti, Danesi, Campa, Del Tacca, & Kelly, 2002); 4. increasing the level of the cellular target (Calderone, 2002; Lupetti, Danesi, Campa, Del Tacca, & Kelly, 2002); and 5. through the formation of biofilms (Chandra et al., 2001); 6. Therefore determining new targets of novel therapeutic agents will be a valuable resource in clinical applications.

ii. Mechanisms of Candida albicans virulence

Virulence in *C. albicans* has been associated with the production of aspartyl proteases (SAPs) and lipases (Calderone, 2002), production of various adhesion macromolecules including the *ALS*, *EAP*, *CSH*, and *HWP* proteins (Fu et al., 2002; Li & Palecek, 2003; Singleton, Fidel, Wozniak, & Hazen, 2005; Staab, Bradway, Fidel, & Sundstrom, 1999; X. Zhao et al., 2004; X. Zhao, Oh,

Yeater, & Hoyer, 2005), and the morphological change from the blastoconidial cell form (yeast-like appearance) to the hyphal form (filament-like appearance) (Calderone, 2002; Staab, Bradway, Fidel, & Sundstrom, 1999). However, there is very limited information available regarding their roles or mechanisms of action in virulence.

II. Overview of *C. albicans* - mediated adhesion

i. Characteristics of adhesion in C. albicans

The ability of pathogenic organisms to establish contact with their respective host depends on cell adhesion molecules. Adherence to mucosal surfaces and subsequent cell–cell aggregation of the infecting cells serve as initial and critical steps in *C. albicans* establishing itself as a commensal inhabitant or pathogen in mammalian hosts. The importance of adherence is reflected by the fact that reductions for both adherence to host substrate and virulence are observed when strains with mutated adhesion genes or with mutations in genes that regulate adhesions are used (Buurman et al., 1998; Fu et al., 2002; X. Zhao et al., 2004). Following adherence, *C*. *albicans* grows in a colonial state. Indeed, in disseminated disease, micro-colonies are present in target organ tissue and are diagnostic for disease (Kamai et al., 2002). The binding specificity of *C. albicans* is extremely broad and includes a variety of diverse substrates such as mammalian cell-surface receptors, extracellular matrix proteins (ECM), various polysaccharides, and lipids, and plastic surfaces (Calderone, 2002). This wide range of binding specificity and interactions between *C. albicans* with its targets suggests the availability of numerous

molecular adhesins. Hence, developing molecular and cellular models for adhesion is essential in understanding the pathogenic as well as the commensal process.

ii. Adhesin molecules expressed by Candida albicans

Molecular genetics has led to the discovery and characterization of several *C. albicans* adhesins each localized on the cell surface. These include Eap1p, Hwp1p, Csh1p, and the proteins of the *ALS* gene family.

A. Eap1p

Eap1p (Enhanced adherence to polystyrene protein) was originally isolated in a screen using *Saccharomyces cerevisiae* cells with a *flo8* mutation expressing a *C. albicans* genomic DNA library. Eap1p mediates adhesion of *C. albicans* or *S. cerevisiae* to polystyrene surfaces and kidney epithelial cells *in vitro* (Li & Palecek, 2003). Eap1p adhesion featured resistance to shear forces generated through controlled laminar flow. Additionally, *EAP1* expression restored diploid pseudohyphal growth and haploid invasive growth to adhesion deficient *S. cerevisiae* strains, therefore demonstrating functional similarity with the *S. cerevisiae* cell wall protein *FLO11* (Li & Palecek, 2003). *EAP1* expression is regulated by the transcription factor Efg1p, which is known to be a key regulator of filamentation in *C. albicans* (Fu et al., 2002; Li & Palecek, 2003).

B. Hwp1p

Hwp1p (Hyphal wall protein) is localized to cell wall upon germ tube formation and is absent during the yeast growth phase (Staab, Bradway, Fidel, & Sundstrom, 1999). As an adhesin, Hwp1p is unique, in that its primary structure

displays a mammalian/fungal hybrid dynamic. The N-terminal domain is similar in both sequence and structure to mammalian SPR (Small Proline Rich) proteins (Staab, Bahn, Tai, Cook, & Sundstrom, 2004). SPRs are substrates for the mammalian transglutaminases (TGases) (Staab, Bahn, Tai, Cook, & Sundstrom, 2004). The C-terminal region contains sequences for GPI anchor modification. As a consequence of the such biophysical similarities, Hwp1p is a substrate for mammalian transglutaminase and causes binding of *C. albicans* to buccal epithelial cells through the formation of covalent crosslinks (Staab, Bahn, Tai, Cook, & Sundstrom, 2004; Staab, Bradway, Fidel, & Sundstrom, 1999). In addition, *C. albicans* strains with mutated *HWP1* genes were less virulent compared to wild type strains (Staab, Bradway, Fidel, & Sundstrom, 1999). Finally, Hwp1p is involved in *C. albicans* conjugation in mating type specific and manner. Hwp1p is specifically localized to the cell wall of **a**/**a** conjugation tubes and is absent on α/α tubes (Daniels, Lockhart, Staab, Sundstrom, & Soll, 2003). In addition, the first daughter cell emerges from the **a**/**a**-cell side of the zygote conjugation bridge (Daniels, Lockhart, Staab, Sundstrom, & Soll, 2003).

C. Csh1p

Cell Surface Hydrophobicity (CSH) is a significant factor in mediating *C. albicans* virulence (Singleton, Fidel, Wozniak, & Hazen, 2005). Hydrophobic strains are more adherent to epithelial, endothelial, and ECM compared to hydrophilic strains (Singleton, Fidel, Wozniak, & Hazen, 2005). The hydrophobic cell wall protein Csh1p is the first protein shown to affect the hydrophobic cell surface phenotype (Singleton, Fidel, Wozniak, & Hazen, 2005). Also, Csh1p

mediates attachment to human umbilical vein endothelial cells (HUVECs) and *C. albicans* cell-to-cell aggregation under shear forces (Singleton, Fidel, Wozniak, & Hazen, 2005). The Als proteins are the most widely expressed adhesins.

III. *C. albicans* adhesion mediated by ALS proteins

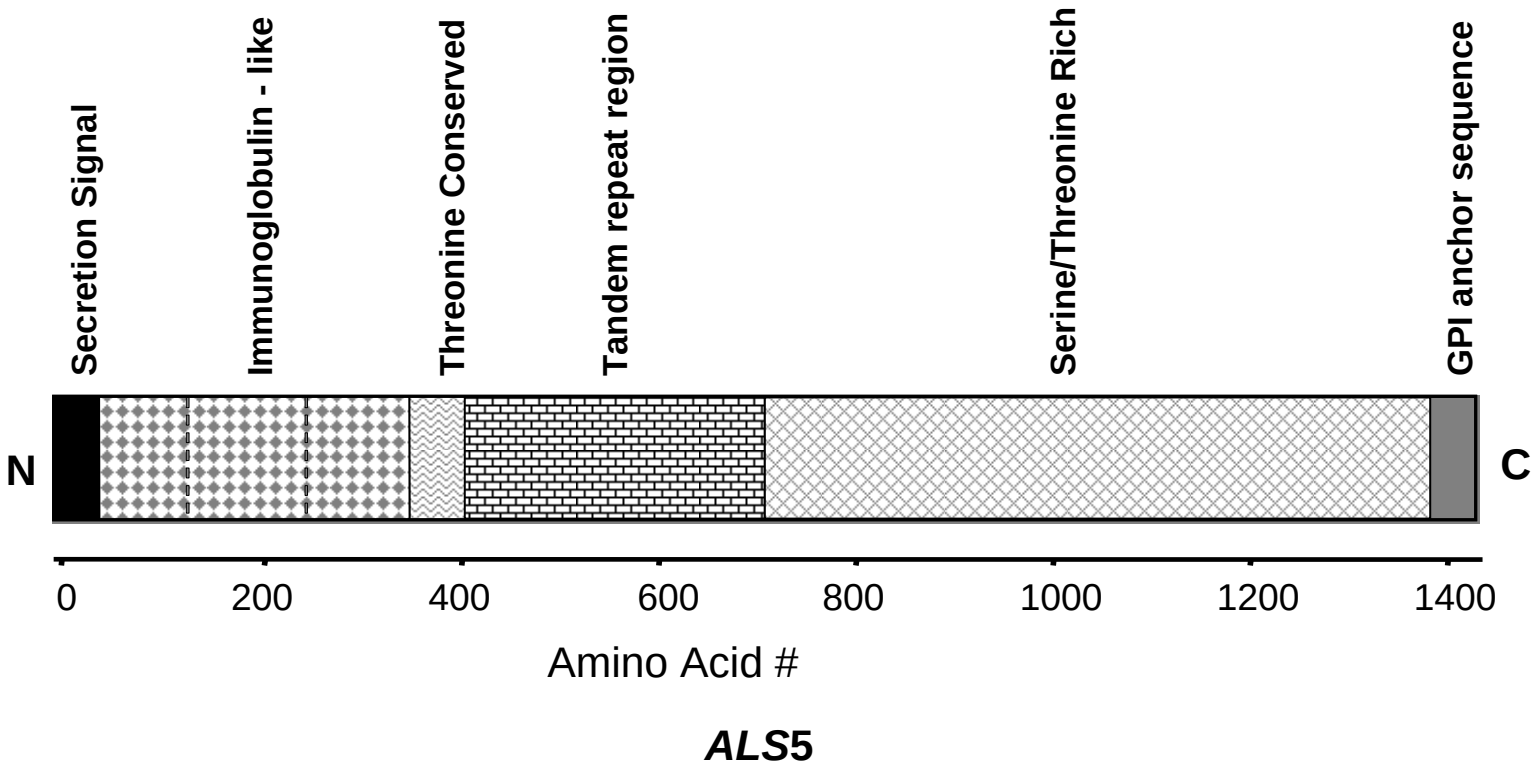
i. The ALS gene family

ALS1 was discovered in a screen for hyphal-specific transcripts (Hoyer, Scherer, Shatzman, & Livi, 1995). The name Agglutinin-Like Sequence has been assigned to this family due to the sequence similarity to the *S. cerevisiae* cell-surface adhesion molecule α-agglutinin, which mediates binding between opposite haploid cell types during mating (Lipke, Terrance, & Wu, 1987; H. Zhao, Shen, Kahn, & Lipke, 2001). The greatest amount of homology observed between *ALS* proteins and α-agglutinin is in the N-terminal domain, which in *S. cerevisiae* has been shown to be a member of the Immunoglobulin superfamily (Chen, Shen, Bobin, Kahn, & Lipke, 1995; Grigorescu, Chen, Zhao, Kahn, & Lipke, 2000; Lipke, Chen, de Nobel, Kurjan, & Kahn, 1995). This sequence homology initially suggested that *ALS* proteins would have an adhesive function. Currently, there are eight members in this family (*ALS1 -7*, and *ALS9*) (Hoyer, 2001b; X. Zhao et al., 2004). Partial *ALS* genes are also found in *Candida dubliniensis* and *Candida tropicalis* (Hoyer et al., 2001). Western blots using *C. albicans* anti-Als serum showed the cross-reactive non-*albicans* proteins to be localized to the cell wall matrix, thus suggesting conserved function between these organisms for processing *ALS* proteins (Fu et al., 2002; Hoyer, Payne, & Hecht, 1998).

ii. Homology and structural similarity of Als proteins

ALS proteins show a high degree of similarity to each other, thus predicting similar three-dimensional structures. The *ALS* protein structure consists of four regions: An N-terminal Immunoglobulin–like (Ig) domain that is 55%-90% identical across the family, a conserved threonine rich region that has 90-98% similarity among *ALS* proteins, a central domain consisting of a motif of 36 amino acids repeated sequentially (ergo, a tandem repeat region), and a heavily glycosylated C-terminal serine/threonine rich region, where the sequence variability is highest between family members (Gaur & Klotz, 1997; Hoyer & Hecht, 2000, , 2001b). Additionally, a signal sequence for transporting the protein outside the cell membrane is encoded within the first 20 residues of the N-terminal domain, and a 13-20 residue sequence at the end of the C-terminal domain signals the attachment point of a glycosylphosphatidylinositol (GPI) anchor, thus allowing cross-linking to the cell wall matrix (Figure 1). Currently, there is no three-dimensional crystal structure available for α-agglutinin or any *ALS* proteins. However, extensive work carried out on α-agglutinin has shown that the “globular head” region composed of three tandem IgV-like domains is rich in anti-parallel β-sheets connected by random coil structures (Chen, Shen, Bobin, Kahn, & Lipke, 1995; de Nobel, Lipke, & Kurjan, 1996; Grigorescu, Chen, Zhao, Kahn, & Lipke, 2000; H. Zhao, Chen, Shen, Kahn, & Lipke, 2001). This domain mediates function and structural properties which are consistent for proteins in the Ig superfamily (Lipke, Terrance, & Wu, 1987; Terrance & Lipke, 1987; Whelan et al., 1990; Wojciechowicz & Lipke, 1989).

Figure 1. Model of Als proteins with Als5p as prototype. *ALS5* features a Signal Sequence (black), Immunoglobulin-like region (solid diamond pattern), Threonine rich region conserved in *ALS* genes (grey zigzag pattern), Tandem Repeat region containing six repeats of 36 amino acids (horizontal brick pattern), a highly glycosylated Serine/Threonine rich region (outlined diamond pattern), and a GPI anchor sequence (grey).


Secretion Signal
Immunoglobulin - like
Threonine Conserved
Tandem repeat region
Serine/Threonine Rich
GPI anchor sequence
N
C
0
200
400
600
800
1000
1200
1400
Amino Acid #

***ALS*5**

The sequence similarity between *ALS* proteins and α-agglutinin proposes that the N-terminal domain also contain similar Immunoglobulin folds. Indeed, secondary structure analyses determined by Circular Dichroism (CD) spectrophotometry, Fourier Transform Infra–Red (FTIR) spectroscopy, and molecular modeling of the Als proteins N-terminal domain confirmed that this region consists mostly of β-sheets, particularly in anti-parallel form interposed by extended regions, homologous to adhesins or invasins of the immunoglobulin superfamily (Hoyer & Hecht, 2001b; Sheppard et al., 2004).

iii. Expression profile and allelic variation of ALS genes

The *ALS* genes are differentially expressed in different growth phases and in different morphological forms (Green, Zhao, Yeater, & Hoyer, 2005; Hoyer, 2001b; Hoyer & Hecht, 2000; Hoyer, Payne, & Hecht, 1998). *ALS1* expression is maximal compared to other family members upon fungal cell inoculation to fresh growth media (X. Zhao et al., 2004). Similarly, *ALS3* expression is maximal when germ tubes are microscopically visible (X. Zhao et al., 2004). In addition, certain *C. albicans* strains can carry *ALS* genes, which are not present in other strains. For example, *ALS5* is absent in *C. albicans* strains B311 and B792 (Hoyer, 2001b; Hoyer & Hecht, 2000). Also, strains can display variations in the number of tandem repeats, thereby resulting in the same *ALS* gene having different sizes between strains and between alleles within the same strain (Hoyer & Hecht, 2000, , 2001b). Studies examining the two *ALS3* alleles in *C. albicans* showed a drastic difference in adhesion to vascular endothelial cells for the larger Als3p allele (containing a greater number of tandem repeats) compared to the smaller

Als3p allele (Oh et al., 2005). Further analysis of *ALS* allelic pairing across five major *C. albicans* clades demonstrated a tendency of *C. albicans* to encode one smaller and one larger *ALS* allele (Oh et al., 2005).

ALS gene activity was shown in *C. albicans* pathogenesis. Reverse–Transcription PCR tests detected *ALS* gene expression in human clinical specimens and in a vaginal candidiasis model (Cheng et al., 2005). Although transcription from all *ALS* genes was observed, *ALS1 - 3,* and *9* were detected most frequently (Cheng et al., 2005). Similar results for *ALS* transcriptional activity were monitored in a murine model of disseminated candidiasis (Green, Zhao, & Hoyer, 2005). Als protein production has been demonstrated both *in vitro* and *in vivo* tests. Immunohistochemical analysis of tissue sections from *C. albicans* infected mice using Als antiserum showed a distribution of Als proteins along the fungal cell wall (Green, Zhao, & Hoyer, 2005; Hoyer, Clevenger, Hecht, Ehrhart, & Poulet, 1999). Also, the gene encoding the yeast - enhanced green fluorescent protein (GFP) was placed under control of *ALS* promoters in *C. albicans*. Flow cytometry and RT- PCR analyses on the P*ALS* – GFP reporter strains implicated that some Als proteins – mainly Als1p and Als3p – are abundant on the *C. albicans* cell surface, while others are produced at lower levels (Green, Zhao, Yeater, & Hoyer, 2005).

iv. Functional properties of Als proteins

Functions of Als proteins have been shown through various adherence assays using *C. albicans* containing mutations in *ALS* genes and through heterologous expression of individual *ALS* genes in *S. cerevisiae*. Als proteins

(Als1p-7p and -9p) mediated binding of *C. albicans* to various substrates such as the ECM proteins fibronectin, laminin, type IV collagen, and gelatin, in addition to several different endothelial and epithelial cell types (Sheppard et al., 2004). Deletion of *ALS1* and *ALS3* resulted in a decrease of *C. albicans* adhesion to both HUVECs and buccal epithelial cells (BEC), along with a decrease in epithelial destruction in a reconstituted human epithelium (RHE) model of oral candidiasis (X. Zhao et al., 2004). Also, Als3p mediated significant endothelial cell invasion (Sheppard et al., 2004). Recent studies demonstrated that deletion of *ALS2* and *ALS4* slowed germ tube formation and slightly reduced *C. albicans* adhesion to vascular endothelial cells (X. Zhao, Oh, Yeater, & Hoyer, 2005). Despite the availability of data assessing the binding activity of Als proteins as well as their involvement in pathogenesis, the broad array of different Als proteins on the *C. albicans* cell wall has made it difficult to study the molecular mechanisms mediating adherence of individual Als proteins and subsequent colonization of *C. albicans.*

IV. Characteristics of Als5p-mediated adhesion and aggregation

i. Isolation and heterologous expression of *ALS5* in *S. cerevisiae*

One particular gene of this family that has been characterized extensively is *ALS5*. Originally, *ALS5* was isolated in a screen which depended on heterologous expression of *C. albicans* genes in *S. cerevisiae*. *ALS5* conferred the ability of *S. cerevisiae* to adhere to ECM proteins and human BECs (Gaur & Klotz, 1997). Under conditions where transformed *S. cerevisiae* cells over-expressed Als5p, an initial rapid **adhesion** step between the cell and the target

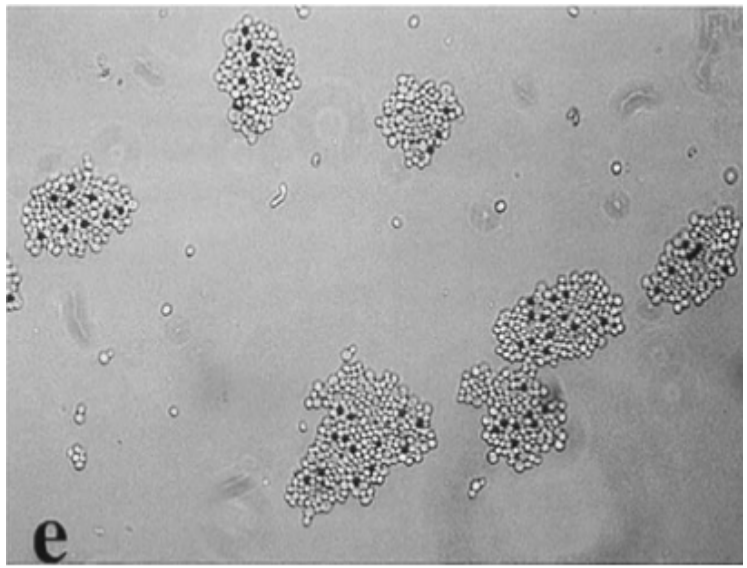
occurred, followed by a slower yeast cell-yeast cell **aggregation** step (Figure 2A) (Gaur, Klotz, & Henderson, 1999). The binding phenotype mimicked that of *C. albicans* cells. The binding interaction occurred over a broad pH range (between pH 2-9), and was resistant to the shear forces generated by vortexing and dissolution by non-chaotropic salt or sugar additives (Gaur, Klotz, & Henderson, 1999). Pre-treatment with hydrogen bond disrupting agents, urea or formamide reduced the adherence phenotype (Gaur, Klotz, & Henderson, 1999), thus demonstrating that Als5p mediated binding occurs primarily through hydrogen bonding (Figure 2B). This binding has been called stable reversible specific (SRS) adherence (Gaur & Klotz, 2004).

ii. Identification of Als5p ligands

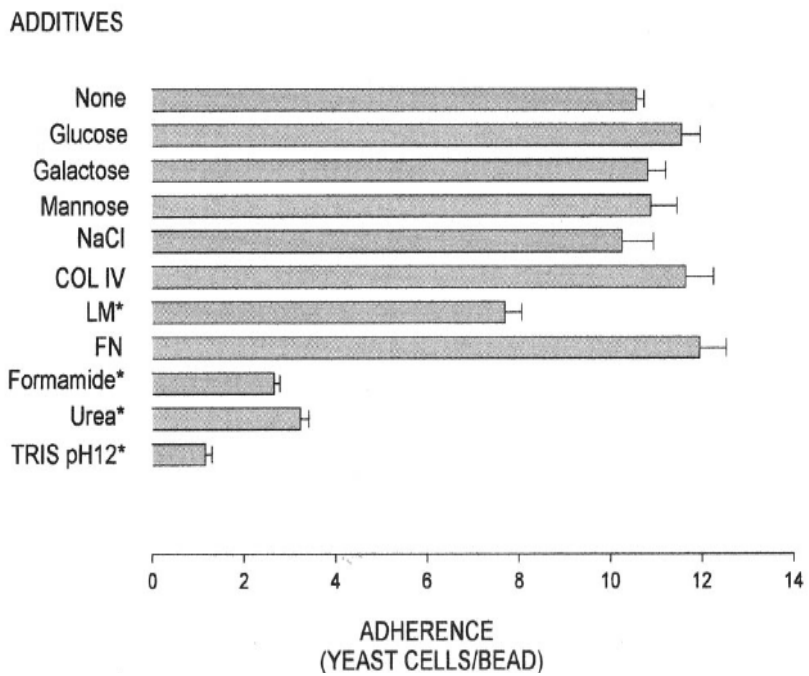
C. albicans adherence to host cells *in vivo* along with adherence to ECM proteins *in vitro* is highly selective. Adherence of *C. albicans* to ECM rich environments such as basement membrane occurs at specific sites. Thus, only specific molecular sites serve as substrates for *C. albicans* attachment. Specific molecular target sequences responsible for the adhesion and aggregation activity of Als5p have been identified. *C. albicans* and *ALS5* transformed *S. cerevisiae* cells adhered and aggregated on bead surfaces coated with amino acid homopolymers of threonine, serine, and alanine. Poly-threonine, poly–serine peptides and a 23 mer peptide derived from fibronectin were effective in inhibiting *C. albicans* cells and *ALS5* – expressing *S. cerevisiae* from binding to fibronectin coated beads and peptide coated Poly Ethylene Glycol beads respectively (Gaur, Smith, & Klotz, 2002; Klotz, Gaur, Rauceo et al., 2004).

Figure 2. Expression of *ALS5* in *S. cerevisiae* and binding characterization (Reprint with permission from Gaur et al., 1999). (A) S. *cerevisiae* overexpressing *ALS5* binds to fibronectin coated beads and forms cellular aggregates. (B) Effects of various additives on Als5p-mediated adhesion.

A.


e

B.

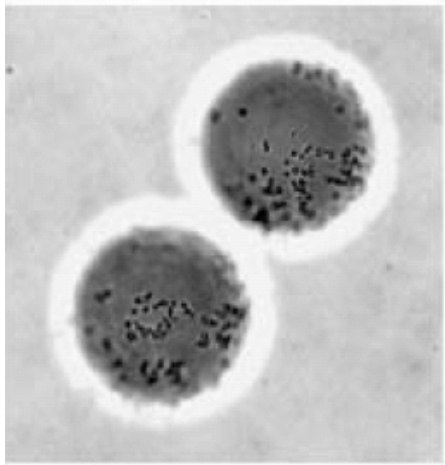

ADDITIVES
None
Glucose
Galactose
Mannose
NaCl
COL IV
LM*
FN
Formamide*
Urea*
TRIS pH12*
0
2
4
6
8
10
12
14
ADHERENCE
(YEAST CELLS/BEAD)

Also, naturally occurring sequences such as fibronectin 10-mer peptides containing 5-7 serine or threonine residues facilitated adherence, whereas adherence was not observed with fibronectin peptides containing too few Ser or Thr residues, or when the same peptides were presented in a closed loop confirmation (Gaur & Klotz, 2004; Gaur, Smith, & Klotz, 2002). These results demonstrated the significance of sequence composition and steric confirmation in mediating Als5p adherence. A screen demonstrating yeast cell adherence to a 7–mer peptide from a library 19^7 unique peptide beads identified specific sequences *ALS5* transformed *S. cerevisiae* cells adhered to in comparison to *S. cerevisiae* transformed with *ALS1* and *C. albicans* cells (Klotz, Gaur et al., 2004). Analysis of the peptides mediating yeast cell adherence showed a $\tau\phi$+ consensus sequence, which consists of successive turnlike, bulky hydrophobic, and cationic Lys or Arg residues (Klotz, Gaur et al., 2004). Taken together these observations indicate that Als5p recognizes certain amino acid sequences and *ALS* proteins recognize unique sequences despite their high degree of sequence identity to each other (Figure 3 and Table 1).

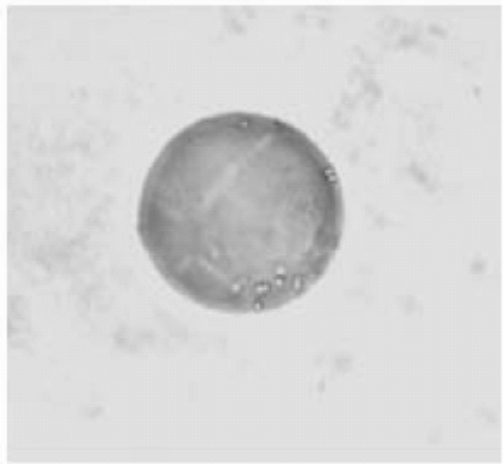
iii. Molecular characteristics of the Als Ig–like domain and Tandem repeat region

Recent studies showed which regions within Als proteins mediate binding. The sequence homology of the N-terminal domain of Als proteins to α-agglutinin and immunoglobulins indicates that Als binding activity may be a consequence of this domain. Indeed, *S. cerevisiae* cells expressing Als1p in which the N–

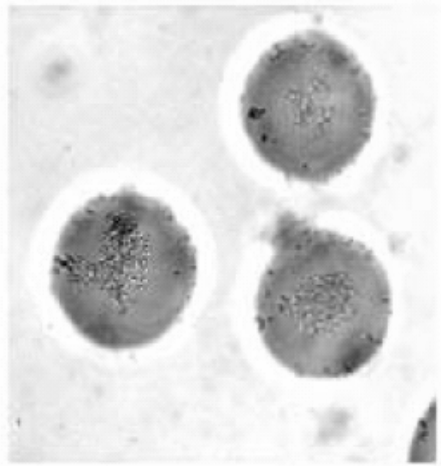
Figure 3. Adhesion of *S. cerevisiae* expressing Als5p or Als1p to peptide coated beads (Reprint with permission from Klotz et al., 2004). The large spheres are beads, and the adherent yeasts are visible as small dots.



S. cerevisiae expressing Als5p. The peptide is **AYK**SLMT



S. cerevisiae expressing Als5p. The peptide is SKMAYTL



S. cerevisiae expressing Als1p. The peptide is VTH**THR**R

Table 1. Peptide sequences mediating fungal adhesion (Reprint with permission from Klotz et al., 2004). Fungi were mixed with 10^2 to 10^3 beads from the 7-mer library. *: these peptide sequences were confirmed after specific synthesis. Bold print: amino acid residues with consensus sequence.

Peptide-beads selected by *C. albicans**	**Peptides selected by *S. cerevisiae* expressing *ALA1/ALS5****	**Peptides selected by *S. cerevisiae* expressing *ALS1***
KTKFLVD	**KTK**FLVD	HLYA**SWR**
VTH**THR**R	VTH**THR**R	VYY**PFK**Q
DK**GWR**AP	DK**GWR**AP	**DLK**LVRP
KLRIPSV	**KLR**IPSV	
AYKSLMT	**AYK**SLMT	

terminal domain was deleted showed approximately a 10-fold decrease in adhesion compared to cells expressing wild type Als1p (Loza et al., 2004). It must be noted that although adherence was significantly reduced, it was not completely abolished. Also, *S. cerevisiae* cells expressing chimeric Als5p–Als6p constructs demonstrated localization of adhesive function to the N–terminal domain (Sheppard et al., 2004).

The central tandem repeat (TR) region is rich in threonine and β-branched residues. In fact, this region has 2-3 times more β –branched residues than globular proteins. This domain is not found in α-agglutinin; however similar regions are found on the surfaces of pathogenic organisms such as *Cryptosporidium parvum*, *Trypanosoma cruzi* and *Pneumocystis carinii* (Barnes et al., 1998). A BLAST search using the tandem repeat region of Als5p as a query yielded approximately 500 "hits" with a low expectation value ($e < 10^{-13}$), and over 8,000 "hits" with expectation value ($e < 1$). Many of the similar sequences found were mucins and other proteins with adhesive properties. Several interesting hits found in BLAST searches are a region in the prion precursor PrPc protein associated with amyloid plaques in Alzheimer's disease, and in uncharacterized ORF's from *Plasmodium* and *Mycobacterium tuberculosis* (Table 2). In *S. cerevisiae*, threonine rich repeats are observed within flocculin (*FLO*) proteins. These proteins facilitate cell/cell interaction to form aggregates or flocs, induce filamentation, and mediate the invasion of agar in the presence of divalent cations (Lo & Dranginis, 1996, , 1998).

Table 2. Selected sequences similar to the Thr-rich region of Als5p (BLAST search). BLAST-P search of a non-redundant database using the TC-TR regions of Als5p as a query.

GI #	Genus	annotation	e value
	Eukaryotes		
Various	Candida spp.	27 ALS and related sequences	e-132 to e-26
7494135	Cryptococcus neoformans	Invasin	1E-16
4505285	Homo sapiens	Mucin 2	9E-11
1019435	Typanosoma cruzi	Mucin-like	4E-10
19114899	Schizosaccharomyces pombe	agglutinin-like	5E-10
2827462	HepatitisA	Receptor	7E-09
17221102	Herpesvirus	Glycoprotein	1E-08
1155358	Brugia	microfilarial sheath	6E-08
17550356	Caenorhabditis elegans	Uncharacterized ORF	1E-07
7494069	Leishmania	promastigote surface antigen	2E-07
28923372	Neurospora crassa	Uncharacterized ORF	3E-07
6322209	Saccharomyces cerevisiae	Flo11p invasion	3E-07
	Prokaryotes		
13277318	Ruminococcus	putative cellulosome anchoring protein	6E-10
25027182	Corynebacterium	Uncharacterized ORFs (2 hits)	1E-9 & 1E-7
22970264	Chloroflexus	Uncharacterized ORFs (3 hits)	6E-9 TO 3E-5
23137706	Cytophaga	Uncharacterized ORFs (2 hits)	3E-6 & 2E-4
23040767	Trichodesmium	Uncharacterized ORFs (2 hits)	8E-06
23024139	Leuconostoc	Uncharacterized ORFs (2 hits)	8E-5 & 1E-4
22991255	Enterococcus	Uncharacterized ORF	4E-04
27469167	Staphylococcus	Hemagglutinin	4E-04
19070694	Streptococcus salivarius	protein A	1E-03

The sequence arrangement in the TR region contain 5 -11 amino acid stretches of β-branched residues (Thr, Val, Ile), which are connected to each other via short loop-like sequences (Gly, Asn, Asp). These β-branched residues are conformationally restricted to form α–helices and especially β-sheets. Secondary structural predictions have identified the TR region to be composed of mainly β-strands for the branched residues and random coil structure for the loop sequences (Eisenberg, Schwarz, Komaromy, & Wall, 1984). Als1p contains 20 tandem repeats and Als5p contains 6 repeats. This is important since *S. cerevisiae* cells expressing Als1p auto-aggregate in contrast to Als5p cells which aggregate only following attachment to ligand (Klotz, Gaur et al., 2004a). Currently, the mechanisms underlying how Als5p aggregates following binding to the ligand are unknown. In addition, the role of the TR region in Als protein function is not well understood.

v. Aim of Thesis Research

In this study, the yeast heterologous expression system was used to present a model for Als5p-mediated aggregation, in which an adhesion-triggered change in the conformation of Als5p propagates around the cell surface, forming ordered aggregation-competent microcolonies. The formation of cellular aggregates is dependent on Als5p activity and independent of metabolic activity and signal transduction. Secondly, the role of the Als5p Ig domain and TR region was determined through cellular aggregation assays and binding assays using soluble Als5 protein constructs. Finally, the structural properties of Als5p Ig domain and TR region were investigated.

Chapter II

Global Cell Surface Conformational Shift Mediated by a *Candida albicans* Adhesion

Introduction

Infections due to fungal species are widespread and can lead to death in immunocompromised individuals. *Candida albicans* is the most common fungal opportunistic pathogen, and can exist in a wide range of associations from a superficial commensal state to systemic infections of its human or animal host. Typical cases of candidiasis in humans include oral and vaginal infections, whereas disseminated *Candida* infections are a significant cause of morbidity and mortality in immunocompromised patients (Calderone, 2002; Klepser, Lewis, & Pfaller, 1998).

Adherence to host and subsequent aggregation of the infecting cells serve as initial and critical steps in *C. albicans* establishing itself as a commensal inhabitant or pathogen. The importance of adherence is reflected in reductions in virulence seen for strains with mutated adhesin genes or with mutations in genes that regulate adhesins (Buurman et al., 1998; Fu et al., 2002; Staab, Bradway, Fidel, & Sundstrom, 1999). Following adherence, *C. albicans* grows in a colonial state. Indeed, in disseminated disease, micro-colonies are present in target organ tissue and are diagnostic for disease (Kamai et al., 2002).

Along with the characterized adhesins Hwp1 and Eap1p (Li & Palecek, 2003; Staab, Bradway, Fidel, & Sundstrom, 1999) , the *C. albicans* genome encodes the Als gene family of cell-surface glycoproteins, which are apparently the most widely expressed adhesins (Hoyer, 2001a; Hoyer & Hecht, 2000; Hoyer, Payne, & Hecht, 1998). Als1p and Als5p are the best characterized of the Als adhesins (Fu et al., 1998; Gaur & Klotz, 1997; Gaur, Klotz, & Henderson,

1999; Gaur, Smith, & Klotz, 2002; Hoyer & Hecht, 2001a; Kamai et al., 2002; Loza et al., 2004; X. Zhao, Pujol, Soll, & Hoyer, 2003)

Heterologous expression of Als5p in *Saccharomyces cerevisiae* has been used to identify several ligands including extracellular matrix (ECM) proteins, human buccal epithelial cells (Gaur & Klotz, 1997), homopolymers of threonine, serine, and alanine (Gaur, Smith, & Klotz, 2002), and a "tφ+" consensus sequence. This last ligand consists of successive a turn-like, bulky hydrophobic, and cationic Lys or Arg residues (Klotz, Gaur et al., 2004). Kinetic analysis of Als5p-mediated adhesion reveals an initial rapid adhesion to ligand, followed by slower yeast cell-yeast cell binding interactions forming macroscopic aggregates (Gaur, Klotz, & Henderson, 1999). In this paper we will refer to binding of cells to ligand-coated beads as "adhesion", and cell-to-cell binding as "aggregation." Both types of interaction occur over a broad pH range (between pH 2-9), and are resistant to the shear forces generated by vortexing and to dissolution by non-chaotropic salt or sugar additives (Gaur, Klotz, & Henderson, 1999). Urea (6M) or 50% formamide reversibly inhibits the adherence and aggregation phenotypes. This binding has been called Stable Reversible Specific adherence (Gaur, Smith, & Klotz, 2002).

Materials and Methods

Cell Transformation. The previously described plasmid pGK114 was used to over-express Als5p in *S. cerevisiae* (Gaur, Klotz, & Henderson, 1999). Plasmid pGK114 was transformed into the non-flocculent *S. cerevisiae* strain W303-1B by the lithium acetate method (Gietz, Schiestl, Willems, & Woods, 1995). Transformants were selected for the experiments reported here.

Adherence Assays. Adherence assays were performed as previously described (Gaur, Klotz, & Henderson, 1999). Briefly, *S. cerevisiae* cells containing pGK114 were grown with shaking at 30^{o}C in YPGal media (10 g/l yeast extract, 20 g/l peptone, 20 g/l galactose) to late logarithmic phase for Als5p expression. For experiments with *Candida albicans*, strain Ca1 cells was grown in YEPD media (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose) to stationary phase (~1.50 x 10^{8} cells/ml (Gaur, Klotz, & Henderson, 1999). Approximately 5mls of the culture was transferred to fresh YEPD media and the cells were incubated for 2 hours to maximize *ALS* expression. Cells were harvested and washed with Tris EDTA (TE) buffer, pH 7.0, three times and resuspended in TE buffer. In a 13 x 100 mm glass tube Als5p–expressing cells were mixed with fibronectin–coated (FN) magnetic beads at a 100:1 cell to bead ratio in TE buffer, briefly vortexed, and incubated at room temperature with gentle shaking for 30 – 45 minutes. Each tube was vortexed briefly and immediately placed into a magnetic separator (Dynal). Adherent and aggregated cells were gently washed three times with TE buffer while the tube remained within the magnet. The cells were resuspended in TE buffer and a sample was placed onto a microscope

slide for examination. Cells were viewed under a Nikon Optiphot-2 microscope equipped with a Sony DK-5000 camera. For assays investigating the effects of additives on adhesion, additives were added to the cell/bead mix at the onset of the adherence assays.

Fluorescence of ANS. Adherence assays were performed as described above except that following cellular incubation, the cells were washed once with TE buffer. Aggregates were wet-mounted, and photographs were taken after a seven-minute exposure to UV light.

Birefringence Assays. Adherence assays were performed as described above. For observation of cell surface birefringence, samples were observed with polarized light under phase with a 20X or 40X objective. A second polarizer (analyzer) was inserted between the objective and the eyepiece of the camera, and rotated to give a completely dark background, as described (http://www.microscopyu.com/articles/polarized/polarizedintro.html). Micrographs were exposed for 2-4 seconds at F 2.8. All micrographs in the figures were recorded under identical conditions.

Antibody inhibition and immunofluorescence assays. Polyclonal Als1p-specific antibodies were raised in rabbits following injection with the N-terminal region Als1p. The antibodies were twice adsorbed overnight with heat killed *S. cerevisiae* W303-1B before use. For adherence assays using antibodies, cells were pre-incubated with anti-Als1p antibodies at 1:40 for one hour before the onset of the experiment.

For immunofluorescence assays, *S. cerevisiae* expressing Als5p (galactose grown) and non-expressing cells (glucose grown or without pGK114) were incubated with anti-Als1p polyclonal antibodies at 1:50 for one hour followed by three successive washes in phosphate buffered saline (pH 7.00). The cells were then incubated with *S. cerevisiae*–adsorbed fluorescein-labeled goat secondary antibodies at 1:100 for one hour and washed as described above. Cells were microscopically examined.

Results

Effects of additives on adherence and aggregation of *ALS5*-transformed *S. cerevisiae* cells

In W303-1B, a non-flocculent strain of *S. cerevisiae,* galactose-induced expression of Als5p led to adhesion of the cells to FN-coated beads and formation of large aggregates that included cell-to-cell associations (Figure 4A). The adherence and aggregation were similar to those seen in *S. cerevisiae* strain YPH499 (Gaur & Klotz, 1997; Gaur, Klotz, & Henderson, 1999; Gaur, Smith, & Klotz, 2002). Neither adhesion to beads nor aggregation was seen in non-transformed cells (Figure 4F), nor in transformed cells grown in glucose (data not shown). Adherence to ligand beads and cellular aggregation were similar for non-treated control cells and for cells incubated with cycloheximide (10μg/ml; Figure 4B) or sodium azide (10 mM; Figure 4C). The signal transduction inhibitors AMPNP (300μM), staurosporine (30μM; (Chai, Hsu, Du, & Laurent, 2002)) or okadaic acid (2 μM) also had no effect (data not shown). There was also adherence and aggregation in experiments where Als5p-expressing cells were heat killed prior to the adherence assay (data not shown). Quantification of adhered and aggregated cells showed similar binding for controls and samples treated with cycloheximide or sodium azide (Figure 5).

Effects of antibodies on adherence and aggregation. When Als5p expression was induced by growth in galactose, the protein was displayed at the surface of intact cells and reacted with a polyclonal antibody raised against Als1p (Figures 6A and B). The pGK114-transformed cells showed no fluorescence after growth in repressing glucose medium (Figures 6C and D).

Figure 4. Effects of additives on adherence and aggregation of *ALS5* transformed *S. cerevisiae* cells. (A) *S. cerevisiae* strain W303-1B expressing Als5p binding to FN-coated beads (dark spheres) and adhering to other cells. (B) Cycloheximide, 10µg/ml, was added to the cell/bead mix at the onset of the experiment. (C) Sodium azide, 1 mM, was similarly added at the onset of the experiment. (D) Adhesion and aggregation in the presence of ANS, 100µM. (E) Cells treated with Congo Red, 30µM. (F) Non-transformed *S. cerevisiae* W303-1B cells. Bar (shown in D) = 10µm

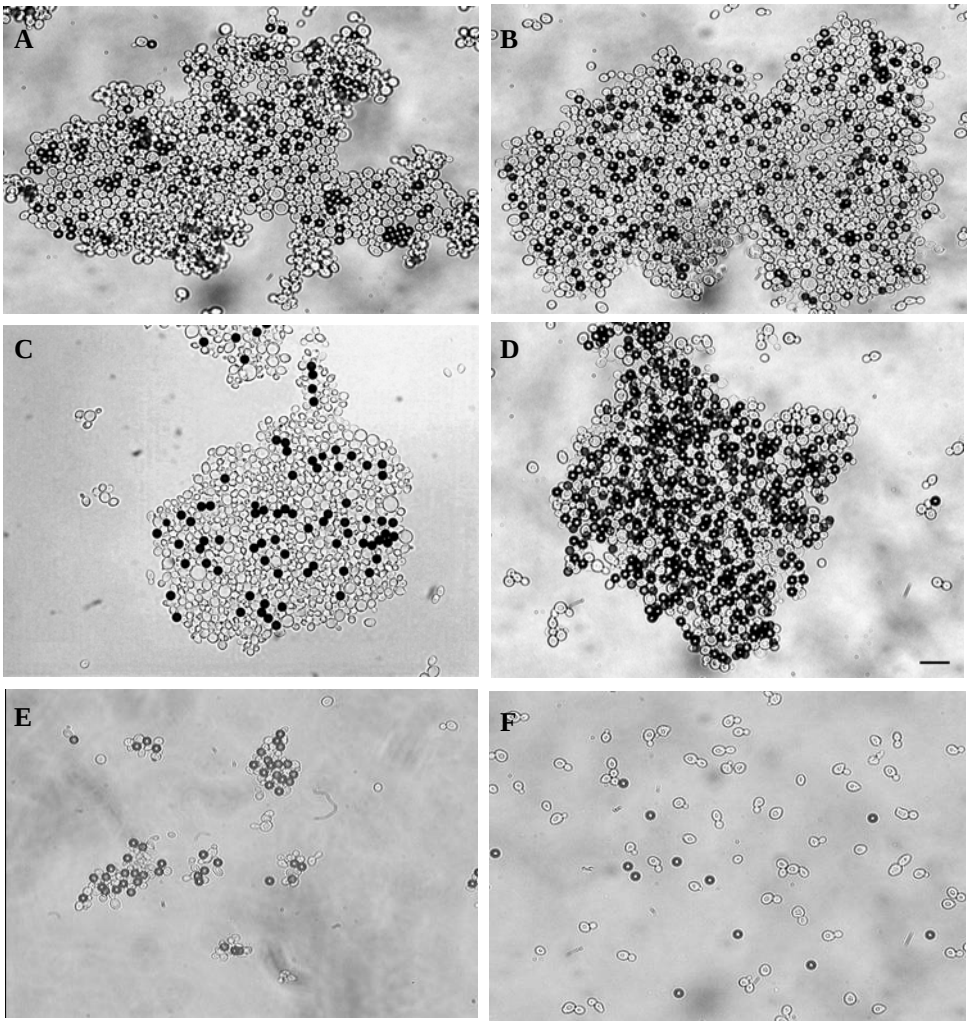

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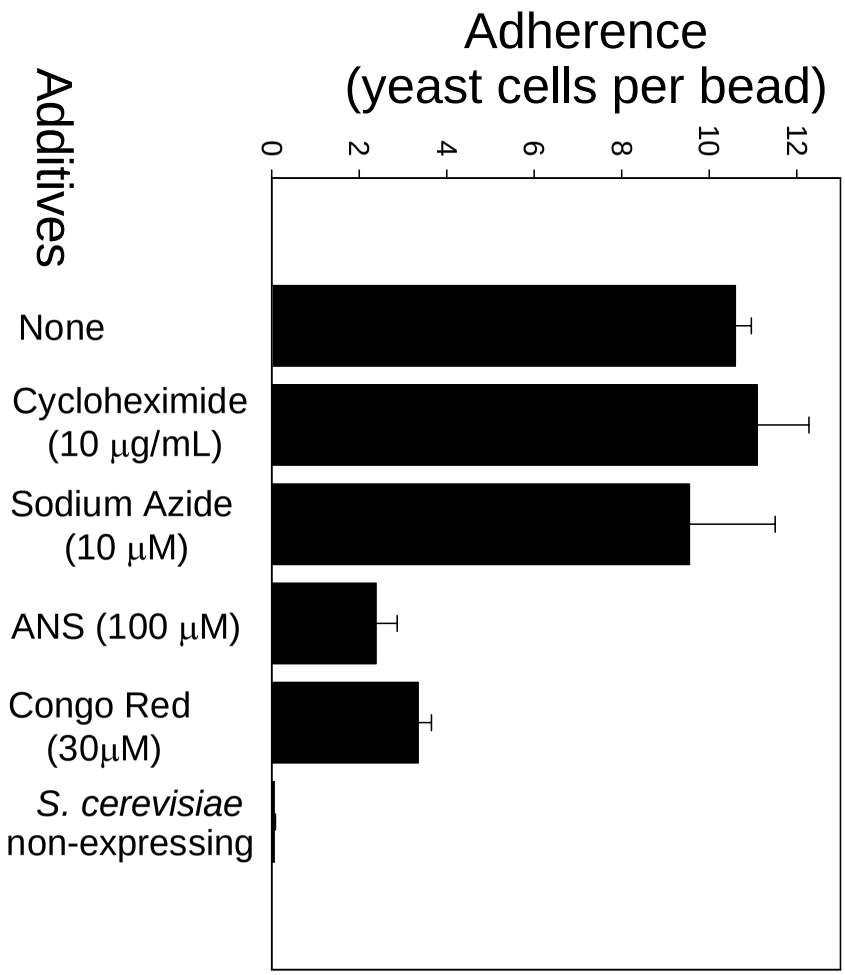
Figure 5. Quantitative Analysis of Adherence/Aggregation. Cellular aggregates were disrupted with the addition of 0.1M NaOH, and cells and beads were counted on a hemocytometer. Data plotted is the mean and standard deviation from three separate experiments.


Adherence
(yeast cells per bead)
0
2
4
6
8
10
12
Additives
None
Cycloheximide
(10 μg/mL)
Sodium Azide
(10 μM)
ANS (100 μM)
Congo Red
(30μM)
S. cerevisiae
non-expressing

Cells not transformed with pGK114 also showed no fluorescence (data not shown). An Als1p-specific monoclonal antibody did not react with Als5p-expressing cells (data not shown).

The polyclonal antibody also inhibited both adherence to beads and cell-to-cell aggregation (Figures 6E and F). This result implied that cell surface Als5p was essential for both the adhesion and the cell-to-cell binding in the Als5p-expressing *S. cerevisiae* cells.

Als5p–mediated aggregation in the presence of ANS and Congo Red. 8–Anilino–1-naphthalene–sulfonic acid (ANS) is fluorescent in hydrophobic environments, so it is commonly used to monitor secondary structure changes during protein folding and unfolding (Bailey, Dunker, Brown, Garner, & Griswold, 2001; Kundu & Guptasarma, 1999, , 2002; Maiti & Surewicz, 2001; Plakoutsi, Taddei, Stefani, & Chiti, 2004). ANS (100μM) reduced aggregation in assays of Als5p-expressing cells (Figures. 4D and 7A), showing a five-fold decrease in cells per bead (Figure 5). There was also a marked increase in fluorescence of aggregating cells compared to non-aggregating *ALS5*-expressing cells and non-transformed *S. cerevisiae* cells (Figures 7A-C). Reduced cellular aggregation and increased fluorescence associated with ANS treatment were also observed in *C. albicans* (Figures 7D, E and 8A, C). The surfaces of adherent cells were uniformly fluorescent or not, rather than fluorescent only in the region next to the ligand bead (Figures 7A-C). This observation implied that Als5p molecules around the entire surface had changed conformation to show similar exposure of hydrophobic regions.

Figure 6. Surface localization of Als5p on *S. cerevisiae* cells and antibody inhibition of cellular aggregation. (A) Cell surface fluorescence of Als5p-expressing *S. cerevisiae* cells. (B) Bright Field –micrograph of the cells seen in panel A. (C) Non- expressing *S. cerevisiae* cells (grown in glucose). (D) Bright Field –micrograph of the cells seen in panel C. (E) Adherence assays with Als5p-expressing *S. cerevisiae* in the presence of Als1p antibodies. (F) Adherence assays in the absence of Als1p antibodies.

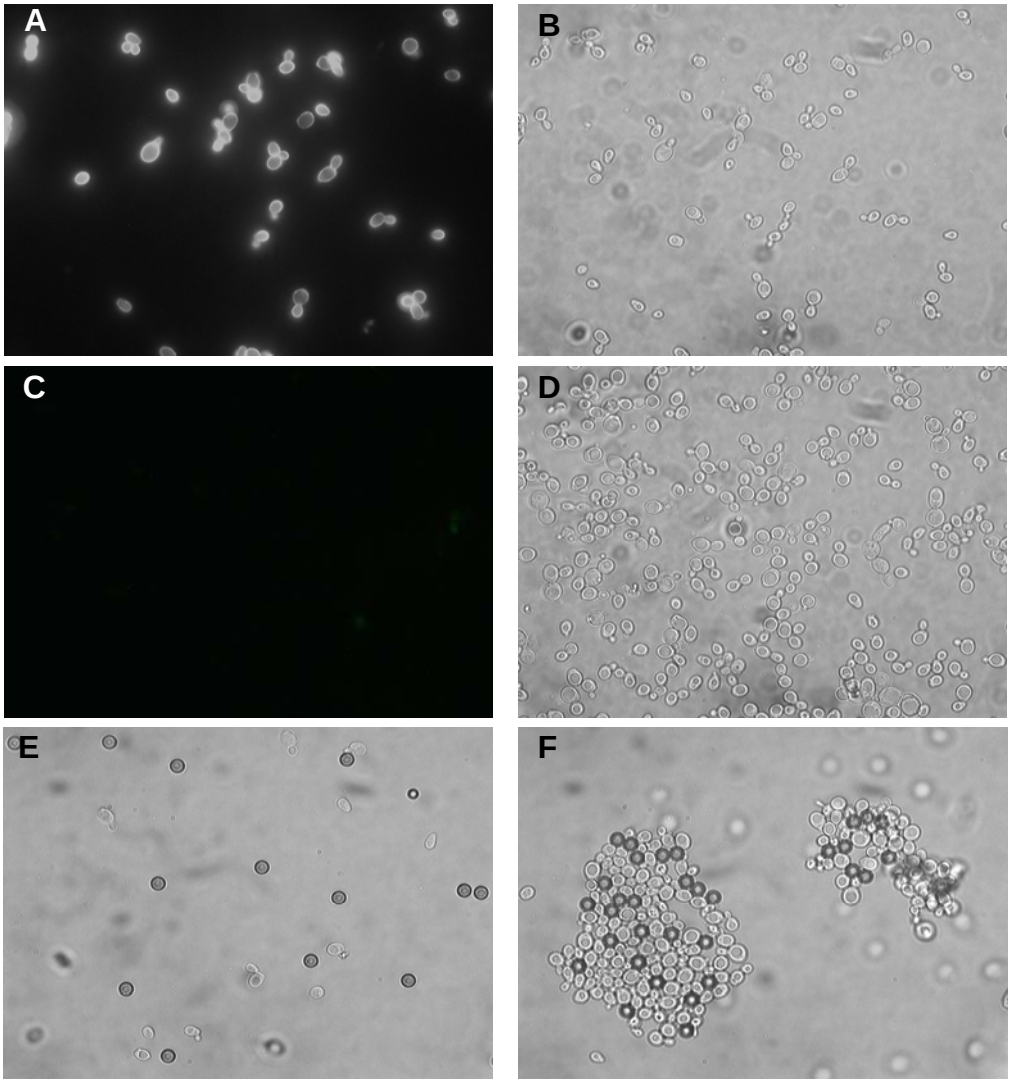

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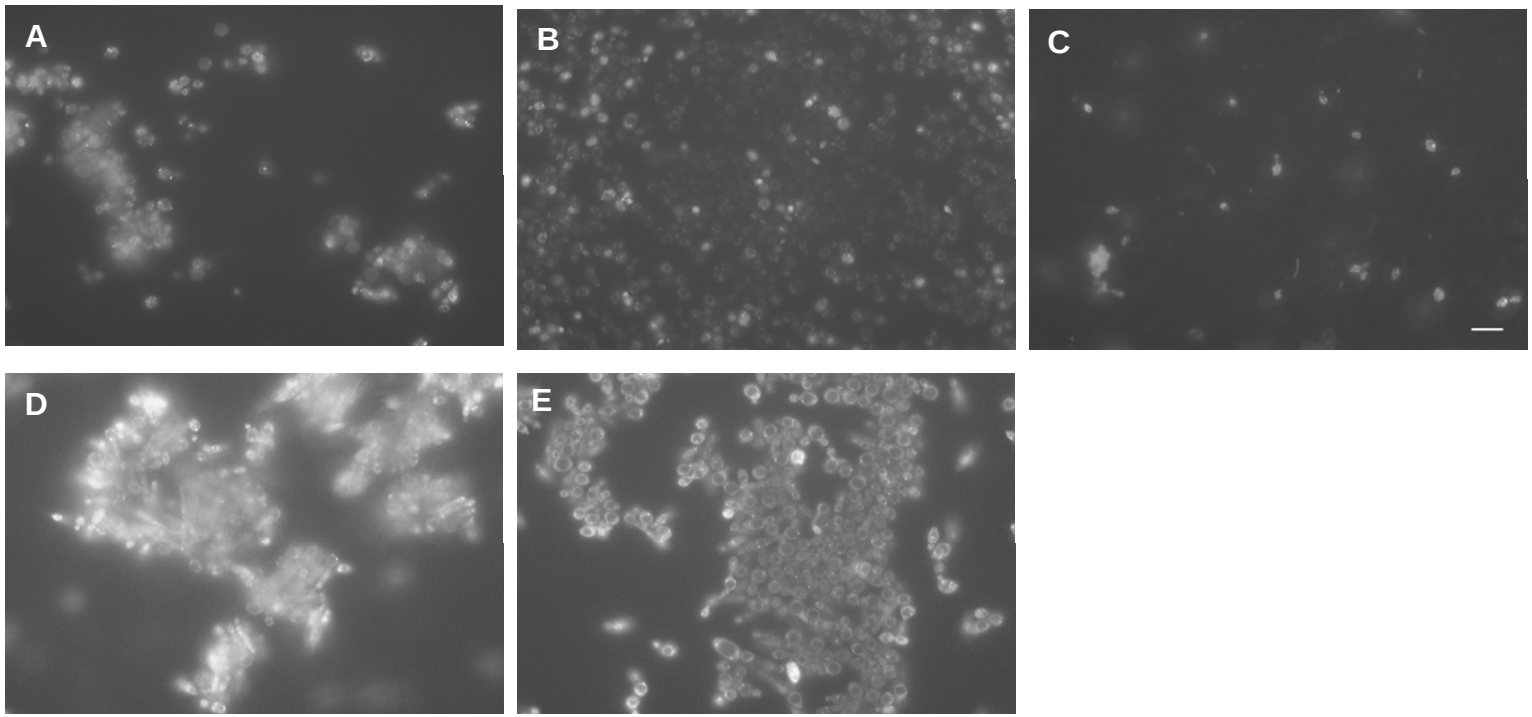
Figure 7. ANS surface fluorescence analysis of Als5p-mediated adhesion and aggregation. Adherence assays were performed with *S. cerevisiae* in the presence of 100μM ANS. (A) Als5p-expressing cells binding to FN-coated beads and to other cells. (B) Als5p-expressing cells in the absence of FN-coated beads. (C) Non–expressing *S. cerevisiae* cells. Bar = 10μm. There are a similar number of cells in panels B and C. (D) Cellular aggregation of *C .albicans* Ca1 cells incubated with FN-coated beads in the presence of 1mM ANS; (E) ANS-treated Ca1 cells in the absence of beads.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The dye Congo Red (CR) stains amyloid structures and inhibits their formation (Chapman et al., 2002; Lorenzo & Yankner, 1994; Plakoutsi, Taddei, Stefani, & Chiti, 2004). Like ANS, CR significantly reduced cellular aggregation in *S. cerevisiae* expressing Als5p (Figures 4E and 5) and in *C. albicans* (Figures 8A, B). CR had no effect in aggregation assays of cells that did not express Als5p.

Surface birefringence of Als5p–aggregates. When Als5p-mediated cellular aggregates of *S. cerevisiae* were viewed between crossed polarizers, the cell surface was clearly birefringent, whereas there was less birefringence in non-aggregating cells (Figures 9A, B). Inspection of Als5p expressing cells throughout the course of the adherence assay showed that cell surface birefringence increased as the aggregates enlarged, but was most apparent after 30 min of the assay (Figures 10A-E). The aggregation-induced birefringence was observed in the presence of metabolic inhibitors (not shown), but was absent from the small adhesive cell groups seen in the presence of ANS or CR (Figures 9C, D). Cell-surface birefringence was also observed in adherence assays using *C. albicans,* and was much greater in the presence of FN-coated beads than in their absence (Figures 9E, F). To determine whether the surface birefringence mediated by Als5p aggregation was a general property of yeast cell-yeast cell binding, we also observed the cell surface of the *S. cerevisiae* strain YIY345 during Flo11p-mediated flocculation and in sexual aggregates of *S*. *cerevisiae* formed through sexual agglutinins. In neither case was there surface birefringence greater than in non-aggregated controls (data not shown).

Figure 8. Effects of ANS and Congo Red on *Candida albicans* adherence and aggregation to FN-coated beads. (A) no additive. B) Congo Red added (300 μM). C) ANS added (1 mM).

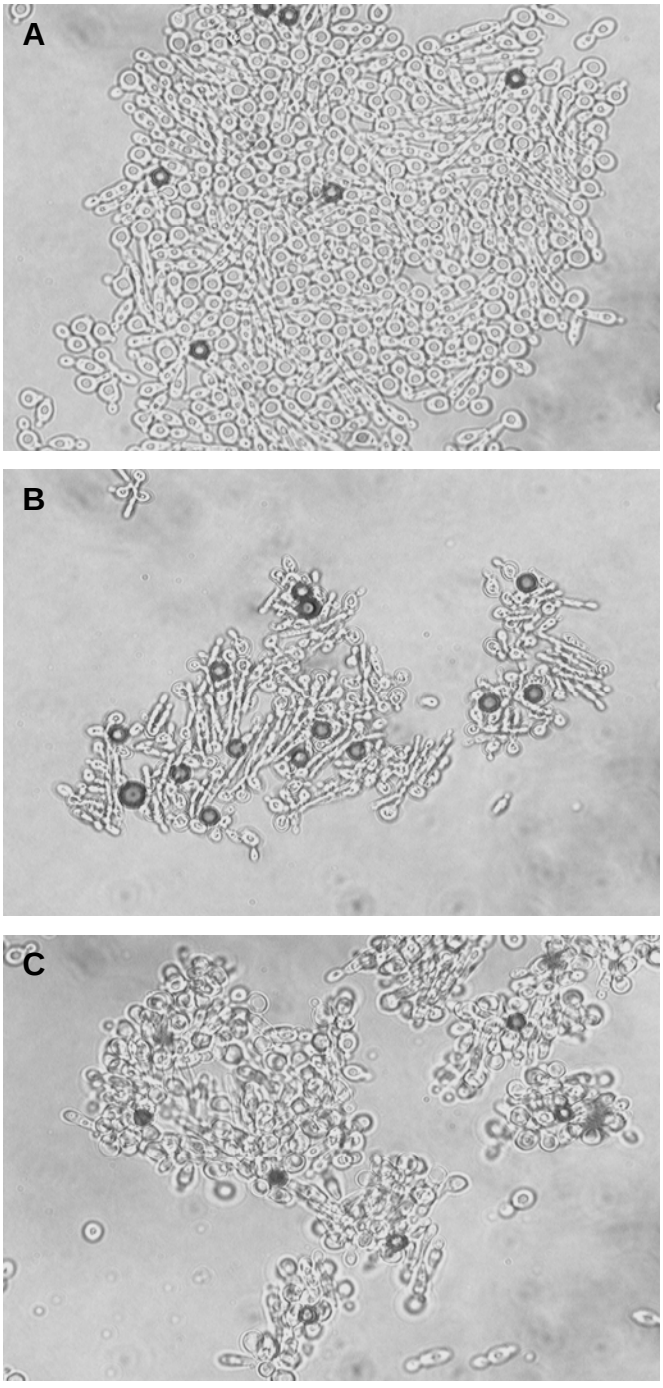
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Figure 9. Cell surface birefringence of Als5p-mediated aggregates. Cells were viewed between crossed polarizers. (A) Cellular aggregation mediated by *S. cerevisiae* expressing Als5p following incubation with FN coated beads. (B) Als5p-expressing cells were incubated with uncoated magnetic beads and centrifuged prior to observation. (C) Als5p-expressing cells incubated in the presence of 100μM ANS. (D) Als5p-expressing cells incubated in the presence of 30μM Congo Red. (E) Cellular aggregation of *Candida albicans* strain Ca1 in the presence of FN-coated beads. (F) Ca1 cells in the absence of FN coated beads. Bar = 50 μm.

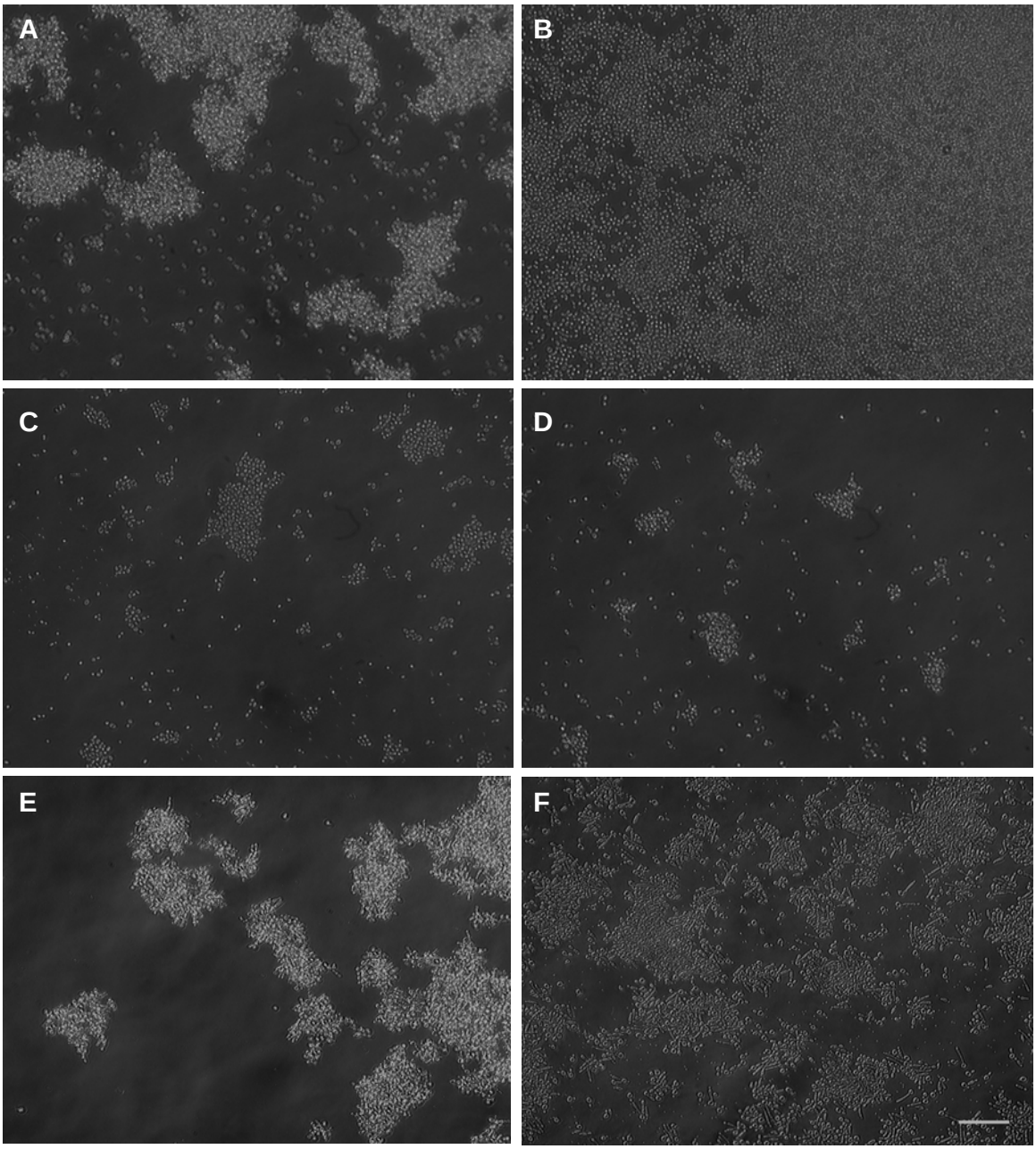
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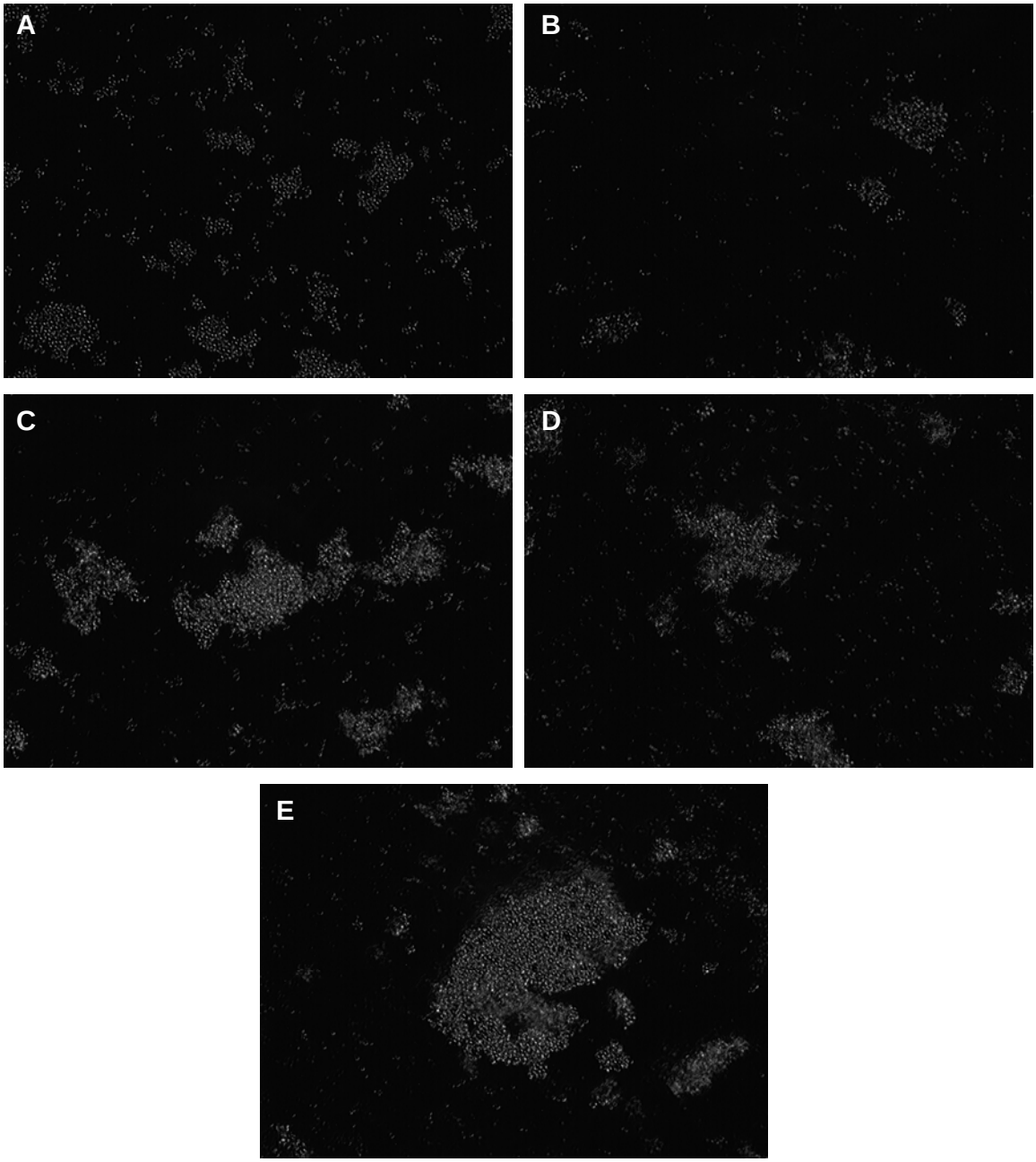
Figure 10. Kinetic analysis of Als5p-mediated cell surface birefringence formation. Aggregation assays were performed and cells were monitored after 5min incubation with FN-coated bead (A), 15min (B), 30min (C-E).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Discussion

Als5p mediates adhesion to fibronectin and cellular aggregation.

Previous studies showed that Als5p expression in *S .cerevisiae* results in a rapid cell-to-ligand adhesion step followed by a slower cell–cell binding, which we call aggregation (Gaur, Klotz, & Henderson, 1999). The biphasic kinetics suggest that following adhesion to ligand, Als5p may undergo a modification that mediates cellular aggregation. Such modification might include covalent modification of the cell surface Als5p, synthesis and display of new adhesin molecules with aggregative properties, or a non-covalent modification of Als5p structure.

Surface display of Als5p resulted in adhesion to FN-coated beads and cellular aggregation. Both interactions were inhibited by a specific antibody that reacts with Als5p. In addition, indirect immunofluorescence confirmed Als5p surface localization. Taken together with galactose-induced adhesion and aggregation, these results imply that cell surface Als5p is necessary and sufficient for binding to FN-coated beads and aggregation.

To eliminate the possibility that the Als5p-mediated aggregation phenotype resulted from adherence-induced expression of an altered, aggregate-competent version of Als5p, or induction of another adhesin altogether, aggregation assays were performed in the presence of various inhibitors. Adhesion and aggregation were similar for non-treated Als5p–expressing cells and cells treated with inhibitors of signal transduction, intermediary metabolism, or protein synthesis, and indeed in heat-killed cells. These results indicated that aggregation occurs independently of cellular metabolic activity.

ANS fluorescence increases upon aggregation. On the other hand, there is evidence to support a ligand-induced change in 3-dimensional structure of Als5p. The fluorescent dye ANS has been extensively used to study protein surface hydrophobicity and changes in exposure of hydrophobic regions after structural transitions (Bailey, Dunker, Brown, Garner, & Griswold, 2001; Kundu & Guptasarma, 1999, , 2002; Maiti & Surewicz, 2001). Indeed, ANS fluorescence reported an increase in cell surface hydrophobicity upon aggregation. Primary and secondary amino acid sequence analyses of Als5p indicate a significant amount of residue hydrophobicity, predicting that a conformational shift would correlate with a change in ANS fluorescence (Eisenberg, Schwarz, Komaromy, & Wall, 1984; Hoyer & Hecht, 2001a). The fluorescence was greater for aggregated cells in comparison to non–aggregating Als5p-expressing cells and untransformed control *S. cerevisiae* cells. The binding and fluorescence of ANS was uniform around the entire cell surface. Additionally, treatment with ANS reduced the Als5p–mediated aggregation. These results in cellular aggregation assays mimicked the effects of ANS in assays of protein unfolding for carbonic anhydrase and other proteins (Bailey, Dunker, Brown, Garner, & Griswold, 2001; Kundu & Guptasarma, 1999, , 2002; Maiti & Surewicz, 2001). In those studies the dye reports increased surface hydrophobicity during denaturation. Furthermore in the presence of ANS, protein aggregation is decreased (Bailey, Dunker, Brown, Garner, & Griswold, 2001; Kundu & Guptasarma, 1999, , 2002; Maiti & Surewicz, 2001). Thus the characteristics of dye binding in the cellular aggregation assays imply that a similar structural transition is involved in the

transition to competence for aggregation, and is dependent on the presence of both Als5p and its ligand fibronectin.

The amyloid-binding dye Congo Red inhibits aggregation. The dye Congo Red (CR) stains amyloid proteins (Chapman et al., 2002; Lorenzo & Yankner, 1994). CR has a strong affinity for proteins with high β-sheet secondary structure content like Als5p, and inhibits formation of amyloid protein aggregates (Hoyer & Hecht, 2001a; Lorenzo & Yankner, 1994). To determine whether CR would inhibit Als5p-mediated cellular aggregates, adherence assays were performed in the presence of 30μM CR. Similar to our results with ANS, Congo Red treatment on Als5p–expressing cells inhibited aggregation. This result is similar to the CR-mediated inhibition of amyloid formation in other systems (Lorenzo & Yankner, 1994). In sum, aggregation of Als5p-expressing cells was not affected by metabolic poisons, but was greatly reduced by two agents that perturb protein secondary structure.

Als5p mediated aggregation results in increased order in cell surface molecules. Molecular birefringence is a qualitative indicator of ordered and regular structure. When viewed under polarized light, depending on the molecular orientation of the material in the light path, anisotropic substances will refract light to yield variations in the brightness and/or color of an image (de Campos Vidal & de Carvalho, 1990; Feitosa, Vidal, & Pimentel, 2002; Inoue, 1987). If the formation of Als5p-mediated cellular aggregates is dependent on the formation of an ordered array of Als5p molecules, then we would expect an increase in cell surface birefringence for aggregating cells compared to non-

aggregating cells. Our results show that Als5-mediated aggregates of *S. cerevisiae* are more birefringent than non–aggregating cells expressing Als5p or cells aggregated by other adhesions. Similar birefringent aggregates were observed in *C. albicans*. (Note that in *C. albicans*, birefringence of *C. albicans* cannot be attributed to be a function of Als5p activity exclusively, since *C. albicans* may also be expressing other Als proteins as well as other surface adhesions (Fu et al., 2002; Hoyer, 2001a)). Congo Red or ANS prevented the development of increased cell surface birefringence. These results are also consistent with the idea that Als5p undergoes a conformational shift to form ordered domains on the cell surface in cellular aggregates. Thus, development of birefringence in *S. cerevisiae* depended on expression Als5p and presence of beads coated with Als5p ligand. In *C. albicans* also, the presence of FN-coated beads in aggregates increased birefringence (Figures 9E vs. 9F).

All of the results suggest a model in which Als proteins bind to a few bead-bound peptide ligands (Klotz, Gaur et al., 2004b), and then undergo a structural change to an aggregative state. This transition propagates around the entire cell surface. The kinetics of aggregation imply that this transition takes place over 5-30 min (Gaur, Klotz, & Henderson, 1999). The aggregative state is characterized by the formation of birefringent and hydrophobic domains on the entire surface of the cell. The increases in both ANS fluorescence and birefringence show long-range order around the entire surface of individual cells. Thus the ordered state appears to encompass the surface of each cell, following ligand binding at a single region of the cell surface.

The development of the birefringence was Als5p-specific in *S. cerevisiae*, dependent on binding to ligand-coated beads, independent of cellular metabolism, and was inhibited by ANS and CR, agents that perturb secondary structure. Furthermore, although ANS and CR compete for aggregative cell-to-cell binding, the increase in ANS fluorescence documents ligand-induced structural changes in Als5p that accompany the transition to the aggregative state. This change is coupled with a global increase in cell-surface hydrophobicity for the aggregative state. The similarity in behavior of Als5p-expressing *S. cerevisiae* and *C. albicans*, which expresses many Als proteins, implies that similar molecular properties mediate adhesion and aggregation in the pathogenic organism. The structural and functional similarities in the Als proteins support this idea (Fu et al., 2002; Gaur, Smith, & Klotz, 2002; Hoyer, 2001a; Klotz, Gaur et al., 2004b).

Chapter III

Structural and Functional Characterization of the Als5p Ig-like Domain and Tandem Repeat region

Introduction

Infections mediated by the opportunistic fungal pathogen *Candida albicans* are a significant cause of death in immunocompromised patients. In addition, *C. albicans* is the fourth most common nosocomial infectious agent (Calderone, 2002). As a commensal organism *C. albicans* commonly resides on mucosal tissues without causing any symptoms of disease. However, changes in the host's immunity usually lead to the onset of candidiasis that can be as mild as superficial mucosal infections or severe disseminated systemic infections (Calderone, 2002). A variety of *C. albicans* virulence factors are known, including the aspartyl proteases (SAPs), lipases, various adhesion macromolecules, and the morphological change from the blastoconidial cell form (yeast-like appearance) to the hyphal form (filament-like appearance) (Calderone, 2002). However, the molecular mechanisms of pathogenesis are not well understood. Adherence to host tissue is the initial key prerequisite step in maintaining a successful relationship as a commensal or pathogenic organism. Also, fungal adherence is critical, since strains with mutated adhesion genes or with mutations in genes that regulate adhesions display reduced virulence (Buurman et al., 1998; Fu et al., 2002; X. Zhao et al., 2004).

The *ALS* (Agglutinin–Like Sequence) gene family encodes cell–surface glycoproteins that mediate adherence to various host's substrates. Currently, there are eight known *ALS* genes, and the Als proteins are the most widely expressed adhesins in *C. albicans* (Hoyer, 2001b; X. Zhao et al., 2004). Als1p, Als3p, and Als5p have been extensively characterized and Als1p and Als3p are

involved in *C. albicans* virulence (Fu et al., 2002; X. Zhao et al., 2004). Als proteins show a high degree of identity and similarity between each other, thus predicting similar three-dimensional structures. The Als protein structure consists of the following: An N-terminal Immunoglobulin–like (Ig) domain that is 55%-90% identical across the family, a conserved threonine rich (TC) region that has 90-98% similarity among *ALS* proteins, a central domain consisting of a tandem repeat (TR) motif of 36 amino acids repeated sequentially, and a heavily glycosylated C-terminal serine/threonine rich region, where the sequence variability is highest between family members (Gaur & Klotz, 1997; Hoyer & Hecht, 2000, , 2001). Additionally, a signal sequence for transporting the protein outside the cell membrane is encoded within the first 20 residues of the N-terminal domain, and a 13-20 residue sequence at the end of the C-terminal domain signals the attachment point of a GPI anchor, thus allowing cross-linking to the cell wall matrix.

Recent studies using Als5p and Als1p have provided significant insight into the molecular mechanisms of Als-mediated adhesion and aggregation as well as the structural design of Als proteins. Expression of Als5p in the nonpathogenic yeast *Saccharomyces cerevisiae* resulted in binding of yeast cells to a variety of substrates such as extracellular matrix coated beads, human buccal epithelial cells and various peptide sequences (Gaur, Klotz, & Henderson, 1999; Gaur, Smith, & Klotz, 2002; Klotz, Gaur et al., 2004). Als5p–mediated adhesion is stable over a broad pH range, resistant to sheer forces, and is reversibly inhibited when treated with urea and formamide (Gaur, Klotz, &

Henderson, 1999). The initial binding of Als5p–expressing cells to ligand triggers a conformational change in Als5p that propagates over the cell surface subsequently leading to the formation of cellular aggregates (Rauceo et al., 2004). Als5p-mediated adhesion and aggregation occurred independently of metabolic activity and signal transduction. In contrast, cellular aggregation was inhibited by treatment with the dyes 8-anilino-1-naphthalene-sulfonic acid (ANS) or Congo Red (CR) (Rauceo et al., 2004). Finally, cells expressing chimeric Als5p–Als6p plasmids demonstrated localization of adhesive function to the N-terminal domain, therefore demonstrating that the Ig domain mediates adherence to substrate (Sheppard et al., 2004). Studies with Als1p showed that mutations in the Ig domain resulted in a loss of adherence (Loza et al., 2004). Also, adherence was significantly reduced when the TR region was deleted (Loza et al., 2004). Secondary structure analyses determined by Circular Dichroism (CD) spectrophotometry and Fourier Transform Infra–Red (FTIR) spectroscopy of the Als1p and Als5p N-terminal Ig domain confirmed that this region consists mostly of anti-parallel β-sheets, homologous to adhesins or invasins of the immunoglobulin superfamily (Hoyer & Hecht, 2001b; Sheppard et al., 2004). Consequently, the current working model of the Als domains in adhesion indicates that the Ig domain mediates adherence to substrate. The TR region is postulated to be cross-linked within the cell wall matrix and stabilizes the Ig-domain in the correct conformation for binding.

In this study I explored the functional and structural properties of the Als5p Ig domain and TR region. Heterologous expression in *S. cerevisiae* of an Als5p

mutant in which the TR region was deleted, showed a significant decrease in cellular aggregation compared to wild type Als5p cells. Soluble Als5p protein fragments containing the Ig domain and TC region alone (IG-TC) and the Ig domain, TC region and TR region (Ig-TC-TR) were purified and physically characterized through SDS-PAGE and dot blots for glycosylation. The TR region was determined to be *O*–glycosylated, in contrast to the Ig domain and TC region which is not glycosylated. Protein expression analysis showed that the TC region was important for protein secretion to the cell surface and outside of the cell. Binding assays revealed a drastic difference between Als5p Ig-TC-TR and Als5p Ig-TC protein fragments in binding to fibronectin (FN). CD spectroscopy of the Als5p Ig-TC–TR regions showed the TR region alters the secondary structural properties of the Ig domain through increasing the amount of α–helical structure and reducing the amount of β-sheet structure.

Materials and Methods

Construction of Plasmid Vectors. The previously described shuttle vector pGK114 (Gaur, Klotz, & Henderson, 1999) containing the *ALS5* ORF facilitated subcloning into the pYES2.1/V5-His-TOPO cloning kit (Invitrogen) (Figure 11). Plasmid pGK114 was used as template for PCR amplifications, and the various plasmid constructs to be made are shown in Table 3.

For plasmids pRL 02, 03, and 09 the corresponding sequences were amplified and ligated to the pYES vector. Following amplification in *E. coli* XL10 - Gold cells (Stratagene), plasmid DNA was extracted using the Qiagen plasmid extraction kit according to the manufacturer's instructions and analyzed by restriction enzyme digestion to verify correct insert orientation. Plasmids containing the correct *ALS5* insert orientation were sequenced, amplified in *E. coli*, and extracted using the Qiagen maxi prep kit. Plasmid DNA was used to transform *S. cerevisiae* W303-1B cells by the lithium acetate protocol (Gietz, Schiestl, Willems, & Woods, 1995).

Plasmid pRL11 is analogous to plasmid pRL02 with the exception of a factor Xa protease cleavage site preceding the first tandem repeat and the first 14 codons of C-Terminal S/T stalk are not included. Two separate PCR reactions were performed. First, the entire *ALS5* N–Terminal region was amplified and the 3' reverse primer contained the factor Xa site followed by the *Bgl II* endonuclease site. This fragment was ligated into the pYES vector to create plasmid pRL011. Plasmid DNA was amplified in *E.coli* and purified as described above. In the second reaction, The *ALS5* central TR region was amplified.

Figure 11. Plasmid pYes 2.1/V5-His-TOPO. Yeast/E.coli shuttle vector used for subcloning *ALS5* constructs. The pYES TOPO shuttle vector features the *GAL1* inducible promoter, sequences for replication in *E. coli* and *S. cerevisiae*, termination of transcription, ampicillin drug resistance, a uracil selectable marker, 6X His tag for metal-affinity purification, and a V5 epitope sequence for protein detection. Finally, a PCR cloning site with the topoisomerase I enzyme covalently bound to overhanging thymidine residues at the 3' ends of the vector is included.

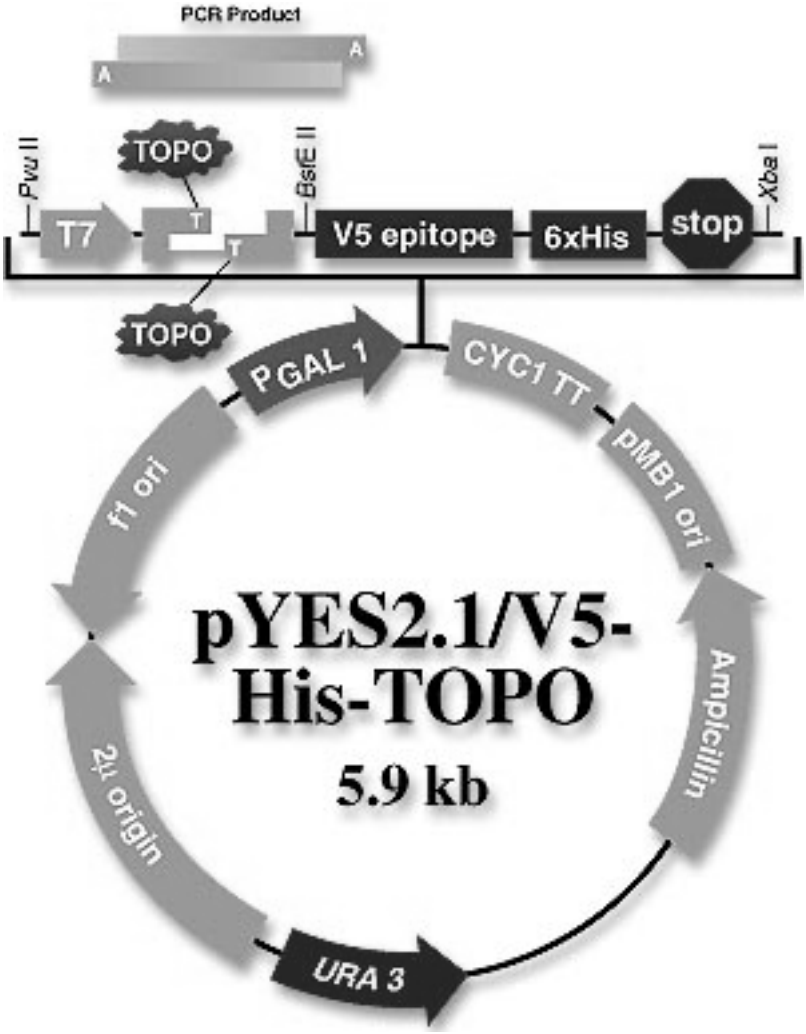
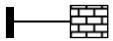
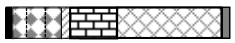
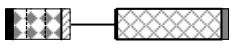
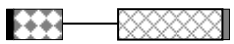

PCR Product
A
A
TOPO
Pvu II
BstE II
Xba I
T7
T
T
V5 epitope
6xHis
stop
TOPO
PGAL 1
CYC1 TT
f1 ori
pMB1 ori
pYES2.1/V5-
His-TOPO
5.9 kb
Ampicillin
2μ origin
URA 3

Table 3. Structure and status of Als5p constructs. *ALS5* plasmid constructs were prepared and protein constructs without the GPI anchored sequences were purified by Nickel chromatography. The description of the various Als5p domains is the same as in figure 1 with the following additions: (——) domain deletion, () Factor Xa protease cleavage site, S=constructs secreted into the growth medium, C=constructs localized onto the cell wall.

Plasmid Name	Designed protein	Plasmid Construction	Protein Expressed	Protein Purified
S-complete /pRL01		-	-	-
S-Ig-TC-TR/ pRL02		Complete	Secreted: 300 mg/L	**Yes**
S-Ig / pRL03		Complete	Intracellular	-
S-Ig-TC/ pRL09		Complete	Secreted:100 mg/L	**Yes**
S-TC-TR / pRL04		Complete	Intracellular	-
S-TC / pRL05		Complete	Intracellular	-
S-TR / pRL06		Complete	Intracellular	-
S-Ig-Tc-X-TR / pRL11		Complete	Secreted: 300 mg/L	**Yes**
C-complete		Complete	Cell surface	n/a
C-Ig-TC-stalk-anchor / pRL10		Complete	Cell surface	n/a
C-TR-Stalk-anchor / pRL08		Complete	Cell Surface	n/a
C-Ig-stalk-anchor / pRL07		Complete	Intracellular	n/a

The 5’ forward primer contained the *Bgl II* endonuclease site and the 3’ reverse primer contained the *BstE II* endonuclease site. Amplicons were ligated to pYES to create plasmid pRL 011’, and amplified as described above. Plasmid pRL 011’ was digested with *Bg II* and *BstE II* to release the ~670bp *ALS5* TR insert, and gel purified using the Qiagen gel extraction kit. Plasmid pRL011 was digested and the ~ 7.3kb *ALS5* Ig/vector fragment was gel purified as described above. The insert and vector fragments were combined via T4 ligase to create plasmid pRL11. All subsequent cloning steps followed the procedure described above.

To create plasmid pRL10 the *ALS5* N-Terminal Ig-TC region was amplified by PCR. The 5’ forward primer contained the *Eco RI* endonuclease site and the 3’ reverse primer contained a 6X Histidine tag for immunodetection followed by the *Bgl II* endonuclease site. Amplicons were ligated into pYES to generate plasmid RL010. In the second PCR reaction, the *ALS5* C–Terminal region containing the Ser/Thr rich stalk,GPI anchor, and stop codon was amplified. 5’ forward primers contained the *Bgl II* endonuclease site and the 3’ reverse plasmids contained the *Xho I* endonuclease site. Amplicons were ligated to pYES vector to form plasmid pRL010’. Plasmids pRL010 and pRL010’ were amplified in *E. coli* and purified. To generate a secretion signal, the chimeric shuttle vector pGL01 was used. This vector is a modification of plasmid pGAL1 423 (ATCC), containing an invertase secretion signal-α–agglutinin–GFP fusion product regulated by a Galactose promoter. The α–agglutinin/GFP fusion protein was shown to be localized at high levels on the yeast cell surface when induced by galactose (personal communication with Marlyn Gonzalez). pGL01 was digested

with *EcoRI* and *Xho*I to release the α–agglutinin/GFP fragment, and the ~7.0 kb vector fragment containing the p423GAL1 backbone and invertase secretion signal was gel purified. pRL 010 was digested with *EcoRI* and *Bgl II*, and the ~1.3kb product containing the *ALS5* Ig-TC fragment was gel purified. pRL010' was digested with *Bgl II* and *Xho I* to release the ~2.0kb *ALS5* C–Terminal fragment and gel purified. The three fragments were then ligated together and subsequent cloning steps followed the procedures described above.

Protein Expression and Purification of Als5p IG-TC-TRR (Als5p$^{1-664}$) and Ig-TC (Als5p$^{1-431}$) protein fragments. Protein expression was induced by growing *S. cerevisiae* cells containing the appropriate plasmid in csm-ura containing galactose as a carbon source until late stationary phase. Protein excretion into the culture supernatant was verified by dot blots as described below. Als5p cell culture supernatants were harvested (1 - 4L) and Phenyl Methyl Sulfonyl Fluoride (PMSF) was added to a final concentration of 10nM. The supernatant was concentrated to approximately 150 ml through a "Millipore" filtration apparatus having a 30kDa molecular weight cutoff (MWco). Concentrated supernatant was raised to pH 7.00 using 1M Tris Base, and Als5 proteins were chromatographed on a Nickel-NTA column (Qiagen) pre-equilibrated with 20mM Imidazole, 300mM NaCl, 50mM NaH_2PO_4, pH 7.00 buffer (Buffer A). The column was washed with Buffer A to remove non–specific proteins. Als5p protein fragments were eluted with 500mM Imidazole, 300mM NaCl, 50mM NaH_2PO_4, pH 7.00 buffer (Buffer B). The presence of Als5p protein fragments was determined by dot blots. Fractions containing Als5p protein

fragments were pooled and dialyzed exhaustively into 20mM NaH_2PO_4, 0.01%NaN_3, pH 7.00 buffer (Buffer C) using a 3,500 kDA MWco membrane. The sample was concentrated by further dialysis in Buffer C supplemented with 10% Poly Ethylene Glycol (PEG) 35,000 MW. Protein concentration was determined by Absorbance at 280 nm readings where an OD of 1.00 is equivalent to 1mg/ml of protein. Approximately mg/L quantities were produced for $Als5p^{1-664}$, $Als5p^{1-650}$ $^{/factorXa}$, and $Als5p^{1-431}$ cultures respectively.

Polyacrylamide gel electrophoresis. Als5p protein samples were electrophoresed in SDS polyacrylamide gels (10% running gel) and stained by Coomassie Blue dye.

Dot Blots of Als5p proteins. Als5p protein samples were immobilized on nitrocellulose membranes and allowed to dry. Membranes were blocked for 1 hour using Phosphate Buffered Saline buffer with 5.0% w/v dry milk (PBSTM :137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.1% Tween-20, pH 7.0) and washed with PBST buffer three times. Next, the membrane was probed for 1 hour using Anti-His6x or Anti–V5-peroxidase-conjugated antibodies (Invitrogen) at a working concentration of 1:5000 and washed. Proteins were detected by incubating the membranes with equal parts of SuperSignal West Pico Stable Peroxide and Super Signal West Pico Luminol/ Enhancer Solution solutions (Pierce). The membrane was exposed to film and developed using the X-OMAT developing apparatus.

Flow Cytometry and Indirect Immunofluorescence Assays. Immunofluoresce assays (IFA) were performed as previously described in

chapter 2. For flow cytometry, antibody–labeled cells were placed into a FACScan (Becton Dickinson) for single color analyses. Flow cytometry data were analyzed using Cell Quest Pro.

Adherence assays. Adherence assays were performed as described in chapter 2. Briefly, *S. cerevisiae* cells containing pGK114 or pRL10 were grown with shaking at 30°C in YPGal medium (10 g of yeast extract per liter, 20 g of peptone per liter, and 20 g of galactose per liter) to the stationary phase for Als5p expression. Cells were harvested and washed with Tris-EDTA (TE) buffer (pH 7.0) three times and then resuspended in TE buffer. In a glass tube (13 by 100 mm) Als5p-expressing cells were mixed with fibronectin (FN)-coated magnetic beads at a cell-to-bead ratio of 100:1 in TE buffer, briefly vortexed, and incubated at room temperature with gentle shaking for 30 to 45 min. Each tube was vortexed briefly and immediately placed into a magnetic separator (Dynal). Adherent and aggregated cells were gently washed three times with TE buffer while the tube remained within the magnet. The cells were resuspended in TE buffer, and a sample was placed onto a microscope slide for examination.

Cells were viewed with a Nikon Optiphot-2 microscope equipped with a Sony DK-5000 camera. For assays in which the effects of additives on adhesion were investigated, additives were added to the cell-bead mixture at the onset of the adherence assays.

Analysis of protein glycosylation. Dot Blots were performed as described above with the exception that blots were probed with 0.5μg/ml Concanavilin A in PBSTM buffer supplemented with 10μM $CaCl_2$ and $MnSO_4$.

Circular Dichroism (CD) Spectroscopy. Far UV spectra of Als5p$^{1-664}$ were recorded using an AVIV spectropolarimeter model 215 instrument using quartz, thermo–regulated cuvettes (HELLMA) with a 0.1 cm path length. The spectra were corrected by subtraction of the 20mM NaH_2PO_4 buffer baseline spectra. CD data was analyzed by the self–consistent method with the SELCON program (Sreerama & Woody, 1993).

Binding Assay for Soluble Als5p protein fragments. In a polystyrene 96-well plate, a 1mg/ml solution of fibronectin (Sigma) was serially diluted across the row so that each column has a different dilution of antigen (Dilution Range = 1mg/ml–10ng/ml in 10 fold increments). All samples were analyzed in duplicate. Each well contained a volume of 50μl and the plate was incubated at room temperature for 2hours or overnight at 4°C. Next, the wells were washed three times with PBST buffer, blocked for one hour with 200 μl of PBST buffer supplemented with fresh native Bovine Serum Albumin (BSA) 1mg/ml and washed. 15μg of the designated Als5p protein fragment was added to the wells and incubated for two hours at room temperature, and washed. The wells were then incubated with primary Anti–V5 antibodies at a working concentration of 1:5000 for two hours, washed, and incubated with secondary anti–mouse alkaline phosphatase conjugated antibodies at a working concentration of 1:10,000 for 1 hour. Finally, after a final wash, 50 μl of a pNPP (para – Nitrophenylphosphate 5mg, 1M diethanolamine, 0.5 mM $MgCl_2$, buffer pH 9.80) solution was added to the wells and the plate was allowed to incubate for 30 min. Development of a yellow color indicated that the Als5p proteins bound to

fibronectin. Negative Controls for the following categories were included: cross reactivity between BSA and primary and secondary antibodies, non–specific binding of Als5p and BSA, non–specific binding of Als5p to the secondary antibodies, and the pNPP substrate constituted as a “blank”. A positive control monitoring the interaction between Als5p and the primary and secondary antibodies was also included.

Quantification was performed by obtaining Optical Density readings at 420nm using a microplate reader after 30 minutes of color development. Data analysis was performed with the Sigma Plot 7.0 and Excel software. The binding data for Als5p proteins was normalized based on absorbance of the Als5p binding to the primary and secondary antibodies.

Results

Construction and Purification of the Als5p Ig-TC and Ig-TC–TR domains.

In previous studies the Als5p Ig domain ($Als5p^{18-329}$) was purified in a *Pichia pastoris* - based expression system (Hoyer & Hecht, 2001b). Here we sought to produce and purify the various Als5p domains. Our initial attempts to purify Als5p protein fragments using *P. pastoris* were unsuccessful. Therefore, purification was performed in a *S. cerevisiae*–based expression system. *ALS5* gene fragments were amplified by PCR, and subcloned into the pYES shuttle vector to create plasmid pRL02, pRL09 and pRL11 respectively (Table 3). $Als5p^{1-431}$, $Als5p^{1-664}$, and $Als5p^{1-650\ /factorXa}$ protein fragments were harvested from yeast culture supernatants and purified by Ni-NTA column chromatography. Dot blots using anti-V5–HRP or anti-His6X antibodies verified Als5p protein secretion (Figure 12A and B). Als5p protein fragments were further resolved on SDS – Polyacrylamide gels (Figures 13).

The *ALS5* TC region is critical for protein secretion. Notably, in contrast to the successful purification of $Als5p^{1-431}$ and $Als5p^{1-664}$, the $Als5p^{1-329}$ protein fragment was not secreted into the culture supernatant (Table 3). Protein production was determined following lysis of $Als5p^{1-329}$ – expressing yeast cells and performing dot blot analyses on the lysate fraction (Compare figure 12B to figures 14A and B). In addition, a deletion mutant of Als5p that lacked the TC and TR (plasmid pRL07) regions did not localize the protein on the cell wall (Table 3), and dot blots of lysed pRL07-expressing cells indicated the protein was being translated (data not shown). In contrast, reinsertion of the TC region (plasmid

Figure 12. Dot blot analysis of Als5p protein fragments. Growth culture media of *S. cerevisiae* expressing Als5p protein fragments was harvested and Als5p protein fragments purified by Ni-NTA chromatography. Proteins secretion was determined by probing blots with anti-His6X antibodies at 1:5000 working concentration. (A) Als5p $^{1\text{-}664}$ (Ig/TC/TRR) elution fractions 6-12. (B) Als5p $^{1\text{-}431}$ (IG/TC) elution fractions 5-9. Bovine Serum Albumin (BSA) 1mg/ml is used as a negative control and purified α–agglutinin is used as a positive control. 2ul of each designated protein sample was spotted onto the nitrocellulose membrane.

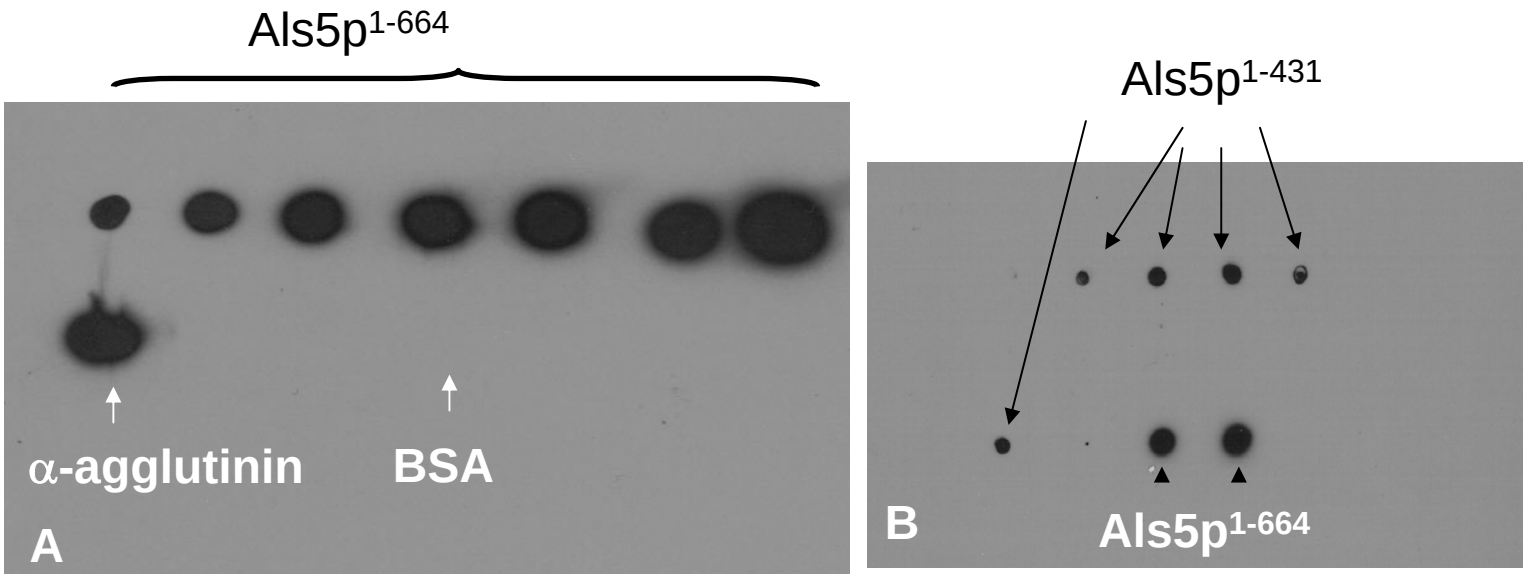

Als5p1-664
α-agglutinin
BSA
A
Als5p1-431
B
Als5p1-664

Figure 13. SDS PAGE analysis of Als5p IG-TC-TR protein fragments. Purified Als5p$^{1-664}$ was electrophoresed on a 10% SDS-PAGE gel followed by staining with Coomassie Blue. Lane 1: Low Molecular weight protein standards were as follows: phosphorylase b, 97,400; bovine serum albumin, 66,200: ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400; Lane 2. Als5p$^{1-664}$ with an apparent molecular mass of >97,400. Note the expected molecular mass is ~ 70kda. The slower migration is due to glycosylation of the TC and TRR regions.

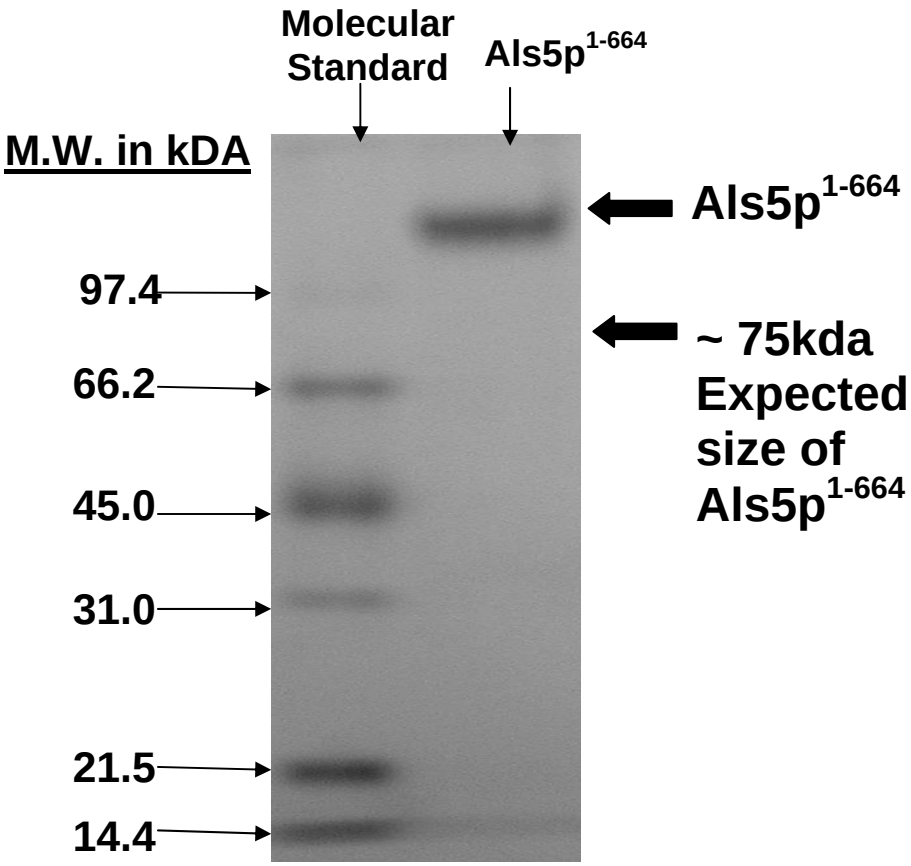
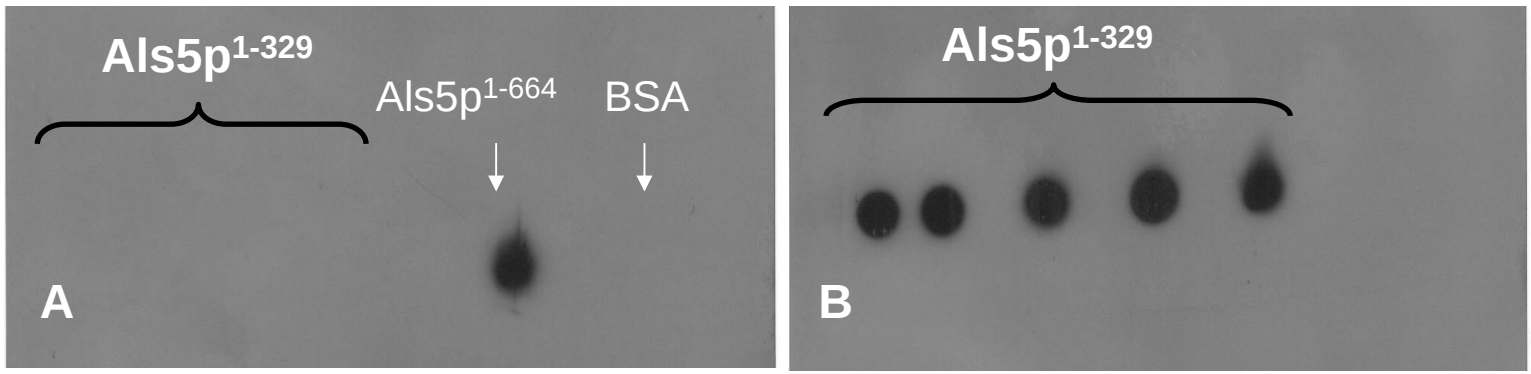

Molecular Standard
Als5p1-664
M.W. in kDA
Als5p1-664
~ 75kda Expected size of Als5p1-664
97.4
66.2
45.0
31.0
21.5
14.4

Figure 14. Protein secretion analysis of Als5p$^{1-329}$ (Ig) - expressing cells. *S. cerevisiae* Als5p$^{1-329}$ (Ig) - expressing cells were harvested following growth in YPGal media. (A) Dot Blot of growth media supernatant for Als5p$^{1-329}$-expressing cells. (B) Blot of Als5p$^{1-329}$ cellular lysate fraction. Approximately $3x10^{10}$ cells were washed, resuspended in 25ml of 1X PBS, and vortexed with glass beads for cell lysis. The lysate was separated from the cellular debris by centrifugation prior to spotting on the nitrocellulose membrane. 1mg/ml BSA is used as a negative control and Als5p$^{1-664}$ is used as a positive control. Blots were probed with anti-His6X antibodies at 1:5000 dilution. 2ul of designated protein sample was spotted onto the nitrocellulose membrane.



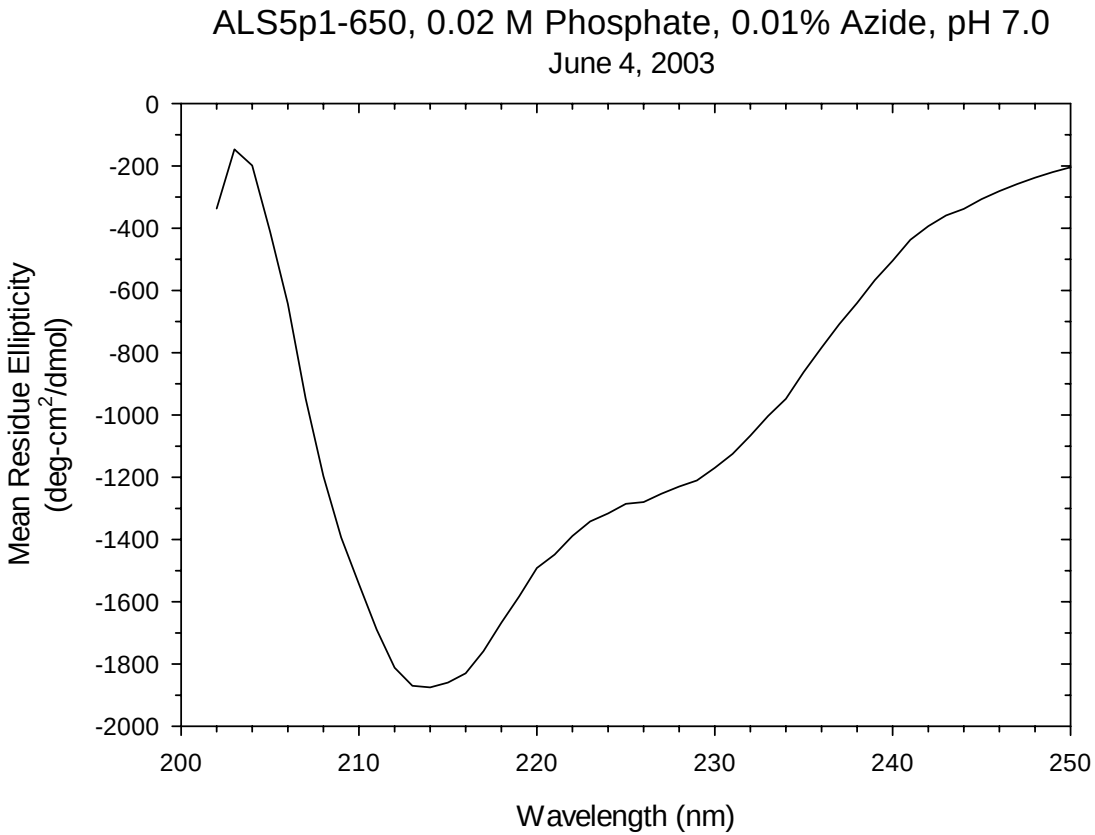
Growth Medium **Cellular Lysate**

pRL10) to this mutant showed the protein was successfully localized on the cell wall (Table 3). Therefore, the TC region is required for proper protein secretion in *S. cerevisiae*.

Structural Characterization of the Als5p N-terminal and Central domains. CD Spectroscopy showed the Als5p$^{1-329}$ protein fragment to be composed of mostly anti–parallel β–sheet with very little α–helical content, and mass spectrometry demonstrated that the protein was not glycosylated (Hoyer & Hecht, 2001b). Additionally, CD spectra of the Als1p$^{1-432}$ N-Terminal protein fragment revealed structural elements rich in anti–parallel β-sheets (Sheppard et al., 2004). In order to understand how the TR region contributes to Als5p structure, CD spectra were obtained for Als5p$^{1-664}$ proteins (Figure 15). SELCON analysis of the spectra yielded the following values: 24% α – helix, 13.9% β – sheet, 13.7% Turns, and 41% unstructured regions.

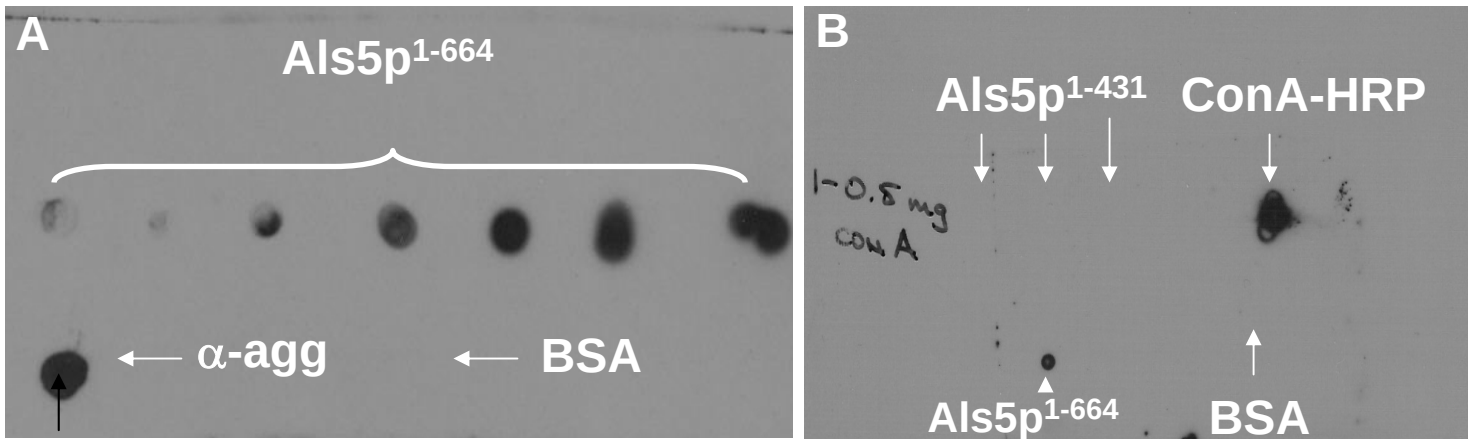
SDS–PAGE analysis of Als5p$^{1-664}$ shows the protein migrates approximately 30KD higher than the predicted size of the protein (Figure 13). Since no *N*–Glycosylation sites exist within Als5p$^{1-664}$, we proposed that Als5p$^{1-664}$ must be *O*-Glycosylated. Dot blots using Concanavilin A–HRP as a probe showed Als5p$^{1-650}$ to be *O*-glycosylated (Figure 16A). Glycoslyation was specifically localized to the TR region, since blots with Als5p$^{1-431}$ indicated this region was not glycosylated (Figure 16B) and SDS PAGE analysis of Als5p$^{1-431}$ showed that the protein migrated at the predicted molecular weight (data not shown). These results are in agreement with previously published results (Hoyer & Hecht, 2001).

Figure 15. Circular Dichroism (CD) spectra of Als5p$^{1-664}$. Far UV Spectra of Als5p$^{1-664}$. CD Spectra were deconvoluted by the SELCON method and secondary structural percentages are seen in the inset table. Deconvolution data from Hoyer et al 2001 are in the bottom row.



Structures	Helix	Sheet	Turns	Unordered	Source
Percentages	24.0	13.9	13.7	41.2	Ig-TR (Rauceo 2003)
	4	60	21	23	Ig (Hoyer 2001)

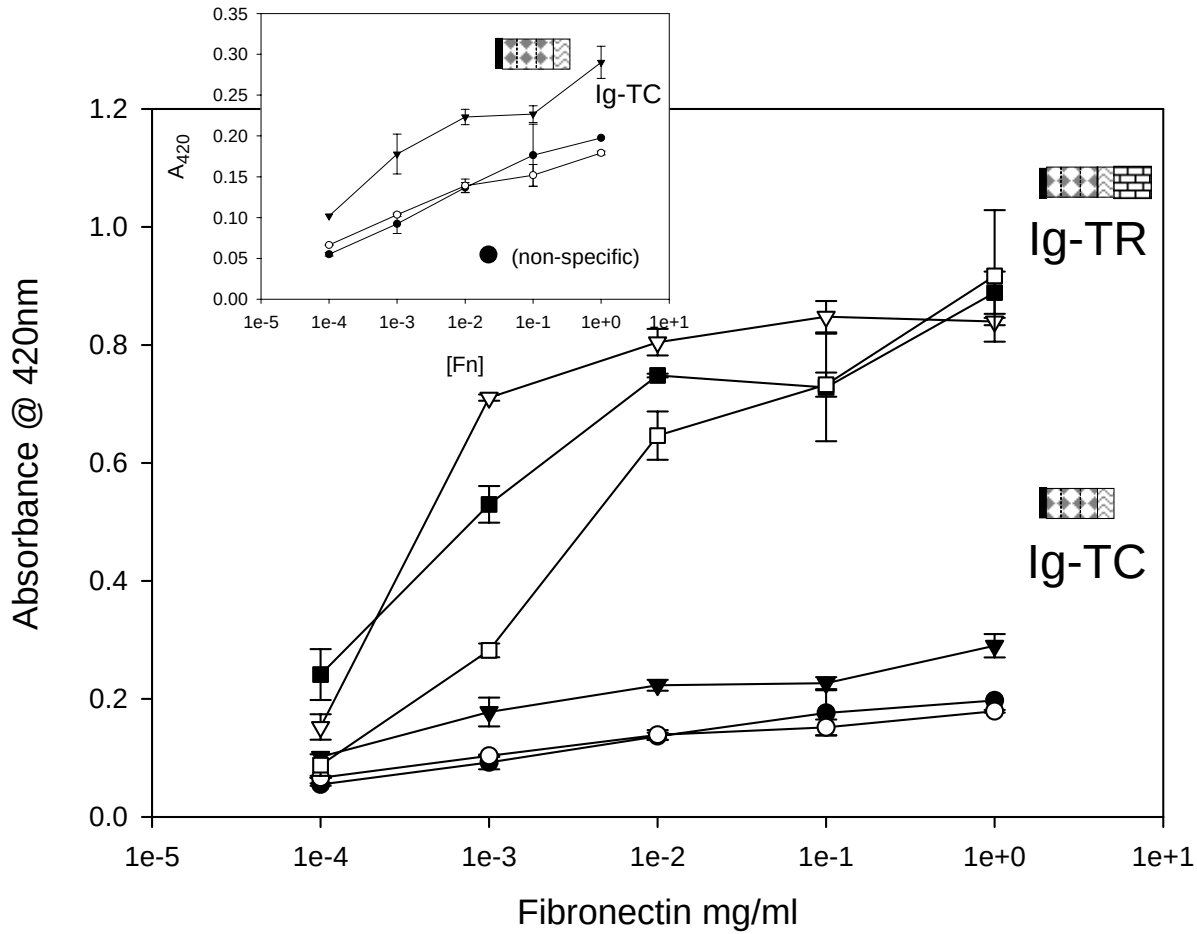
Figure 16. Glycosylation Analysis of Als5p protein fragments. Dot Blots were probed with 0.5μg/ml Concanavilin-A conjugated to horse radish peroxidase antibodies. (A) Dot Blot of Ni-NTA purified Als5p$^{1-664}$ elution fragments 6-10. 1mg/ml BSA is used as a negative control and α–agglutitnin is used as a positive control. (B) Als5p $^{1-431}$ elution fragments 5-9. BSA is used as a negative control and Als5p$^{1-664}$ and Concanavilin A are used as positive controls. 2ul of designated protein sample was spotted onto the nitrocellulose membrane.


A
Als5p1-664
α-agg
BSA
B
Als5p1-431
ConA-HRP
1-0.5 mg
ConA
Als5p1-664
BSA

Binding of Als5p IG-TC and Als5p IG-TC-TR protein fragments to fibronectin. The drastic differences observed in secondary structural properties of Als5p $^{1-431}$ and Als5p $^{1-664}$ implies that there may be significant differences in function. In a modified ELISA test where Als5p protein fragments were incubated with FN coated wells, Als5p$^{1-664}$ bound to fibronectin with apparent nM affinity and approximately 10-fold greater than Als5p$^{1-431}$ (Figure 17). All relative negative controls for the categories listed in methods showed absorbance values that were not statistically different from "blank" baseline values. Positive controls assessing the binding between Als5 protein fragments and antibodies showed a 1.0-1.5 fold difference for Als5p$^{1-664}$ compared to Als5p$^{1-431}$, therefore indicating that this result was not due to better cross reactivity of anti-V5 antibodies with Als5p$^{1-664}$. The presence of the factor Xa cleavage site did not affect activity, since similar results were obtained using Als5p$^{1-650\ /factorXa}$ (data not shown). Taken together, these results show that the Als5p Ig-TC -TR regions bind to fibronectin better than the Ig-TC regions alone and the TR region modifies Ig domain function.

Mutant *ALS5* proteins are localized on the cell surface. In order to further understand the role of the Als5p domains in mediating adhesion and cell–cell aggregation, a cell surface construct containing a deletion of the TR region (pRL10/Als5pΔTR) was prepared. Plasmid pRL10 was transformed into *S. cerevisiae* W303–1B cells and *ALS5* expression was induced by growth in YPGal. Protein surface localization was monitored by immunofluorescence microscopy.

Figure 17. The interaction of Als5p protein fragments with Fibronectin. Fibronectin was incubated into microtiter wells at various concentrations followed by incubation with $Als5p^{1-431}$ (black circles, black triangles, and white circles) or $Als5p^{1-664}$ (white triangles, and black and white rectangles), anti-V5 antibodies and anti mouse alkaline phosphatase conjugated antibodies. PNP was added to the wells to verify binding. Absorbance values were recorded on a microplate reader and the data was exported into Sigma Plot for analysis. Data from three independent experiments was plotted. Experiments were performed in duplicate. Error bars represent the range for duplicate samples.


Absorbance @ 420nm
Fibronectin mg/ml
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1e-5
1e-4
1e-3
1e-2
1e-1
1e+0
1e+1
Ig-TR
Ig-TC
A420
[Fn]
(non-specific)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35

Micrographs revealed uniform fluorescence around the cell surface for both Als5p (ΔTR) and (wt) cells (Figure 18A-D).

Als5p cell to cell aggregation in the absence of the tandem repeat region. To determine the phenotype of the Als5p (ΔTR) cells, adhesion assays were performed. Als5p (ΔTR) cells adhered to FN coated beads similarly to Als5p (wt) cells (Figure 19A). However, cell to cell binding was drastically reduced for Als5p (ΔTR) cells compared to Als5p (wt) cells (Figures 19B-D). Post-treatment of cellular aggregates with anti-Als1p antibodies revealed similar levels of protein on the cell surface (Figures 19 E and F). Therefore, the Tandem Repeats significantly contributes to Als5p mediated cellular aggregation.

Figure 18. Cell surface localization of Als5p (ΔTR)-expressing cells. Anti-Als1p N-Terminal domain antibodies were used to assess Als5p (ΔTR) cell surface localization. (A) Cell surface fluorescence of Als5p-expressing *S. cerevisiae* cells. (B) Bright Field micrograph of the cells seen in panel A. (C) Cell surface fluorescence of Als5p (ΔTR) -expressing cells. (D) Bright Field micrograph of the cells seen in panel C.

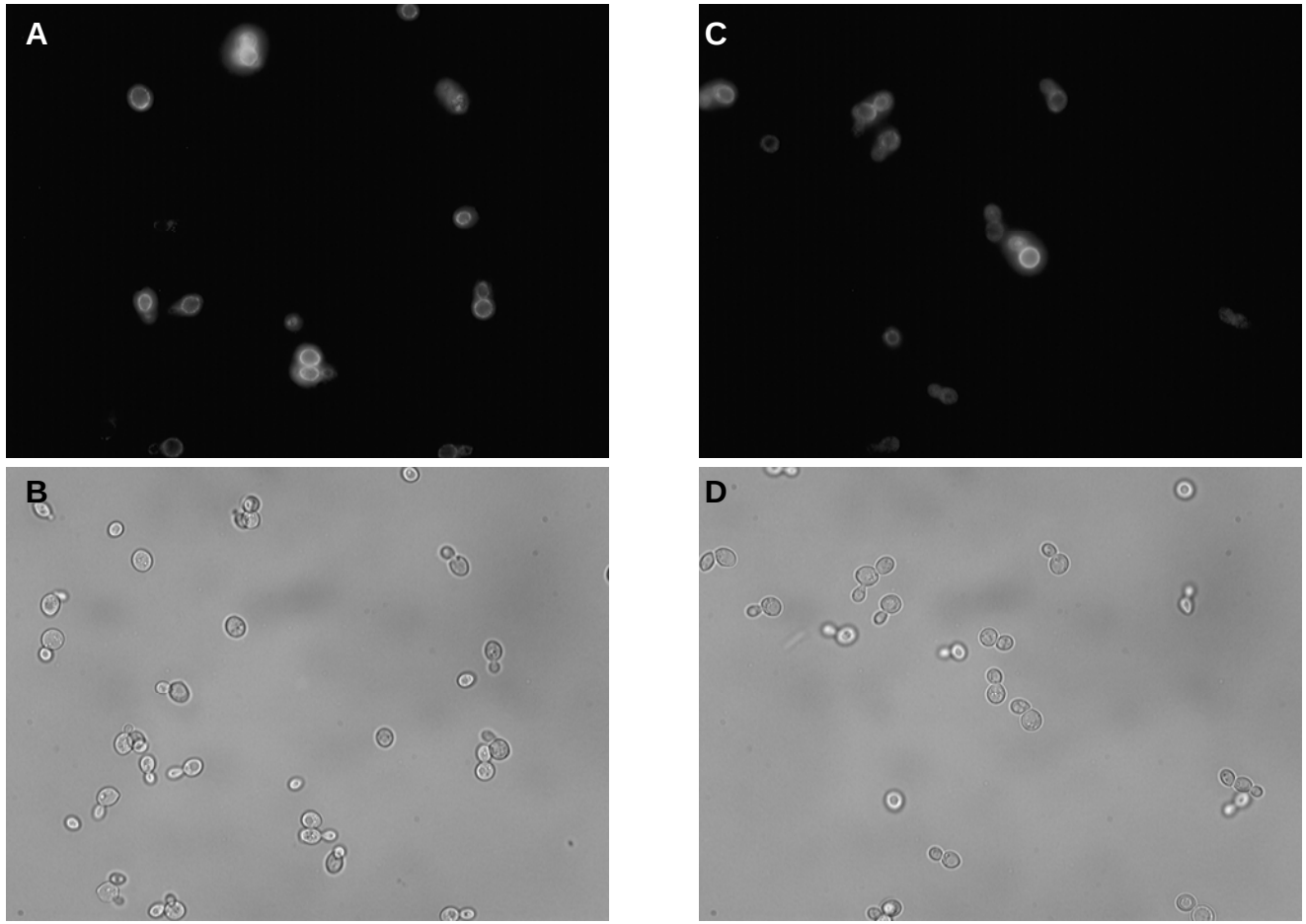
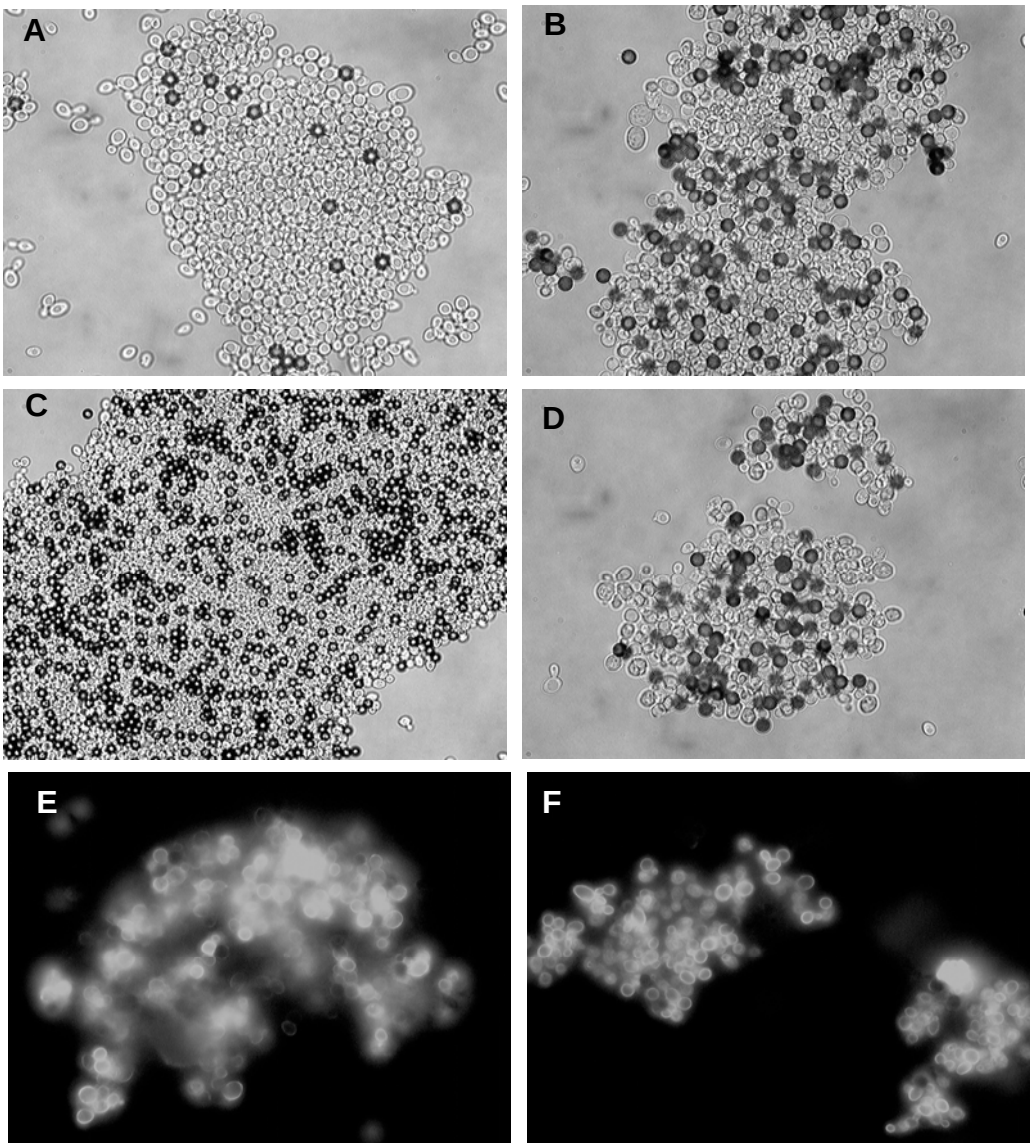

A
C
B
D

Figure 19. Adhesion Assays of Als5p (ΔTR)-expressing cells. (A) Als5p (wt)-expressing cells. (B-D) Als5p (ΔTR)-expressing *S. cerevisiae* cells. (E) Cell surface fluorescence of Als5p (ΔTR) aggregates. (F) Cell surface fluorescence of Als5p (wt) aggregates.


A
B
C
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E
F

Discussion

Biophysical properties of Als5 Ig-like domain and Tandem Repeat Region.

Mature Als proteins migrate significantly slower than their predicted molecular weight on SDS–polyacrylamide gels, therefore implicating that these proteins are highly glycosylated (Kapteyn et al., 2000). CD and Mass spectroscopy confirmed Als5p$^{18-329}$ (Ig domain) is not glycosylated (Hoyer & Hecht, 2001b). Primary sequence analysis of Als5p indicates an elevated number of threonine residues in the TC region, TR region, and serine and threonine residues in the C–Terminus. Since there are not any *N*-Glycosylation sites, these regions may be heavily *O*-Glycosylated and will result in protein migration at a higher molecular size than predicted. In this study, Als5p protein constructs containing the N-Terminal Ig domain and TC region (Ig-TC/Als5p$^{1-431}$) and the Ig domain, TC region, and central TR region (Ig-TC-TR/Als5p$^{1-664}$) were produced. SDS PAGE confirmed successful purification of Al5p$^{1-431}$ and Als5p$^{1-664}$. Als5p$^{1-431}$ migrated at the predicted molecular weight of 50kda (data not shown). In comparison, Als5p$^{1-664}$ migrated at a higher molecular weight (>100kda) than the predicted size of 76kda, thus suggesting that the TR region is *O*-Glycosylated.

The protein Concanavilin A binds to mannose carbohydrate moieties. In order to directly demonstrate that the TR region is *O*-glycosylated, dot blot analysis of Als5p$^{1-431}$ using Concanavilin A -HRP showed that this region is not glycosylated despite the presence of the TC region, of which, 35% are threonine residues. On the other hand, similar tests with Als5p $^{1-664}$ confirmed this region is

glycosylated, thereby demonstrating that the TR region is glycosylated and the Ig-domain and TC region is not. The presence of glycosylation in the TR region may serve in protecting the TR region from proteolysis, or aid in the adhesive nature of Als proteins. Indeed, cleavage of Als5p$^{1\text{-}650/FactorXa}$, where the factor Xa protease cleavage site is between the TC region and TR region was unsuccessful (data not shown). In addition, Als proteins containing a greater number of repeats such as Als1p and Als3p are more adhesive and are involved in tissue invasion and cellular colonization (X. Zhao et al., 2004).

The *ALS5* TC region is important for protein secretion. The initial isolation and primary sequence profiling of *ALS5* described the region in between the Ig domain and TR region as the Threonine Conserved (TC) (Gaur & Klotz, 1997). However, models of Als proteins include the TC region as part of the Ig domain despite the fact that this region does not display structural or sequence similarity to immunoglobulin domains (Sheppard et al., 2004).

Previous studies using a *Pichia pastoris* expression system showed that Als5p residues 1-329 (Ig) are sufficient for protein excretion outside of the cell (Hoyer & Hecht, 2001b). Expression of the identical Als5p sequences in *S. cerevisiae* was unsuccessful, and protein expression was only verified following cell lysis. This result was consistent in an initial attempt at producing an Als5p cell surface mutant which lacked the TC and TR regions (plasmid pRL07/ΔTC-TR). Cells expressing pRL07 did not localize Als5p on the cell surface (Table 3). Only after modification of the vector with the addition of the TC region (plasmid pRL10/ΔTR), was protein surface localization observed (Table 3 and Figure

18C). Since Als5p was successful in being exported outside of the cell in both cases where the TC region was present, we conclude that the TC region is important for protein excretion by most likely enabling the protein fragment to form a properly folded tertiary structure.

Secondary Structural profile of the Als5p Ig-like domain and Tandem Repeat region. The initial delineation of the name Agglutinin like sequence to ALS proteins was based on the observation that the N-terminal region contains sequence similarity to the *S. cerevisiae* adhesin α-agglutinin N-terminal Immunoglobulin-like domain. Such sequence similarities predict structural similarities. Indeed, secondary structural analysis of Als5p and Als1p Ig-domains confirmed that this protein fragment is composed mainly of anti-parallel β-sheets and very little α-helix, consistent with Immunoglobulin proteins (Hoyer & Hecht, 2001b; Sheppard et al., 2004). Additionally, molecular models predict that the N-terminal region of other *ALS* family members form Ig domains (Sheppard et al., 2004). Secondary structural predictions suggest that the Thr-rich tandem repeat region adopts an extended conformation comprised primarily of extended β–sheets connected by unstructured regions with significant hydrophobicity at the surface. The sequence composition is described as “low complexity”, meaning that a well-defined three-dimensional structure is not present. This feature is similar to the native forms of casein, clusterin and “molten globule” intermediates (Bailey, Dunker, Brown, Garner, & Griswold, 2001).

CD analysis of Als5p$^{1\text{-}664}$ indicated that the protein contains a higher percentage of alpha helical structure than beta sheet structures. The high

percentage of α–helix structure was significantly higher than the 4% value reported for spectra on Als5p$^{1-329}$. Also, there was a significant decrease in β–sheet from the Als5p$^{1-329}$ reported value of 60% (Hoyer & Hecht, 2001b). Finally, 41% of Als5p$^{1-664}$ was unstructured. These results show that the Als5p TR region drastically affects the overall structure of Als5p.

The Als5p Tandem Repeat region significantly enhances binding to Fibronectin. The *ALS* central tandem repeat region is rich in threonine and β-branched residues. This sequence composition is found on cell surfaces of several pathogenic organisms, mucin proteins, and in *S. cerevisiae* are common on flocculin (FLO) proteins. Expression of Flo11p causes the formation of cellular aggregates in the presence of divalent cations (Lo & Dranginis, 1996, , 1998). We hypothesized that the TR region in *ALS5* may mediate cellular aggregation, and deletion of the tandem repeats would be accompanied by a loss of cellular aggregation. Our strategy was to combine biochemical and cellular analyses to characterize the functional aspects of the TR region.

Previous studies demonstrated purification of Ig-like domains of Als1p and Als5p respectively. Subsequent analyses focused on determining the secondary structural characteristics as well as constructing predictive three –dimensional models (Hoyer & Hecht, 2001b; Sheppard et al., 2004). However, there are no reports currently available that demonstrate functional behavior of Als proteins in solution. The biophysical differences between Al5p$^{1-431}$ and Als5p$^{1-664}$ suggest there may also be functional differences. Microplate assays show that Als5p$^{1-664}$ binds to immobilized fibronectin with affinity at least 10-fold greater than Al5p$^{1-431}$

(Figure 17). Binding curves with Als5p$^{1-664}$ show that saturation was not observed although the concentration of Als5p$^{1-664}$ exceeded the amount of immobilized fibronectin. Thus, suggesting that Als5p$^{1-664}$ may be binding to both fibronectin and other Als5p molecules (Figure 17). On the other hand, this result is not observed using Als5p$^{1-431}$ at equal concentrations. Taken together, these results show that under well-defined experimental conditions the Als5p TR region significantly enhances binding to fibronectin.

Als5p cell-surface expression in *S. cerevisiae* mediates adhesion to ligand followed by a conformational shift propagated over the cell surface to form cellular aggregates. In studies using chimeric Als5p-Als6p constructs, adhesion was localized to the N-terminal Ig domain (Sheppard et al., 2004). Mutations in the N-terminus of Als1p caused a loss of adherence to endothelial cells, and there was a 93% loss of adherence to endothelial cells compared to wild type Als1p upon complete deletion of the Als1p tandem region, thus demonstrating that Als1p-mediated adherence is localized in the N-terminal domain (Loza et al., 2004). Furthermore, the tandem repeats were postulated to be cross linked in the cell wall matrix in order to keep the Ig domain accessible to interact with various substrates. In these experiments, adherence was reported by counting the number of colony forming units (CFUs) following incubation of yeast with endothelial cells and the addition of YPD agar. As a consequence of this experimental design, it is unknown whether cells formed cellular aggregates. Nevertheless, the high sequence homology between Als1p and Als5p Ig domain suggests similar results would be expected in Als5p. Here we report on the

effects of deleting the tandem repeat of Als5p on cell–cell aggregation. Als5p mutants were detected on the cell surface by indirect immunofluorescence. Micrographs of aggregation assays showed that Als5p (ΔTR) cells adhered to FN–coated beads similar to wild type Als5p-expressing cells, however cell to cell aggregation was significantly reduced. Thus, our results show that deletion of the TR region causes a reduction in cell-cell interactions instead of a total eradication as postulated. The TRs may contribute to the formation of stable cell-cell aggregates through by serving as a binding site for other Als5p molecules. Future binding experiments using purified TRs are needed to verify this hypothesis.

Collectively, this study presents a successful attempt to characterize the physical properties and analyze the functional role of the Als5p Ig-like domain and TR region respectively. Binding tests and structural analyses demonstrates how the TR region influences Als5p structure and function. Our results are in agreement with previous studies in that the Ig domain mediates adherence to substrate. On the other hand, our findings show the TR region is directly involved in adhesion and aggregation. Future studies will test the binding activity of purified Als5pTR protein fragments. These findings are important as it provides valuable insight for a region conserved across the ALS family that has not been studied as well as Thr-rich sequences found in other pathogenic organisms.

Chapter IV

Conclusions and Summary of Results

Summary of Results

A yeast heterologous expression system was used to study the functional and structural characteristics of the *Candida albicans* adhesin Als5p. The usage of *Saccharomyces cerevisiae* as a host was based on the premise that there are no *ALS* functional homologs in *S. cerevisiae*. Therefore, the functional characteristics of Als5p could be studied exclusively without the interference of other Als proteins through studies using *C. albicans*. Cellular and biochemical assays determined the mechanism of Als5p mediated adhesion and cellular aggregation.

Indirect Immunofluorescence (IFA) and antibody inhibition assays verified that Als5p mediated adherence to fibronectin and cellular aggregation was a direct consequence of Als5p activity.

Als5p-expressing *S. cerevisiae* cells bound to fibronectin coated beads, initiating an Als5p-structural change to form cellular aggregates. Treatments with various types of inhibitors demonstrated that Als5p-mediated aggregation occured independent of metabolic activity. The adherence to ligand beads and cellular aggregation were similar for non-treated control cells and for cells incubated with cycloheximide, sodium azide, AMPNP, staurosporine, or okadaic acid, thus indicating that aggregation occurs independently of protein synthesis and metabolic activity.

Adhesion assays in which Als5p expressing cells were pretreated with the fluorescent dye ANS show reduced cell-cell aggregation, coupled with an increase in fluorescence for aggregation-induced cells compared to non-

aggregating cells and untransformed *S. cerevisiae* cells. These results indicate that an increased surface hydrophobicity accompanies aggregation. To determine that the increase in fluorescence was not a consequence of selecting the highest expressing Als5p cells these experiments were performed using *C. albicans* cells and similar results were obtained. Additionally, the amyloid-staining dye Congo Red bound to Als5p expressing *S. cerevisiae* cells and inhibited aggregation. This observation is consistent with previous published studies with amyloid proteins.

Cell surface birefringence is clearly observed with Als5p expressing cells in an aggregated state, and is much greater than birefringence of expressing cells in a non-aggregated state, cells that are not expressing Als5p, and cells treated with ANS or Congo Red. These results were consistent with the idea that Als5p undergoes a conformational shift to form ordered domains on the cell surface in cellular aggregates and are observed.

Als5p protein fragments containing the N-terminal Ig-domain and central TC /TR regions (Als5p$^{1\text{-}664}$ and Als5p$^{1\text{-}650\text{factorXa}}$) and an Als5p protein fragment containing the Ig-domain and TC region (Als5p$^{1\text{-}431}$) were constructed. These proteins were purified in milligram quantities. Biophysical analyses showed that the TR region is *O*-glycosylated and the Ig domain and TC region are not. Glycosylation may serve as a means of protecting the TR region from proteolytic cleavage. We have identified that the *ALS5* TC region residues 330-431 are important for protein secretion.

Previously published CD spectra of the Als5p$^{1-329}$ Ig-domain protein fragment showed predominantly β–sheet structure with relatively very little α–helices. Secondary structural analysis of Als5p$^{1-664}$ using CD spectroscopy revealed that a significant amount of α–helical and β-sheet structures. Selcon deconvolution analysis of CD spectra showed Als5p$^{1-664}$ was composed of 24% α–helix, 13.9% β–sheet, 13.7% Turns, and 41% unstructured. The increase in –α-helix structure is not due to the presence TR region alone, since there are not enough amino acids to account for the 6-fold increase in α-helix. Therefore, these results show that the TR region alters the Als5p structure.

Als5p$^{1-664}$, Als5p$^{1-431}$, and Als5p$^{1-650factorXa}$ bound fibronectin in a microplate binding assay. Quantitatively, Als5p$^{1-664}$ and Als5p$^{1-650factorXa}$ bound to fibronectin 10-fold greater quantities than Als5p$^{1-431}$. Thus the TR region significantly enhanced Als5p binding to fibronectin.

A mutant Als5p cell-surface variant containing the N-terminal region and C-Terminal cell wall anchorage domains (Als5p/ΔTR) has been successfully expressed in yeast. IFA analysis showed localization of the protein on the yeast cell wall. Aggregation assays showed that Als5p (ΔTR)-expressing cells bind to fibronectin-coated beads similar to Als5p (wt) cells, but the cell-cell aggregation phenotype is reduced. Localization of ligand binding to the Als5p Ig domain is in agreement with published results using Als1p.

These findings are important for several reasons. First, through cellular analyses I have shown that following adhesion to protein ligands, Als5p undergoes a structural transition to form multicellular aggregates independently

of metabolic activity or protein synthesis. This process is global, in that the conformational change of Als5p propagates over the entire cell surface and is accompanied by an increase of cell surface hydrophobicity and ordered domains. The behavior similarities of *S. cerevisae* expressing Als5p and *C. albicans* expressing multiple Als proteins strongly implies that conformational switching mediates fungal adherence and colonization in pathogenesis. The localization of adhesive function in the Ig domain is consistent with previous studies in Als1p and Als6p, therefore illustrating conserved function between ALS family members. Second, through biochemical and biophysical characterization of the Als5p Ig-domain and TR region, it is evident that the TR significantly enhances binding to ligand and modifies the structure of the Ig domain, therefore demonstrating a role for a sequence motif that has been poorly characterized amongst the Als proteins.

Chapter V

Supplementary Information on Als5p structure and function

Through cellular assays and binding assays using soluble Als5p protein fragments, the properties ofAls5p mediated cellular adhesion and subsequent cellular aggregation was determined, and the role of the Ig domain and TR region in Als5p function was explored. Throughout the course of developing and performing the bioassays presented, there were several notable observations which may provide insight and evidence to validate our ideas and increase our understanding of the structural and functional relationships of Als5p.

Construction of an *ALS5* mutant library

A key component in our strategy to determine Als5p structure and function was to synthesize numerous *ALS5* domain deletion mutants. Our plasmid-based expression system was designed to facilitate expression of *ALS5* cell-surface mutants and cell-surface anchorage defective mutants. I described construction of several of these mutants in chapter 3 and table 3 summarizes all of the *ALS5* constructs prepared.

Als5p-mediated cell-cell interactions are homotypic

Als5p cell-surface expression causes binding to FN coated beads followed by the formation of cellular aggregates. Adhesion to FN beads is Als5p dependent, since non-expressing cells do not adhere. However, cellular aggregation may be due to Als5p binding other Als5p molecules (homotypic binding) or other *S. cerevisiae* cell wall epitopes to form cellular aggregates. To determine that cell-cell interactions are a direct consequence of Als5p homotypic interactions, I designed a microplate binding assay to screen binding of Als5p cells and non-expressing cells with various ligands. If cellular aggregation is due

to Als5p homotypic interactions, then Als5p-expressing cells should bind to Als5p coated surfaces and non-expressing cells should not bind. Soluble Als5p$^{1-664}$ was coated onto the surface of a 96-well microtiter plate. Fibronectin and denatured BSA were used as positive controls. In addition, nonspecific binding to uncoated wells was screened. After coating the wells with the designated ligand, Als5p expressing or non-expressing cells were incubated in the wells for 1hr. The wells were then gently washed 3-6 times and the plate was microscopically examined to determine binding. Our results confirmed our expectations in that Als5p expressing cells bound to wells coated with soluble Als5p and non-expressing cells did not bind (Figure 20A). In addition, we treated Als5p aggregated cells with Anti-Als1p antibodies. All cells within the aggregate displayed bright green fluorescence (Figures 20B and C). Taken together, these results demonstrate cell-cell binding is due to Als5p expression.

Physical properties of Als5p cellular aggregates

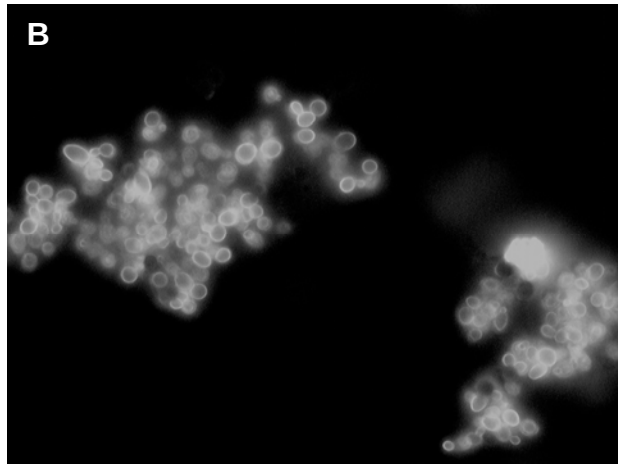
The initial characterization of Als5p activity focused mainly on the properties of cells binding to peptide-coated beads (Gaur, Klotz, & Henderson, 1999). The term SRS (Stable, Reversible, and Specific) was assigned to describe Als5p-mediated adhesion to beads based on the following observations: 1. the ability of cells to bind FN beads in the presence of various chemical additives such as EDTA, Triton, Sodium Chloride, and various sugars 2. resistance to shear forces generated through vortexing, and 3.reversible binding to FN beads following treatment with 6M urea or 50% formamide. Here, we wanted to expand on the characteristics of Als5p adherence by exploring the

Figure 20. Homotypic Binding of Als5p. (A) Table of results for the microplate binding assay using Als5p-expressing cells with various protein-coated surfaces. (B) Cell surface fluorescence of Als5p-aggregating cells. (C) Bright- Field micrograph of cells seen in (B).

A

Experimental Condition	Adherence to Microplate Well
Sc/Als5p + Fibronectin	++++
Sc/Als5p + Als5p	+++
Sc/Als5p + BSA	++
Sc/Als5p + Empty Well	+/-
Sc (wt) + FN	-
Sc (wt) + Als5p	-
Sc (wt) + BSA	-
Sc (wt) + Empty Well	-

B



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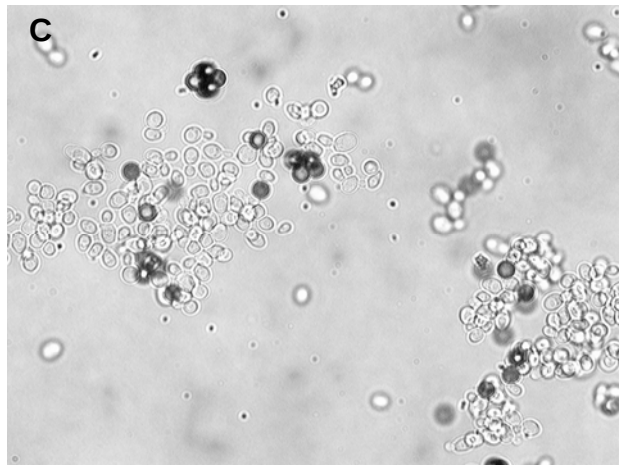


physical properties of Als5p cellular aggregates.

To determine the stability of Als5p cellular aggregates, we treated aggregated cells with various molecular agents. Aggregate resistance to proteolysis was screened by treating cellular aggregates with trypsin. We hypothesized that proteolytic sites on Als5p may be exposed as Als5p undergoes ligand-induced conformational switching. Adhesion assays were performed as outlined in chapter 1. Following the cell/FN bead incubation step, cellular aggregates were treated with trypsin at 100ug/ml or 1ug/ml. The results shown that at a concentration of 1ug/ml, adherence to FN bead was consistent with control samples. However, cellular aggregation was significantly reduced (data not shown). Additionally, post treatment of cellular aggregates ANS and CR yielded similar results. Disruption of aggregates with Anti-Als1p antibodies was not as severe compared to the agents listed above (data not shown). Finally, cellular aggregates are disrupted with prolonged vortexing, in contrast to cells bound directly to beads which are not dissociated. Collectively, these results demonstrate that Als5p binding to FN is characterized by high affinity tight binding. On the other hand, cell-cell binding occurs with much weaker affinity and can be easily disrupted. Interestingly, the reduction of aggregation by anti-Als1p antibodies indicates that the Als5p Ig domain may be involved in cell-cell binding.

Structural characteristics of the Als5p TR region

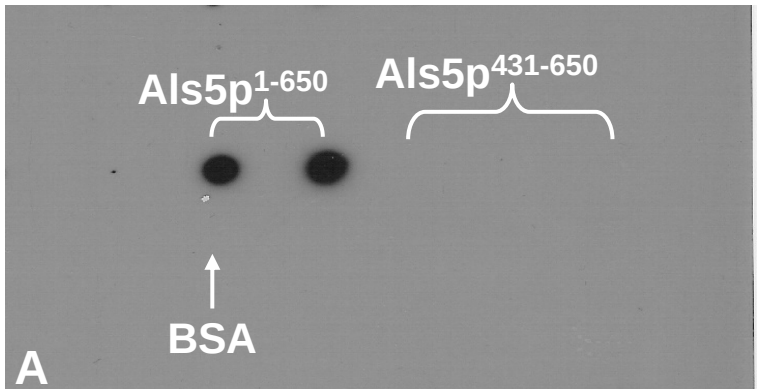
In order to determine the role of the Als5p TR region in aggregation, I proposed to purify a soluble fragment of the TR region ($Als5p^{469-650}$). The TRs were ligated to the invertase signal sequence and subcloned into the pYES

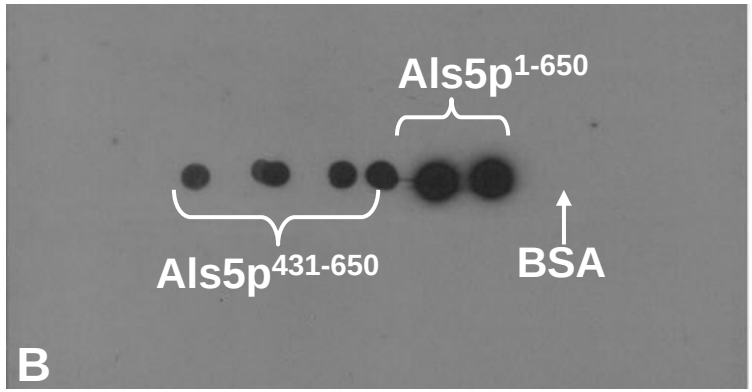
TOPO vector to form plasmid RL06 (Table 3) as outlined in chapter three. Additionally, the same strategy was applied to produce the Als5p protein fragment containing the TC and TR region (plasmid pRL04, Als5p$^{361-650}$). Subsequent expression in *S. cerevisiae* showed that the protein was not secreted into the growth media rather protein production was detected following lysis of Als5p expressing yeast cells (Figure 21A and B). These results suggest that the protein may not be correctly folded.

Sequences rich in β–branched amino acids are found in mucin proteins and are characteristic in the *FLO* and *ALS* gene family. That these regions would possibly mediate cell-cell interactions can be attributed to the conformational restriction along with the neutral hydrophobicity of this region. These characteristics would not promote the formation of a tightly packed domain, but rather advocate the formation of loose sheets with regular secondary structure and no tertiary structure.

Alternatively, these protein fragments may form aggregates thereby inhibiting protein secretion. We hypothesized that Als5p tandem repeats can associate with each other through the formation of initial weak inter and intra-molecular bonds mediated by hydrogen bonding and the hydrophobic effect. At high concentrations a conformational shift to a more compact structure may form through associations from β–branch sequences from different aggregating molecules. Currently we are determining the optimal conditions to purify the TR region through cleavage of Als5p$^{1-650/FactorXa}$, in order to facilitate binding and structural analyses.

Figure 21. Dot Blot analysis of Als5p$^{361-650}$ (TR) protein fragments. *S. cerevisiae* Als5p$^{361-650}$ (TR) - expressing cells were harvested following growth in YPGal media. (A) Dot blot of growth culture supernatant. (B) Cellular lysate fraction. Approximately 3x10^{10} cells were washed, resuspended in 25ml of 1X PBS, and vortexed with glass beads for cell lysis. The lysate was separated from the cellular debris by centrifugation prior to spotting on the nitrocellulose membrane. 1mg/ml BSA is used as a negative control and Als5p$^{1-664}$ is used as a positive control. Blots were probed with anti-V5 antibodies at 1:5000 dilution. 2ul of designated protein sample was spotted onto the nitrocellulose membrane.





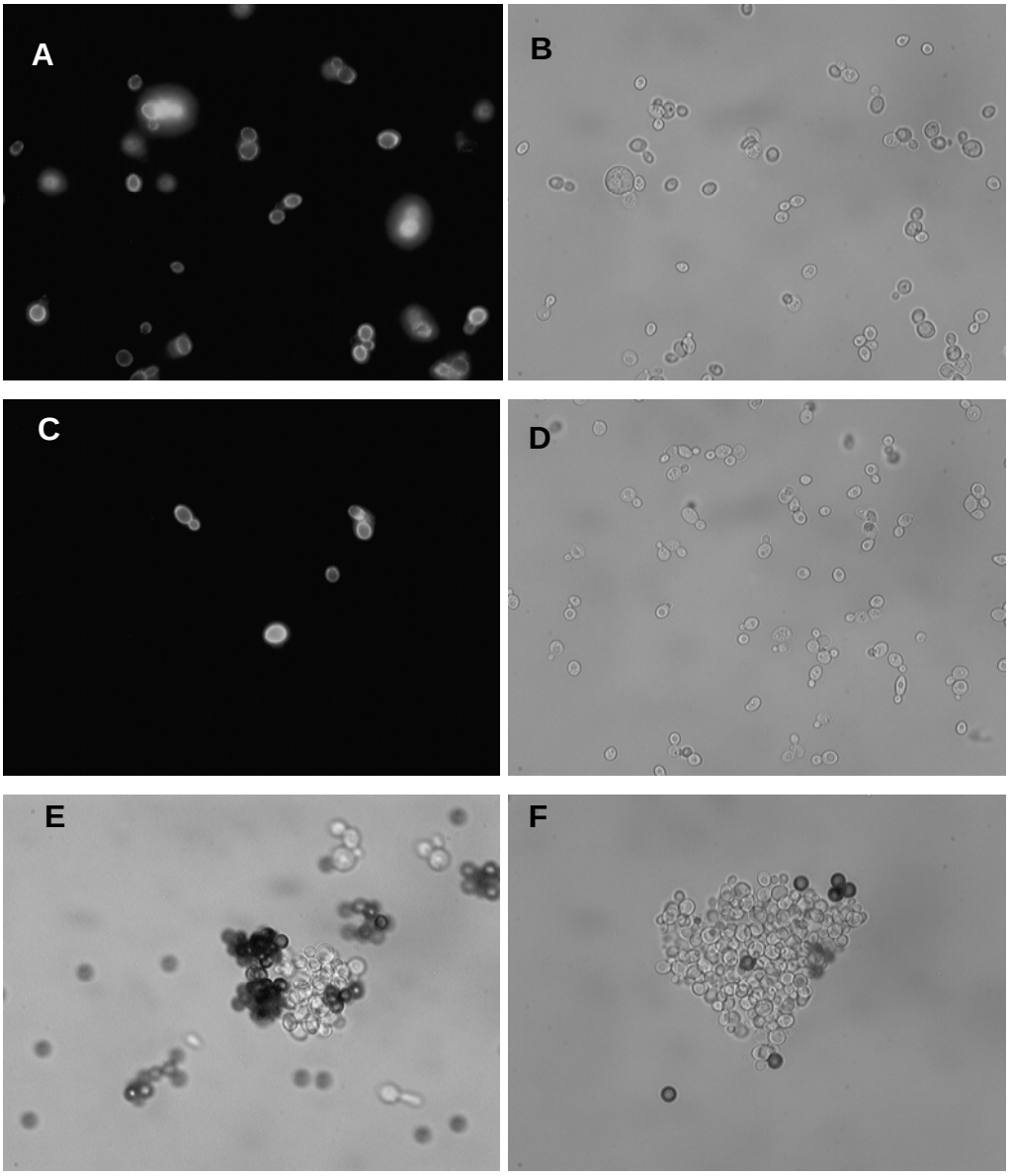
Growth Medium

Cellular Lysate

Analysis of an *ALS5* cell surface variant with an IG domain deletion

To further assess the function of the *ALS5* TRR region, a cell surface variant construct encoding the GPI anchored protein without the *ALS5* Ig domain (plasmid RL08/ALS5 ΔIG-TC) was prepared by PCR-mediated directional cloning as described in chapter 3 for plasmid pRL10. A V5 epitope sequence was engineered at the N-Terminus for immunodetection of the protein on the cell surface. Indirect Immunoflourescence and flow cytometry assays verified protein cell surface localization. Micrographs revealed uniform fluorescence around the cell surface with the same intensity as compared to Als5p (wt) cells (Figure 22 A-D). Fluorescence was not observed for the non-transformed cells. Flow cytometry indicated that a greater number of cells expressing Als5p wt (45%) localized the protein on the cell surface compared to cells expressing Als5p (ΔIG-TC) (8%) and non-transformed cells (0.25%) (data not shown). However, the relative fluorescence intensity was equal in both Als5p-expressing populations, thus strongly indicating that approximately the same number of Als5p molecules were present on the cell surface of the (ΔIG-TC) and (wt)-expressing cells. Initial data obtained from aggregation assays show Als5p (ΔIG-TC) expressing cells binding to FN coated beads and forming small cellular aggregates (Figure 22 E and F). This result strongly suggests the TR region can mediate binding. We are currently attempting to generate greater surface localization through integration of the gene construct into the *S. cerevisiae* chromosomal DNA.

Figure 22. Expression and functional analyses of Als5p Ig-TC domain deletion strain. (A) Cell surface fluorescence of Als5p-expressing *S. cerevisiae* cells using anti-Als1p primary antibodies as a probe. (B) Bright Field –micrograph of the cells seen in panel A. (C) Cell surface fluorescence of Als5p (∆IG-TC)-expressing cells. Cells were probed with anti-V5-FITC conjugated antibodies. (D) Bright Field –micrograph of the cells seen in panel C. (E–F) Adhesion assays Als5p (∆IG-TC)-expressing cells.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Chapter VI

References

Arnow, P. M., Quimosing, E. M., & Beach, M. (1993). Consequences of intravascular catheter sepsis. *Clin Infect Dis, 16*(6), 778-784.

Bailey, R. W., Dunker, A. K., Brown, C. J., Garner, E. C., & Griswold, M. D. (2001). Clusterin, a binding protein with a molten globule-like region. *Biochemistry, 40*(39), 11828-11840.

Barnes, D. A., Bonnin, A., Huang, J. X., Gousset, L., Wu, J., Gut, J., et al. (1998). A novel multi-domain mucin-like glycoprotein of Cryptosporidium parvum mediates invasion. *Mol Biochem Parasitol, 96*(1-2), 93-110.

Buurman, E. T., Westwater, C., Hube, B., Brown, A. J., Odds, F. C., & Gow, N. A. (1998). Molecular analysis of CaMnt1p, a mannosyl transferase important for adhesion and virulence of Candida albicans. *Proc Natl Acad Sci U S A, 95*(13), 7670-7675.

Calderone, R. A. (2002). *Candida and Candidiasis*. Washington, D.C.: ASM Press.

Chai, B., Hsu, J. M., Du, J., & Laurent, B. C. (2002). Yeast RSC function is required for organization of the cellular cytoskeleton via an alternative PKC1 pathway. *Genetics, 161*(2), 575-584.

Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T., & Ghannoum, M. A. (2001). Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance. *J Bacteriol, 183*(18), 5385-5394.

Chapman, M. R., Robinson, L. S., Pinkner, J. S., Roth, R., Heuser, J., Hammar, M., et al. (2002). Role of Escherichia coli curli operons in directing amyloid fiber formation. *Science, 295*(5556), 851-855.

Chen, M. H., Shen, Z. M., Bobin, S., Kahn, P. C., & Lipke, P. N. (1995). Structure of Saccharomyces cerevisiae alpha-agglutinin. Evidence for a yeast cell wall protein with multiple immunoglobulin-like domains with atypical disulfides. *J Biol Chem, 270*(44), 26168-26177.

Cheng, G., Wozniak, K., Wallig, M. A., Fidel, P. L., Jr., Trupin, S. R., & Hoyer, L. L. (2005). Comparison between Candida albicans agglutinin-like sequence

gene expression patterns in human clinical specimens and models of vaginal candidiasis. *Infect Immun, 73*(3), 1656-1663.

Daniels, K. J., Lockhart, S. R., Staab, J. F., Sundstrom, P., & Soll, D. R. (2003). The adhesin Hwp1 and the first daughter cell localize to the a/a portion of the conjugation bridge during Candida albicans mating. *Mol Biol Cell, 14*(12), 4920-4930.

de Campos Vidal, B., & de Carvalho, H. F. (1990). Aggregational state and molecular order of tendons as a function of age. *Matrix, 10*(1), 48-57.

de Nobel, H., Lipke, P. N., & Kurjan, J. (1996). Identification of a ligand-binding site in an immunoglobulin fold domain of the Saccharomyces cerevisiae adhesion protein alpha-agglutinin. *Mol Biol Cell, 7*(1), 143-153.

Eisenberg, D., Schwarz, E., Komaromy, M., & Wall, R. (1984). Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J Mol Biol, 179*(1), 125-142.

Feitosa, V., Vidal, B. C., & Pimentel, E. R. (2002). Optical anisotropy of a pig tendon under compression. *J Anat, 200*(Pt 1), 105-111.

Fu, Y., Ibrahim, A. S., Sheppard, D. C., Chen, Y. C., French, S. W., Cutler, J. E., et al. (2002). Candida albicans Als1p: an adhesin that is a downstream effector of the EFG1 filamentation pathway. *Mol Microbiol, 44*(1), 61-72.

Fu, Y., Rieg, G., Fonzi, W. A., Belanger, P. H., Edwards, J. E., Jr., & Filler, S. G. (1998). Expression of the Candida albicans gene ALS1 in Saccharomyces cerevisiae induces adherence to endothelial and epithelial cells. *Infect Immun, 66*(4), 1783-1786.

Gaur, N. K., & Klotz, S. A. (1997). Expression, cloning, and characterization of a Candida albicans gene, ALA1, that confers adherence properties upon Saccharomyces cerevisiae for extracellular matrix proteins. *Infect Immun, 65*(12), 5289-5294.

Gaur, N. K., & Klotz, S. A. (2004). Accessibility of the peptide backbone of protein ligands is a key specificity determinant in Candida albicans SRS adherence. *Microbiology, 150*(Pt 2), 277-284.

Gaur, N. K., Klotz, S. A., & Henderson, R. L. (1999). Overexpression of the Candida albicans ALA1 gene in Saccharomyces cerevisiae results in aggregation following attachment of yeast cells to extracellular matrix proteins, adherence properties similar to those of Candida albicans. *Infect Immun, 67*(11), 6040-6047.

Gaur, N. K., Smith, R. L., & Klotz, S. A. (2002). Candida albicans and Saccharomyces cerevisiae expressing ALA1/ALS5 adhere to accessible threonine, serine, or alanine patches. *Cell Commun Adhes, 9*(1), 45-57.

Gietz, R. D., Schiestl, R. H., Willems, A. R., & Woods, R. A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast, 11*(4), 355-360.

Green, C. B., Zhao, X., & Hoyer, L. L. (2005). Use of green fluorescent protein and reverse transcription-PCR to monitor Candida albicans agglutinin-like sequence gene expression in a murine model of disseminated candidiasis. *Infect Immun, 73*(3), 1852-1855.

Green, C. B., Zhao, X., Yeater, K. M., & Hoyer, L. L. (2005). Construction and real-time RT-PCR validation of Candida albicans PALS-GFP reporter strains and their use in flow cytometry analysis of ALS gene expression in budding and filamenting cells. *Microbiology, 151*(Pt 4), 1051-1060.

Grigorescu, A., Chen, M. H., Zhao, H., Kahn, P. C., & Lipke, P. N. (2000). A CD2-based model of yeast alpha-agglutinin elucidates solution properties and binding characteristics. *IUBMB Life, 50*(2), 105-113.

Hoyer, L. L. (2001). The ALS gene family of Candida albicans. *Trends Microbiol, 9*(4), 176-180.

Hoyer, L. L., Clevenger, J., Hecht, J. E., Ehrhart, E. J., & Poulet, F. M. (1999). Detection of Als proteins on the cell wall of Candida albicans in murine tissues. *Infect Immun, 67*(8), 4251-4255.

Hoyer, L. L., Fundyga, R., Hecht, J. E., Kapteyn, J. C., Klis, F. M., & Arnold, J. (2001). Characterization of agglutinin-like sequence genes from non-albicans Candida and phylogenetic analysis of the ALS family. *Genetics, 157*(4), 1555-1567.

Hoyer, L. L., & Hecht, J. E. (2000). The ALS6 and ALS7 genes of Candida albicans. *Yeast, 16*(9), 847-855.

Hoyer, L. L., & Hecht, J. E. (2001). The ALS5 gene of Candida albicans and analysis of the Als5p N-terminal domain. *Yeast, 18*(1), 49-60.

Hoyer, L. L., Payne, T. L., & Hecht, J. E. (1998). Identification of Candida albicans ALS2 and ALS4 and localization of als proteins to the fungal cell surface. *J Bacteriol, 180*(20), 5334-5343.

Hoyer, L. L., Scherer, S., Shatzman, A. R., & Livi, G. P. (1995). Candida albicans ALS1: domains related to a Saccharomyces cerevisiae sexual agglutinin separated by a repeating motif. *Mol Microbiol, 15*(1), 39-54.

Inoue, S. (1987). *Video Microscopy*. New York: Plenum Press.

Kamai, Y., Kubota, M., Kamai, Y., Hosokawa, T., Fukuoka, T., & Filler, S. G. (2002). Contribution of Candida albicans ALS1 to the pathogenesis of experimental oropharyngeal candidiasis. *Infect Immun, 70*(9), 5256-5258.

Kapteyn, J. C., Hoyer, L. L., Hecht, J. E., Muller, W. H., Andel, A., Verkleij, A. J., et al. (2000). The cell wall architecture of Candida albicans wild-type cells and cell wall-defective mutants. *Mol Microbiol, 35*(3), 601-611.

Klepser, M. E., Lewis, R. E., & Pfaller, M. A. (1998). Therapy of Candida infections: susceptibility testing, resistance, and therapeutic options. *Ann Pharmacother, 32*(12), 1353-1361.

Klotz, S. A., Gaur, N. K., Lake, D. F., Chan, V., Rauceo, J., & Lipke, P. N. (2004). Degenerate peptide recognition by Candida albicans adhesins Als5p and Als1p. *Infect Immun, 72*(4), 2029-2034.

Klotz, S. A., Gaur, N. K., Rauceo, J., Lake, D. F., Park, Y., Hahm, K. S., et al. (2004). Inhibition of adherence and killing of Candida albicans with a 23-Mer peptide (Fn/23) with dual antifungal properties. *Antimicrob Agents Chemother, 48*(11), 4337-4341.

Kundu, B., & Guptasarma, P. (1999). Hydrophobic dye inhibits aggregation of molten carbonic anhydrase during thermal unfolding and refolding. *Proteins, 37*(3), 321-324.

Kundu, B., & Guptasarma, P. (2002). Use of a hydrophobic dye to indirectly probe the structural organization and conformational plasticity of molecules in amorphous aggregates of carbonic anhydrase. *Biochem Biophys Res Commun, 293*(1), 572-577.

Li, F., & Palecek, S. P. (2003). EAP1, a Candida albicans gene involved in binding human epithelial cells. *Eukaryot Cell, 2*(6), 1266-1273.

Lipke, P. N., Chen, M. H., de Nobel, H., Kurjan, J., & Kahn, P. C. (1995). Homology modeling of an immunoglobulin-like domain in the Saccharomyces cerevisiae adhesion protein alpha-agglutinin. *Protein Sci, 4*(10), 2168-2178.

Lipke, P. N., Terrance, K., & Wu, Y. S. (1987). Interaction of alpha-agglutinin with Saccharomyces cerevisiae a cells. *J Bacteriol, 169*(2), 483-488.

Lo, W. S., & Dranginis, A. M. (1996). FLO11, a yeast gene related to the STA genes, encodes a novel cell surface flocculin. *J Bacteriol, 178*(24), 7144-7151.

Lo, W. S., & Dranginis, A. M. (1998). The cell surface flocculin Flo11 is required for pseudohyphae formation and invasion by Saccharomyces cerevisiae. *Mol Biol Cell, 9*(1), 161-171.

Lorenzo, A., & Yankner, B. A. (1994). Beta-amyloid neurotoxicity requires fibril formation and is inhibited by congo red. *Proc Natl Acad Sci U S A, 91*(25), 12243-12247.

Loza, L., Fu, Y., Ibrahim, A. S., Sheppard, D. C., Filler, S. G., & Edwards, J. E., Jr. (2004). Functional analysis of the Candida albicans ALS1 gene product. *Yeast, 21*(6), 473-482.

Lupetti, A., Danesi, R., Campa, M., Del Tacca, M., & Kelly, S. (2002). Molecular basis of resistance to azole antifungals. *Trends Mol Med, 8*(2), 76-81.

Maiti, N. R., & Surewicz, W. K. (2001). The role of disulfide bridge in the folding and stability of the recombinant human prion protein. *J Biol Chem, 276*(4), 2427-2431.

Masia Canuto, M., & Gutierrez Rodero, F. (2002). Antifungal drug resistance to azoles and polyenes. *Lancet Infect Dis, 2*(9), 550-563.

Oh, S. H., Cheng, G., Nuessen, J. A., Jajko, R., Yeater, K. M., Zhao, X., et al. (2005). Functional specificity of Candida albicans Als3p proteins and clade specificity of ALS3 alleles discriminated by the number of copies of the tandem repeat sequence in the central domain. *Microbiology, 151*(Pt 3), 673-681.

Plakoutsi, G., Taddei, N., Stefani, M., & Chiti, F. (2004). Aggregation of the Acylphosphatase from Sulfolobus solfataricus: the folded and partially unfolded states can both be precursors for amyloid formation. *J Biol Chem, 279*(14), 14111-14119.

Rauceo, J. M., Gaur, N. K., Lee, K. G., Edwards, J. E., Klotz, S. A., & Lipke, P. N. (2004). Global cell surface conformational shift mediated by a Candida albicans adhesin. *Infect Immun, 72*(9), 4948-4955.

Sheppard, D. C., Yeaman, M. R., Welch, W. H., Phan, Q. T., Fu, Y., Ibrahim, A. S., et al. (2004). Functional and structural diversity in the Als protein family of Candida albicans. *J Biol Chem, 279*(29), 30480-30489.

Singleton, D. R., Fidel, P. L., Jr., Wozniak, K. L., & Hazen, K. C. (2005). Contribution of cell surface hydrophobicity protein 1 (Csh1p) to virulence of hydrophobic Candida albicans serotype A cells. *FEMS Microbiol Lett, 244*(2), 373-377.

Sobel, J. D., Faro, S., Force, R. W., Foxman, B., Ledger, W. J., Nyirjesy, P. R., et al. (1998). Vulvovaginal candidiasis: epidemiologic, diagnostic, and therapeutic considerations. *Am J Obstet Gynecol, 178*(2), 203-211.

Sreerama, N., & Woody, R. W. (1993). A self-consistent method for the analysis of protein secondary structure from circular dichroism. *Anal Biochem, 209*(1), 32-44.

Staab, J. F., Bahn, Y. S., Tai, C. H., Cook, P. F., & Sundstrom, P. (2004). Expression of transglutaminase substrate activity on Candida albicans germ tubes through a coiled, disulfide-bonded N-terminal domain of Hwp1 requires C-terminal glycosylphosphatidylinositol modification. *J Biol Chem, 279*(39), 40737-40747.

Staab, J. F., Bradway, S. D., Fidel, P. L., & Sundstrom, P. (1999). Adhesive and mammalian transglutaminase substrate properties of Candida albicans Hwp1. *Science, 283*(5407), 1535-1538.

Terrance, K., & Lipke, P. N. (1987). Pheromone induction of agglutination in Saccharomyces cerevisiae a cells. *J Bacteriol, 169*(10), 4811-4815.

Ueta, E., Tanida, T., Yoneda, K., Yamamoto, T., & Osaki, T. (2001). Increase of Candida cell virulence by anticancer drugs and irradiation. *Oral Microbiol Immunol, 16*(4), 243-249.

Whelan, W. L., Delga, J. M., Wadsworth, E., Walsh, T. J., Kwon-Chung, K. J., Calderone, R., et al. (1990). Isolation and characterization of cell surface mutants of Candida albicans. *Infect Immun, 58*(6), 1552-1557.

Wojciechowicz, D., & Lipke, P. N. (1989). Alpha-agglutinin expression in Saccharomyces cerevisiae. *Biochem Biophys Res Commun, 161*(1), 46-51.

Zhao, H., Chen, M. H., Shen, Z. M., Kahn, P. C., & Lipke, P. N. (2001). Environmentally induced reversible conformational switching in the yeast cell adhesion protein alpha-agglutinin. *Protein Sci, 10*(6), 1113-1123.

Zhao, H., Shen, Z. M., Kahn, P. C., & Lipke, P. N. (2001). Interaction of alpha-agglutinin and a-agglutinin, Saccharomyces cerevisiae sexual cell adhesion molecules. *J Bacteriol, 183*(9), 2874-2880.

Zhao, X., Oh, S. H., Cheng, G., Green, C. B., Nuessen, J. A., Yeater, K., et al. (2004). ALS3 and ALS8 represent a single locus that encodes a Candida albicans adhesin; functional comparisons between Als3p and Als1p. *Microbiology, 150*(Pt 7), 2415-2428.

Zhao, X., Oh, S. H., Yeater, K. M., & Hoyer, L. L. (2005). Analysis of the Candida albicans Als2p and Als4p adhesins suggests the potential for

compensatory function within the Als family. *Microbiology, 151*(Pt 5), 1619-1630.

Zhao, X., Pujol, C., Soll, D. R., & Hoyer, L. L. (2003). Allelic variation in the contiguous loci encoding Candida albicans ALS5, ALS1 and ALS9. *Microbiology, 149*(Pt 10), 2947-2960.