Threonine-Rich Repeats Increase Fibronectin Binding in the *Candida albicans* Adhesin Als5p

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Commensal and pathogenic states of *Candida albicans* depend on cell surface-expressed adhesins, including those of the Als family. Mature Als proteins consist of a 300-residue N-terminal region predicted to have an immunoglobulin (Ig)-like fold, a 104-residue conserved Thr-rich region (T), a central domain of a variable number of tandem repeats (TR) of a 36-residue Thr-rich sequence, and a heavily glycosylated C-terminal Ser/Thr-rich stalk region, also of variable length (N. K. Gaur and S. A. Klotz, Infect. Immun. 65: 5289–5294, 1997). Domain deletions in Als5 were expressed in *Saccharomyces cerevisiae* to excrete soluble protein and for surface display. Far UV circular dichroism indicated that soluble Ig-T showed a single negative peak at 212 nm, consistent with previous data indicating that this region has high β-sheet content with very little α-helix. A truncation of Als5p with six tandem repeats (Ig-T-TR6) gave spectra with additional negative ellipticity at 200 nm and, at 227 to 240 nm, spectra characteristic of a structure with a similar fraction of β-sheet but with additional structural elements as well. Soluble Als5p Ig-T and Ig-T-TR6 fragments bound to fibronectin in vitro, but the inclusion of the TR region substantially increased affinity. Cellular adhesion assays with *S. cerevisiae* showed that the Ig-T domain mediated adherence to fibronectin and that TR repeats greatly increased cell-to-cell aggregation. Thus, the TR region of Als5p modulated the structure of the Ig-T region, augmented cell adhesion activity through increased binding to mammalian ligands, and simultaneously promoted fungal cell-cell interactions.

In *Candida albicans*, as in other microorganisms that associate with animals, adherence to host tissues is a key factor in the maintenance of commensal and pathogenic states. Strains with mutated adhesin genes or with mutations in genes that regulate adhesins display reduced virulence (1, 6, 37). The genes in the *ALS* (agglutinin-like sequence) family encode cell surface glycoproteins that mediate adherence to various host substrates. Currently, there are eight known *ALS* genes, and the Als proteins are the most widely expressed adhesins in *C. albicans* (16, 37). Als1p, Als3p, and Als5p have been extensively characterized, and Als1p and Als3p are involved in *C. albicans* virulence (6, 37). Als proteins show a high degree of identity and similarity between each other, thus predicting similar three-dimensional structures (32). Each Als protein has an N-terminal secretion signal of ~20 residues, a ~300-residue region with sequence and structures similar to those of tandem immunoglobulin (Ig) domains (the Ig-like region; 55% to 90% identical across the family), and a 104-residue conserved threonine-rich region (T) that has 90 to 98% similarity among Als proteins. There is a central domain consisting of a variable number of tandem copies of a Thr-rich 36-residue sequence (TR). There is also a variable-length C-terminal Ser/Thr-rich region which has multiple N- and O-glycosylation sites. In this region, the sequence variability is highest between family members (8, 17, 18). A 13- to 20-residue C-terminal sequence signals the attachment point of a glycosylphosphatidylinositol (GPI) anchor, which is processed to cross-link the adhesins to the cell wall polysaccharide matrix (21, 25).

Because multiple Als adhesins are expressed on *C. albicans*, most of our knowledge of the activities of individual Als proteins derives from a *Saccharomyces cerevisiae* surface display model. In this system, expression of Als5p results in binding of yeast cells to a variety of substrates, such as extracellular matrix-coated beads, human buccal epithelial cells, and various peptide sequences (9, 10, 22, 28, 32, 34). In an assay measuring adherence to protein-coated magnetic beads, Als5p-mediated adhesion is stable over a broad pH range, persists after vortexing, and is reversibly inhibited when treated with urea or formamide (9). The initial binding of Als5p-expressing cells to ligand triggers a conformational change in Als5p, leading to the formation of cellular aggregates (30). Als5p-mediated adhesion and aggregation are independent of metabolic activity and signal transduction, but cellular aggregation is inhibited by agents which perturb protein secondary structure or by H-bond disruptants (10, 30).

Domain deletions and swaps in the *S. cerevisiae* surface display model show that the Ig-like region mediates much of the substrate binding. Mutations or deletion of the Ig-like region of Als1p resulted in inhibition of activity (28). Adherence was significantly reduced when the TR region was deleted (28). Also, a swap of the Ig-like regions of Als5p and Als6p showed...
that the binding specificity of the chimeras mimicked the specificity of the Als proteins from which the Ig-T region came (32).

Two studies of the Ig-like region showed that this domain is a β-sheet-rich structure. Hoyer and Hecht reported a circular dichroism (CD) spectrum characteristic of very high β-sheet content in the Ig-like region of Als5p excised and purified from *Pichia pastoris* (17). CD spectrometry and Fourier transform infrared spectroscopy of a soluble Als1p Ig-T fragment also showed an antiparallel β-sheet structure, similar to those of adhesins and invasins of the immunoglobulin superfamily (17, 32). Comparative modeling studies of Als proteins and the homologous *S. cerevisiae* sexual adhesin α-agglutinin show that this region is compatible with Ig-like structures (13, 24, 32).

A small amount of binding activity remains in Als1p if most of the Ig-like region (residues 29 to 285) is deleted, and there is a reduction in activity with deletion of the tandem repeats (28). This reduction was attributed to the loss of the extended peptide stalk that elevates the active site away from the wall surface. Three arguments question this interpretation. First, the *S. cerevisiae* sexual adhesins are fully active on the cell surface with much shorter stalks. Second, the aggregation activity of Als proteins appears to be proportional to the number of tandem repeats and not the size of the stalk; the Als1p stalk is shorter than Als5p, and the former has greater adherence, although the length of the proteins is around 1,200 residues regardless of the number of repeats. In addition, the lengths of Als proteins vary between 900 and 2,000 residues, and forms of various lengths are active (32). Third, the sequence of the repeats is conserved, in contrast to known stalk sequences in Als and other fungal adhesins (16, 26). This conservation argues for the preservation of a function strongly dependent on a specific sequence or conformation. Therefore, we designed and tested Als5p with and without the TR regions to determine whether there were activities dependent on the presence of the TR region.

### MATERIALS AND METHODS

#### Construction of plasmid vectors

The previously described shuttle vector pGK114 (9) containing the *ALS3* open reading frame facilitated subcloning into the pYES1.1VS-His-TOP cloning kit (Invitrogen), which adds the simian virus 5 (SV5) epitope- and His6-encoding sequences just before the stop codon in cloned open reading frames. Plasmid pGK114 was used as a template for PCR amplifications.

The PCR and sequencing primers used in this study are listed in Table 1, and the various plasmid constructs made are shown in Fig. 1.

For plasmids pRL02, pRL03, and pRL09, the corresponding sequences were amplified and ligated into the pYES vector. Following amplification in *Escherichia coli* XL10-Gold cells (Stratagene), plasmid DNA was extracted using a 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 a QIAGEN maxiprep kit. Plasmid DNA was used to transform *S. cerevisiae* W303-1B cells by the lithium acetate protocol (11).

To create plasmid pRL10, the *ALS5* N-Terminal Ig-T region was amplified by PCR. The 5′ forward primer contained an EcoRI endonuclease site, and the 3′ reverse primer contained a six-histidine tag for immunodetection, followed by a BgII endonuclease site. Amplicons were ligated into pYES to generate plasmid RL10. In the second PCR, the *ALS5* C-terminal region containing the Crr/Thr-rich stalk, GPI anchor, and stop codon was amplified. The 5′ forward primer contained a BgII endonuclease site, and the 3′ reverse primer contained an XhoI endonuclease site. Amplicons were ligated to pYES vector to form plasmid pRL10. Plasmids pRL02 and pRL09 were amplified in *E. coli* and purified. To generate a secretion signal, the chimera shuttle vector pGL01 was used. This vector is a modification of plasmid p423GAL1 (ATCC), containing an invertase secretion signal α/β-agglutinin/green fluorescent protein (GFP) fusion product regulated by a GAL1 promoter. The α-agglutinin/GFP fusion protein was shown to be localized at high levels on the yeast cell surface when induced by galactose (M. Gonzalez, personal communication). pGL01 was digested with EcoRI and XhoI to release the α-agglutinin/GFP fragment, and the ~7.0-kb vector fragment containing the pGL2GAL1 backbone and invertase secretion signal was gel purified. pRL10 was also digested with EcoRI and BglII, and the ~1.3-kb product containing the *ALS5* Ig-T fragment was gel purified. pRL10 was digested with BglII and XhoI to release the ~2.0-kb *ALS5*-Terminal fragment and gel purified. The three fragments were then ligated together, and subsequent cloning steps followed the procedures described above. A similar procedure was followed to create plasmid pRL08, with a V5 epitope added immediately after the signal sequence.

Plasmids pDG100 (containing two tandem repeats) and pDG102 (containing four tandem repeats) were derived from pGK114 through homologous recombination between *ALS5* tandem repeats in *S. cerevisiae* (23). pGK114 was digested with BglII at a unique restriction site located in the second tandem repeat of *ALS5*. The linearized plasmid was transformed into *S. cerevisiae*, and the yeast cells were plated onto synthetic complete-Trp plates and grown at 28°C for 2 days. Only colonies with recircularized recombinant plasmids grow under these conditions. Colonies were scraped using a sterile cell scraper and suspended in 400 μl sterile H2O. Total DNA was isolated by glass beads and phenol-chloroform (31) and transformed into *E. coli*, and transformants were selected on ampicillin plates. Plasmids isolated from the *E. coli* transformants were analyzed by restriction digestion with *Clal* and *Sphl*. The wild-type *ALS5* gives a DNA fragment of approximately 1,200 bp, and mutant plasmids with four or two tandem repeats have approximately 1,000- or 800-bp fragments, respectively. The *ALS5*-NTR construct of two tandem repeats with 1,000-bp fragments and 800-bp fragments had the expected sequences and were transformed into *S. cerevisiae*. The members of each pair of similar plasmids resulted in similar phenotypes, and one of each pair was used in subsequent experiments.

### Expression and purification of Als5p

Protein expression and purification of Als5p (Ig-T) and Als5p (Ig-T) fragments. The growth of *S. cerevisiae* cells containing the appropriate plasmid in defined medium without uracil with galactose as a carbon source resulted in expression of soluble proteins. Similarly, surface-displayed proteins were expressed through growth in defined medium without tryptophan (pGK114, pDG100, and pDG102) or histidine (pRL08 and pRL10). Protein expression into the culture supernatant was verified by dot blot analysis as described below. Als5p cell culture supernatants (1 to 4 liters) were harvested, and phenylmethyl-sulfonyl fluoride was added to a final concentration of 10 μM. The supernatant was concentrated to approximately 150 ml through a Millipore filtration apparatus fitted with a 30-kDa molecular mass cutoff membrane. The concentrated supernatant was adjusted to pH 7.0 using 1 M Tris and chromatographed on a nickel-nitrilotriacetic acid column (QIAAGEN) preequilibrated with 20 mM imidazole, 300 mM NaCl, and 50 mM sodium phosphate buffer, pH 7.0 (buffer A). The column was washed with buffer A to remove nonspecific proteins. Als5p fragments were eluted with 500 mM imidazole, 300 mM NaCl, and 50 mM sodium phosphate buffer, pH 7.0 (buffer B). The Als5p fragments were pooled and dialyzed exhaustively into 20 mM sodium phosphate and 0.01% NaN3, pH 7.0 buffer (buffer C), using a 3,500-kDa molecular mass cutoff membrane. The sample was concentrated by further dialysis against buffer C.
supplemented with 10% polyethylene glycol 35000 (molecular mass). Protein concentration was determined as $A_{280}$. Several milligrams of Als5p 1-664 and Als5p 1-431 were purified.

**Polyacrylamide gel electrophoresis.** Als5p samples were electrophoresed in sodium dodecyl sulfate (SDS) polyacrylamide gels (4 to 20% precast gels; Bio-Rad Laboratories) and stained with Coomassie blue dye. Immunoblot analyses were performed by standard procedures (14).

**Dot blot analyses of Als5p.** Als5p samples were immobilized on nitrocellulose membranes and allowed to dry. Membranes were blocked for 1 h using phosphate-buffered saline (PBS) with 5.0% (wt/vol) dry milk (PBSTM) (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, 0.1% Tween 20, pH 7.0) and washed with PBS-Tween 20 (PBST) buffer three times. Next, the membrane was probed for 1 h using anti-His6 or anti-V5 peroxidase-conjugated antibody (Invitrogen) at a working concentration of 1:5,000 and washed. Proteins were detected by incubating the membranes with equal parts of SuperSignal West Pico stable peroxide and SuperSignal West Pico luminol/enhancer solutions (Pierce). The membrane was exposed to film and developed using an X-OMAT developing apparatus.

**Indirect immunofluorescence assays.** Immunofluorescence assays were performed as previously described with a polyclonal antibody raised against Als1p (30).

**Fibronectin binding assays.** Adherence assays were performed as described previously (9). Briefly, *S. cerevisiae* cells containing plasmids encoding wall-bound adhesins 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 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 13- by 100-mm glass tube, 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. Adherent cells were quantified by hemacytometer counting after dissociation with 0.1 M NaOH (9). Experiments were performed at least three independent times.

**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 blot analyses were performed as described above with the exception that blots were probed with 0.5 μg/ml concanavalin A (ConA) in PBSTM buffer supplemented with 10 μM each of CaCl$_2$ and MnSO$_4$. Hexose was quantified by the phenol-sulfuric acid method (5).

**CD spectroscopy.** Far UV spectra of Als5p 1-664 (Ig-T-TR6) and Als5p 1-431 (Ig-T) were recorded using AVIV model 215 spectropolarimeters, using quartz, thermo-regulated cuvettes (Hellma) with a 0.1-cm path length. Protein solutions (0.4 mg/ml) in 20 mM sodium phosphate buffer, pH 7.0, were multiplied scanned, averaged, smoothed, and corrected by subtraction of the 20 mM NaH$_2$PO$_4$ buffer baseline spectra. Molar ellipticities were calculated based on the amino acid composition and molar extinction coefficient for each fragment (12). CD data were analyzed by the self-consistent method with CONTINLL, using data between 205 and 250 nm (35).

**Fibronectin binding assay for soluble Als5p fragments.** In a polystyrene 96-well plate, a 1-mg/ml solution of fibronectin (Sigma) was serially diluted across the row (1 mg/ml to 10 ng/ml in 10-fold decrements). All samples were analyzed in duplicate. Each well contained a volume of 50 μl, and the plate was incubated

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FIG. 1. Expressed versions of Als5p. The regions of Als5p are marked: S, signal sequence (residues 1 to 17); Ig, tandem Ig-like domains (residues 18 to 327); T, conserved Thr-rich region (residues 328 to 431); TR, tandem Thr-rich repeats (residues 432 to 664); Stalk, Ser-Thr-rich glycosylated stalk; GPI, glycosylphosphatidylinositol addition signal; V, V5 epitope tag; H, His$_6$ tag. For the protein names, the N-terminal-expressed residues are numbered, and “W” denotes that residues 650 to 1419 (stalk and GPI) are also expressed. The results of immunofluorescence assays (IFA) for each cell-bound construct next to each surface-bound construct are shown. The primary antibodies were anti-Als1p for all constructs except Als5p$^{1-17,432-662-W}$, which was labeled with anti-V5.

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for 2 h at room temperature or overnight at 4°C. Next, the wells were washed three times with PBST buffer, blocked for 1 h with 200 μl of PBST buffer supplemented with fresh native bovine serum albumin (BSA) at 1 mg/ml, and washed. A total of 15 μg of the designated Als5p fragment was added to the wells, incubated for 2 h at room temperature, and washed. The wells were then incubated for 2 h with primary anti-V5 antibodies at a working concentration of 1:5,000, washed, and incubated with secondary anti-mouse alkaline phosphatase-conjugated antibodies at a working concentration of 1:10,000 for 1 h. Hydrolysis of p-nitrophenol was monitored as A\textsubscript{420} in a microplate reader after 30 min or more of color development.

RESULTS

Construction and purification of the Als5p Ig-T and Ig-T-TR\textsubscript{6} domains. We constructed S. cerevisiae plasmids that encoded and expressed both cell surface-bound and soluble forms of Als5p. \textit{ALS5} gene fragments were amplified by PCR and subcloned into the pYES shuttle vector to create plasmids pRL02 and pRL09, respectively (Fig. 1). The proteins produced by the various plasmids were named for the amino acid residues encoded. For example, Als5p\textsubscript{1-431-W} has Als5p residues 1 to 431 and 650 to 1419 (“-W”) but lacks residues 432 to 649 in the TR region.

Als5p\textsubscript{1-431} (Ig-T) and Als5p\textsubscript{1-664} (Ig-T-TR\textsubscript{6}) fragments, each without the wall-anchoring residues but with C-terminal V5 and His\textsubscript{6} tags, were harvested from yeast culture supernatants, and dot blot analysis verified secretion (Fig. 2). The proteins were purified by Ni-nitrilotriacetic acid column chromatography, and SDS-polyacrylamide gels showed that they were the major stainable proteins present (Fig. 3). Immunoblotting showed the presence of the V5 epitope in the isolated protein (data not shown).

The Als5p T region is critical for protein secretion. Both the Als5p\textsubscript{1-431} (Ig-T) and Als5p\textsubscript{1-664} (Ig-T-TR\textsubscript{6}) fragments contain the T region of the protein. In contrast, Als5p\textsubscript{1-329} (Ig) without the T region was not secreted into the culture supernatant (Fig. 2). Dot blot analysis demonstrated that the protein was translated and was present in the lysate of Als5p\textsubscript{1-329}-expressing yeast cells. Extracts of control cells without plasmid showed no immunoreactivity. In addition, Als5p\textsubscript{1-329-W}, a deletion mutant of Als5p that lacked the T and TR regions, did not localize the protein on the cell wall (Fig. 1). When the T region was reinserted (Als5p\textsubscript{1-431-W}), the protein was successfully localized on the cell wall (Fig. 1 and see below). Therefore, the T region was required for proper secretion of Als5p N-terminal fragments in \textit{S. cerevisiae}.

Structural characterization of the Als5p N-terminal and central domains. CD spectroscopy studies have reported that Als5p\textsubscript{1-329} (Ig regions) and Als1p\textsubscript{1-432} (Ig-T regions) fragments have far UV CD spectra with a single minimum close to 212 nm, near the characteristic negative peak for proteins with mostly antiparallel β-sheet and very little α-helical content (17, 32). There was a similar spectrum for Als5p\textsubscript{1-431}, the Als5p fragment containing the Ig and T regions (Fig. 4). CD spectra

FIG. 2. Expression and secretion of soluble forms of Als5p. Equal aliquots of His-TRAP elution fractions were spotted on nitrocellulose and probed with peroxidase-conjugated anti-His\textsubscript{6}. White vertical arrows denote antigen-negative fractions. Horizontal arrowheads denote positive controls (black arrowhead, purified Als5p\textsubscript{1-664} or His\textsubscript{6}-labeled α-agglutinin [36]) and negative controls (white arrowhead, BSA).

FIG. 3. Purified soluble proteins. Purified Als5p\textsubscript{1-431} (lane 1) and Als5p\textsubscript{1-664} (lane 4) were electrophoresed on a 4 to 20% gel and stained with Coomassie blue. The apparent molecular masses for standard proteins (lane 3) are on the left. Lane 2 is empty.

FIG. 4. Purified soluble proteins. Purified Als5p\textsubscript{1-431} (lane 1) and Als5p\textsubscript{1-664} (lane 4) were electrophoresed on a 4 to 20% gel and stained with Coomassie blue. The apparent molecular masses for standard proteins (lane 3) are on the left. Lane 2 is empty.
were also obtained for Als5p<sup>1-664</sup>, which contains six repeats in the TR region as well. These spectra showed substantive differences, including more-negative ellipticity around 200 nm, less negativity at the 212-nm peak, and additional negative shoulders near 227 nm and in the region >235 nm (Fig. 4). These shoulders are also present in the Als5p<sup>1-431</sup> spectrum, but overlap with the greater negative ellipticity of the 212-nm band makes them less distinct.

Despite the differences, CONTINLL analyses of the spectra showed that the Ig-T-TR<sub>6</sub> and Ig-T proteins had similar fractions of each secondary structure, that is, the TR region contributed to the β-sheet, turn, and aperiodic conformations but had few, if any, residues in helical conformation (Table 2). The peaks at 227 to 235 nm are characteristic of CD contributions from aromatic amino acid side chains in an asymmetric environment (20).

SDS-polyacrylamide gel electrophoresis showed that Als5p<sup>1-431</sup> had an apparent molecular mass of 66 kDa, about 12 kDa greater than the calculated size of 53.6 kDa. This lower mobility is common in proteins with a high content of hydroxy amino acids (29). Als5p<sup>1-664</sup> had a much greater discrepancy: its apparent molecular mass was 125 kDa, 52% greater than the expected size of 81.7 kDa (Fig. 3). Since no N-glycosylation sites exist within Als5p<sup>1-664</sup>, we tested whether the protein was O glycosylated. Dot blot analysis using ConA-horseradish peroxidase as a probe showed a positive reaction for Als5p<sup>1-664</sup>, an indication of the presence of α-linked mannose or glucose (Fig. 5). Glycosylation was specifically localized to the TR region, because ConA blots with Als5p<sup>1-431</sup> were negative, in agreement with published results (17). Quantification of the hexoses associated with Als5p<sup>1-664</sup> demonstrated approximately 90 mol of hexose per mol of protein, or ~1.5 molecules of hexose per Ser or Thr residue in the TR<sub>6</sub> region.

### Effect of TR on Als5p Ig-T fragment binding to fibronectin.

The differences between Als5p<sup>1-431</sup> (Ig-T) and Als5p<sup>1-664</sup> (Ig-T-TR<sub>6</sub>) implied that there might be differences in function as well. Therefore, we tested binding ability in a semiquantitative assay. The two Als5p fragments were bound to FN-coated microtiter plate wells, and the amount bound was determined by an enzyme-linked immunosorbent assay using antibody to V5. Both Als5p fragments bound to the FN at levels above those of binding to wells coated only with native BSA (Fig. 6). Negative controls omitting primary antibodies, FN, or Als5p showed absorbance values that were not statistically different from those of blank wells.

![Figure 4. Far UV circular dichroism spectra for Als5p<sup>1-431</sup> and Als5p<sup>1-664</sup>. Multiple spectra were averaged, smoothed, and baseline corrected for 0.4-mg/ml solutions of each protein, as described in Materials and Methods. The buffer was 20 mM sodium phosphate, pH 7.0.](http://ec.asm.org/)

![Figure 5. Dot blots with horseradish peroxidase-conjugated concanavalin A. Aliquots of His-TRAP elution fractions were spotted on nitrocellulose and probed with peroxidase-conjugated concanavalin A. White vertical arrows denote antibody-negative fractions. Horizontal arrowheads denote positive controls (black arrowhead, purified His<sub>6</sub>-labeled α-agglutinin [36]) and negative controls (white arrowhead, BSA).](http://ec.asm.org/)
from baseline values from wells in which the secondary antibody or p-nitrophenol had been omitted. Als5p1-664 bound with much greater affinity than Als5p1-431 (Fig. 6). The difference in apparent affinities was not due to differences in antibody binding, because both Als5p fragments bound similar amounts of antibody at similar concentrations in assays which measured antibody binding to immobilized Als5p (data not shown). Estimation of the FN concentration at maximal binding (Fig. 6) implied that Als5p1-664 had a dissociation constant in the nanomole range.

To determine whether the two forms of Als5p had similar amounts of active protein, we assayed various concentrations of Als5p binding to excess immobilized FN. In these assays, there were similar ratios of bound to free protein for Als5p1-431 and Als5p1-664 (data not shown). Therefore, similar amounts of active protein were bound at each concentration of Als5p, implying equivalent molar fractions of active protein. The results confirmed the affinity differences shown in Fig. 6, because 200-fold-higher concentrations of FN were required for binding of the Als5p1-431 Ig-T fragment than for the Als5p1-664 Ig-T-TR6 fragment. Taken together, these results show that Als5p1-664 had a dissociation constant in the nanomole range.

The TR region enhances cell-to-cell aggregation. In order to further understand the role of the Als5p domains in mediating adhesion and cell-cell aggregation, we expressed a cell surface construct containing a deletion of the TR region (pRL10/Als5p1-431-W) (Fig. 1). After Als5p expression was induced by growth in YPGal, immunofluorescence microscopy of intact cells and aggregates revealed uniform fluorescence (Fig. 1 and 7F).

Adhesion assays demonstrated the adhesion and aggregation activity of cells expressing Als5p1-431-W (Ig-T-W). Als5p1-431-W cells adhered to FN-coated beads similarly to Als5p cells. However, cell-to-cell binding was drastically reduced for Als5p1-431-W cells compared to Als5p (wild-type) cells (Fig. 7, compare panel A with panels C to E). Posttreatment of cellular aggregates with anti-Als1p antibodies (28, 30) revealed similar levels of protein on the cell surface (Fig. 7B and F). Therefore, the TR region contributed significantly to Als5p-mediated cellular aggregation.

The contribution of the TR region was confirmed in adhesion/aggregation assays with surface-displayed Als5p with zero, two, four, or six copies of the repeats. We quantified adhesion to beads and cell-to-cell aggregation for Als5p1-431-W (Ig-T-W), Als5p1-431-505-W (Ig-T-TR2-W), Als5p1-431-577-W (Ig-T-TR4-W), and complete Als5p (Ig-T-TR6-W) (Fig. 8). The intensity of cell surface immunofluorescence with anti-Als1p antibody was similar for each of these proteins (Fig. 1), implying similar cell surface concentrations. Although cells expressing any of these versions of Als5p adhered well to FN-coated beads, the number of cell-to-cell interactions, shown in Fig. 8 as cell-to-bead ratios, increased with each increase in the number of repeats.

Adherence properties of the Als5p Thr-rich repeat region. Plasmid pRL08 encodes Als5p1-17-431-664-W, a surface-bound V5 epitope-tagged version of Als5p without the Ig-like region (Fig. 1). YPGal cultures of cells carrying this plasmid showed variable expression of detectable surface-bound antigen. Positive cultures, such as that shown in Fig. 1, were tested in the adherence assay (Fig. 7G). These cells showed poor binding to beads but had more cell-to-cell binding than similar cells grown in glucose or than cells without plasmid grown in YPGal (Fig. 7H). Thus, the TR and stalk regions alone can mediate cell-cell interactions. There was also limited binding to FN-coated beads (not shown).

Substrate specificity of surface-bound Als5p. The surface display model was used to test substrate specificity of Als5p binding. When Als5p-expressing cells were incubated in microtiter wells coated with various proteins, the cells bound to wells coated with FN or with Als5p1-431-664 (Ig-T-TR6). Cells bound poorly to uncoated wells or to wells coated with native BSA (Table 3). Cells not displaying Als5p did not bind to wells coated with any tested substrate. Thus, Als5p1-664 was a ligand for surface-displayed Als5p. Therefore surface-displayed Als5p mediated binding to protein ligands but not to uncoated polystyrene.

**DISCUSSION**

The TR region of Als5p consists of six copies of a 36-mer repeat that is rich in Thr and other β-branched amino acids (8). Such repeats are common to all Als proteins and have been implicated in activity; those Als proteins with more repeats are often associated with a stronger adherence phenotype (16, 32). However, much of the adherence activity resides in the Ig-T region, because deletions in this region abrogated >90% of the binding activity in surface-displayed Als1p, and the adherence characteristics of surface-displayed Als5p/Als6p chimeras were determined by which Ig-T region was expressed (28, 32). These results and sequence analyses have led to suggestions that the TR region was either a spacer region or a site for cross-linking to the wall matrix (16, 19, 28, 32). In contrast, we find that the TR region has a defined secondary structure.
and contributes positively to the activity of the Als5p Ig-T region in vitro. In the surface display model, the repeats have adherence activity and, in addition, they augment the binding affinity and aggregation activity of surface-displayed Als5p.

**Glycosylation in the tandem repeats.** In Als5p, the Ig-like domains of C. albicans, there was substantial glycosylation in the fragment with the TR repeats but not in the Als5p Ig-T region that lacks the repeats. This glycosylation was reflected in the reduced mobility on gel electrophoresis, the reactivity with ConA, and the hexose content of the purified protein fragment. The glycosylation must be O linked because there are no N-glycosylation sites in the Ig, T, or TR region of Als5p (although some are present in the TR regions of other Als proteins). It is likely that this O glycosylation is important for structural and functional properties of this region. The O glycosylations in C. albicans and S. cerevisiae are similar, except that the C. albicans glycans lack an α-1,3-mannosyl residue present at the nonreducing end in S. cerevisiae (1, 15).

The lack of glycosylation in Als5p implies that the T region is not O glycosylated, despite its high Thr content (35%). This finding is consistent with its inclusion in models of Ig-like domains of Als5p (32). The occurrence of O glycosylations in only some domains is common with the homologous adhesin α-agglutinin of S. cerevisiae. In this protein, Ser and Thr residues N terminal to Ser282 are unglycosylated, but all scale comparison, the magnetic beads have a diameter of 4.5 μm. Error bars show standard deviations for quadruplicate samples. Surface expression levels were similar for all constructs (Fig. 1).

The T region of Als5p is important for protein secretion. The initial isolation and primary sequence profiling of ALS5 identified the region with high β-sheet potential in between the Ig domain and TR region as the conserved threonine region (8). Models of Als proteins include the T region as part of the Ig domain despite the fact that this region does not display structural or sequence similarity to immunoglobulin domains (32). We find that this region is essential for the secretion of soluble forms of the N-terminal regions of Als5p and for secretion and cell wall anchorage in the surface display model. Because proper folding is essential for secretion, it is likely that the T region forms a conserved part of the compact Ig-like region of Als proteins.

**The T region contributes to the secondary structure of the Als5p Ig-T region.** CD spectroscopy shows that Als Ig and Ig-T regions have β-sheet-rich structures, as expected for tandem Ig-like domains (17, 32). The CONTINLL analyses of CD spectra for Als5p (Ig-T-TR) also show a β-sheet-rich structure (Table 2). Therefore, the structure of the TR fragment has the same average conformation as the Ig-T fragment alone. This result is in accord with secondary structure predictions for this sequence, which is rich in β-branched amino acids with high β-sheet potential (4).

Because the TR region changed the CD spectrum somewhat, it must have a structure distinct from that of the Ig-T region. This difference is not due to differences in secondary structure content, so there must be differences in tertiary structure and glycosylation. Also, Als5p has different conformational states at different temperatures (data not shown), a characteristic not shared by the Als5p fragment.

**The Als5p TR region significantly enhanced binding to fibronectin.** The versions of Als5p that included the TR showed greater activity than those with TR deleted. Specifically, the relative affinity in vitro of the Als5p soluble fragments showed

![FIG. 8. Adhesion to FN-coated beads and aggregation of S. cerevisiae cells expressing Als5p with different numbers of tandem repeats. Binding in the bead aggregation assay was quantified for Als5p containing six repeats (Als5p), four repeats (Als5p), two repeats (Als5p), or no tandem repeats (Als5p). Error bars show standard deviations for quadruplicate samples. Surface expression levels were similar for all constructs (Fig. 1).](Image 70x562 to 274x722)

<table>
<thead>
<tr>
<th>Protein on cell surface</th>
<th>Protein on substrate</th>
<th>Adhesion</th>
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<tr>
<td>Als5p</td>
<td>FN</td>
<td>++</td>
</tr>
<tr>
<td>Als5p</td>
<td>Ig-T-TR6</td>
<td>+++</td>
</tr>
<tr>
<td>Als5p</td>
<td>BSA</td>
<td>++</td>
</tr>
<tr>
<td>Als5p</td>
<td>None</td>
<td>±</td>
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</table>

*The indicated proteins were coated onto the surface of microtiter wells, washed, and then incubated with cells as indicated. ++++, cells cover bottom of well; ++, cells cover >90% of well; +++, cells cover <50% of well; ±, a few bound cells; -, no significant binding.*

TABLE 3. Binding of Als5p-expressing S. cerevisiae to proteins

** FIG. 7. Adhesion to FN-coated beads and yeast cell aggregation. S. cerevisiae cells expressing Als5p (A and B), Als5p (C to F), Als5p (G), or no Als (H) were incubated with FN-coated magnetic beads (orange-brown), and the aggregates were separated and photographed (9). Panels B and F show immunofluorescence with anti-Als1p to demonstrate surface expression of Als5p in the aggregates. For scale comparison, the magnetic beads have a diameter of 4.5 μm.**

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that the TR region modifies binding activity independently of any role in extension from the wall (16) or cell wall anchorage (32). The FN titration assays with a constant Als5p concentration showed that Als5p$^{1-664}$ (Ig-T-TR$\alpha$) had affinity that was several orders of magnitude higher than that of Als5p$^{1-431}$ (Ig-T) (Fig. 6). Titrations with increasing Als5p concentrations at a constant FN concentration confirmed the difference in affinity and also showed that Als5p$^{1-664}$ binding was complex and not saturable at excess Als5p (data not shown). This non-saturability was consistent with the finding that Als5p can act as its own ligand, as illustrated in Table 2 and Fig. 7, which shows TR-dependent cell-to-cell binding. The overall conclusion is that the TR region of Als5p alters the structure of the binding regions and increases affinity for FN and for binding to itself as well. In the surface display model, the TR region itself had adherence activity.

Like the T region, the Als TR region is rich in Thr and other $\beta$-branched residues. This composition is found in adhesins of several pathogenic organisms, mucin proteins, and S. cerevisiae flocculins (26, 27). There may be analogous functional consequences to the Thr-rich regions in these proteins.

**Cellular consequences of Als structure.** Our results modify the idea that Als binding activity is localized to the Ig region alone and demonstrate a positive role for the TR in Als5p activity. This role could well have been missed in previous studies because CFU counting assays used to assess adherence are insensitive to aggregation of the fungal cells: an adherent single cell or a large aggregate will give rise to a single colony. Also, there must be some adherence activity in regions other than the Ig-T region, because ALS1 strains with deletions in the T region show residual ability to bind endothelial cells (28). We have specifically documented that the TR repeats in Als5p have a defined secondary structure and are highly O glycosylated. The repeats themselves have limited cell-to-cell binding activity, and they greatly increase cell-to-cell binding when coexpressed with the Ig-T regions. Versions of Als5p that included TR$\alpha$ also have increased affinity for FN in vitro. Thus, the activity of Als5p is described by a model in which the Ig-T region is necessary for most binding, with the T region being essential for folding and secretion. The TR region positively modulates adherence, increasing avidity for FN and facilitating the cell-to-cell binding that is a characteristic of C. albicans (2, 33).

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