Accessibility of the peptide backbone of protein ligands is a key specificity determinant in *Candida albicans* SRS adherence

Nand K. Gaur¹ and Stephen A. Klotz¹,²

Correspondence
Stephen A. Klotz
sklotz@u.arizona.edu

Research Service¹, Southern AZ VA Healthcare System and Department of Medicine²,
University of Arizona, Tucson, AZ, USA

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*Candida albicans* displays a high degree of specificity in selecting and adhering to targets *in vivo*. The features of target recognition are poorly understood and likely to involve more than the mere chemical composition of the ligand. Using an adherence assay in which protein and peptide ligands are covalently coupled to magnetic beads, the authors have previously described a new adherence mechanism in *C. albicans*, henceforth referred to as SRS (stable, reversible, specific) adherence. It was previously demonstrated that *C. albicans* and *Saccharomyces cerevisiae* expressing agglutinin-like sequence 5 protein (Als5p, previously referred to as Ala1p or Ala1/Als5p) adhere to peptides containing patches of threonine, serine and alanine residues when these are located in the free end of immobilized peptides. The interaction with protein ligands in SRS adherence predominantly involves the formation of hydrogen bonds. Accordingly, this interaction may occur (1) to the peptide backbone of the protein ligand or (2) to the amino acid side chain with an appropriate functional group. Evidence is provided that the primary interaction occurs with the peptide backbone and the secondary interaction occurs with the side chain. The primary interaction with the peptide backbone is sufficient for adherence to occur, whereas the secondary interaction with a side chain possessing an appropriate functional group stabilizes the interaction. In agreement with these results, it is also demonstrated that proteins lacking secondary and tertiary structure, wherein the peptide backbone is sterically accessible, interact with *C. albicans* and *S. cerevisiae* expressing Als5p. *C. albicans* Als proteins are resistant to denaturation by harsh conditions that kill the yeast cells. The proposed interactions in SRS adherence have striking similarities with those of the molecular chaperone Hsp70, which specifically binds to non-native proteins and resists denaturation.

**INTRODUCTION**

A remarkable relationship exists between *Candida albicans* and the human host. A minimum number of fungal cells reside indefinitely in the host without causing any apparent harm (Calderone & Braun, 1991; Pendrak & Klotz, 1995). This commensal relationship provides *C. albicans* with an unrestricted supply of food in exchange for keeping its population to a minimum. However, the relationship changes to a pathogenic one when host defences are compromised, in which case there is often proliferation of fungi at mucosal surfaces. We believe that understanding the mechanism of adherence in the commensal as well as the pathogenic state may lead to the development of novel therapeutic strategies.

The ability of *C. albicans* to establish and maintain residency at mucosal surfaces in the host is likely to be governed by mechanisms allowing it to adhere rapidly in a stable but reversible manner to host surfaces. A number of mechanisms of adherence have been described in *C. albicans*, although most involve relatively weak interactions and thus are less likely to be primary mechanisms of adherence *in vivo* (Calderone & Braun, 1991; Pendrak & Klotz, 1995). One stable adherence mechanism is the covalent coupling of a fungal cell surface protein, Hwp1, to host cells by the host enzyme transglutaminase (Stabb et al., 1999; Sundstrom, 2002). This mechanism provides for lasting adherence but it is not clear that it can be reversed. Furthermore, the expression of Hwp1 is restricted to the hyphal form, whereas both hyphal and yeast forms of *C. albicans* are known to adhere *in vivo* and are found in infected tissues. We have previously characterized a mechanism of adherence in *C. albicans* which displays properties such as stability to shear forces, adherence in acidic and neutral pH, resistance to the presence of various

**Abbreviations:** CM, carboxylate modified; FN, fibronectin; dFN, denatured fibronectin; LM, laminin; dLM, denatured laminin; SRS, stable, reversible, specific (adherence); TA, tosyl activated.
biological molecules and sensitivity to reagents known to disrupt hydrogen bonds (Gaur et al., 1999). To distinguish this mechanism of adherence from other previously described C. albicans adherence mechanisms we will henceforth refer to it as SRS (stable, reversible and specific) adherence. In contrast to Hwp1-mediated adherence, all morphological forms of C. albicans exhibit SRS adherence (Gaur et al., 1999, 2002).

A large gene family, the agglutinin-like sequence (ALS) family, has been described in C. albicans, where all members share similarities in the amino acid sequence at their N-termini and have similar predicted structural motifs (Hoyer, 2001). At least two members of the ALS family, Als5p and Als1p, have been shown to confer upon Saccharomyces cerevisiae properties similar to that of C. albicans SRS adherence (Gaur & Klotz, 1997; Fu et al., 1998; Gaur et al., 1999). It is expected that other Als proteins will have similar properties as they conserve all the structural features of Als5p.

It is widely accepted that the characterization of molecular changes occurring in the host as well as in C. albicans during the transition from commensal to pathogenic relationship is essential for the complete understanding of Candida pathogenesis (Casadevall & Pirofski, 2000; Van Burik & Magee, 2001; Soll, 2002). Accordingly, we have pursued a host-centred view in which the susceptible host may acquire new functions that are not prominent in the normal host. In this view of Candida pathogenesis, the generation of new SRS adherence target sites in the susceptible host may in part be responsible for the increased proliferation of C. albicans during candidiasis. This assumption necessitates the characterization of molecular features of target recognition in SRS adherence for the understanding of Candida pathogenesis. We have developed an adherence assay in which small synthetic peptides are immobilized in a controlled manner to orient them in specific configurations on the surface of magnetic beads (Gaur et al., 2002). These studies have suggested that in addition to chemical composition of the ligand, the accessibility of patches of certain amino acids is also important.

We continue to define the molecular features of SRS adherence targets in this study and report on the nature of the interaction of C. albicans and S. cerevisiae expressing Als5p with protein and peptide ligands. These studies have suggested functional similarities between Als5p interactions in SRS adherence and those of the molecular chaperone Hsp70, with the peptide backbone of protein ligands. We discuss the significance of the accessibility of the peptide backbone of protein ligands as an important specificity determinant in defining SRS adherence targets.

METHODS

Micro-organisms, plasmids and growth media. A wild-type C. albicans strain (CA1) originally isolated from a human source was used in these studies (Klotz et al., 1983; Gaur & Klotz, 1997). S. cerevisiae YPH499 (MATa ara3-52 lys2-801amber ade2-101 ochre trp1-d63 his3-d200 leu2-d1) was obtained from the American Type Culture Collection and used as a heterologous host for the expression of C. albicans adhesin Als5p. The low-copy and stable plasmid pGK114 was used for the expression of Als5p under the control of wild-type GAL1 inducible promoter (Gaur et al., 1999). C. albicans and S. cerevisiae YPH499 were grown in one of the following media: YPD (1 % yeast extract, 2 % peptone, 2 % glucose), YPRG (1 % yeast extract, 2 % peptone, 1 % raffinose, 1 % galactose) and SC (0-67 % yeast nitrogen base, 2 % glucose) with appropriate amino acid supplements at 50 μg ml⁻¹ final concentrations.

Peptides and magnetic beads. Peptides were chemically synthesized and purity determined by mass spectroscopy (Research Genetics, Huntsville, AL, USA). Tosyl-activated magnetic beads were purchased from Dynal and were coupled with fibronectin that had been denatured by boiling for 10 min (Gaur & Klotz, 1997). Carboxylate-modified magnetic beads were purchased from Seradyme and peptides were coupled to them using the carbodiimide coupling method as described before (Gaur et al., 2002).

Adherence assay. C. albicans yeast cells were grown in YPD and harvested from the late exponential phase of growth and S. cerevisiae harbouring pGK114 were grown in YPRG and harvested from the stationary phase for the adherence assay as described before (Gaur et al., 1999). Both yeast cells were washed and stored in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.0). The quantitative adherence assay using fibronectin (FN)-coated tosyl-activated (TA) magnetic beads and the qualitative adherence assay using peptide-coated carboxylate-modified (CM) magnetic beads were performed as described before (Gaur et al., 2002). Briefly, an excess of yeast cells was mixed with magnetic beads and incubated with shaking at room temperature for 30 min. Magnetic beads and adhered yeast cells were collected by a magnet and washed three times each with 1 ml TE buffer. For microscopic observation, adherent yeast cells were suspended in 0-1 ml TE buffer. Adherent yeast cells were dissociated from magnetic beads by suspending in an appropriate amount of 0-1 M NaOH, and beads and cells were counted using a haemocytometer. Experimentally consistent results of peptides or proteins incubated with yeast cells for 30 min prior to adding FN-coated TA magnetic beads. Denatured proteins were prepared just before use by incubating the required amount in a tube in a boiling water bath for 10 min. The stability of adherence was measured by preparing a microscope slide with cells adherent to polythreonine- or polyalanine-coated CM magnetic beads; the cover slip was sealed and the preparation observed under the light microscope. The slide was left at 4 °C or 25 °C for 24 h before observing it again under the microscope. Magnetic beads remained bound to yeast cells after 24 h incubation when the interaction was stable. When the interaction was less stable, the magnetic beads separated from yeast cells with less than 24 h, resulting in an abundance of free beads and yeast cells.

Statistical analysis. The adherence assay for each experiment was performed a minimum of three times. Results are presented as means and standard deviations and were analysed by using a paired Student t test with a P value of <0.05 considered significant.

RESULTS

Adherence to threonine and alanine peptides

We have reported previously that adherence of C. albicans and S. cerevisiae expressing Als5p occurs to 10-mer homopolymers of threonine, serine and alanine and to peptides
containing ‘patches’ of threonine residues (Gaur et al., 2002). The primary interaction involved in SRS adherence is through the formation of hydrogen bonds (Gaur & Klotz, 1997; Gaur et al., 1999, 2002). The side chains of threonine and serine contain a hydroxyl group, which is capable of forming a hydrogen bond, whereas the side chain of alanine lacks any group capable of forming a hydrogen bond. This observation implies that the primary interaction is not occurring through the side chain of amino acids but through the peptide backbone, which is the only other place where a hydrogen bond can be formed in a peptide. To investigate this possibility further we synthesized peptides containing four, six and eight threonine or alanine residues. These peptides were coupled to carboxylate-modified (CM) magnetic beads and were used in an adherence assay using C. albicans yeast cells and S. cerevisiae expressing Als5p. CM beads coated with 4-mers of threonine or alanine exhibited barely detectable adherence of C. albicans yeast cells and S. cerevisiae expressing Als5p, whereas 6-mers of either threonine or alanine exhibited maximum adherence of C. albicans and S. cerevisiae expressing Als5p. No significant increase in adherence of yeast cells was observed with 8-mers of threonine or alanine. These results suggest that for both threonine and alanine, six-residue peptides are sufficient to serve as targets for maximal adherence.

Adherence to threonine peptides is more stable than to alanine peptides

Since threonine and alanine side chains are chemically different and adherence occurring to peptides containing six residues of either threonine or alanine is phenotypically indistinguishable, we compared the stability of adherent yeast cells to threonine and alanine peptides. As shown in Fig. 1, the polyalanine-coated CM beads dissociated from C. albicans yeast cells within 24 h, whereas polythreonine-coated CM beads remained attached to the yeast cells. It is interesting to note that the dissociation of polyalanine-coated CM beads from the yeast cells also caused aggregates to disintegrate, resulting in free beads and cells. Similar results were obtained with S. cerevisiae expressing Als5p (results not shown). These results suggest that although the primary interaction occurs with the peptide backbone in alanine and threonine peptides, the interaction with the side chain of threonine and not alanine provides stability to the primary interaction.

Competition of adherence by small synthetic peptides

We have demonstrated previously that certain peptides serve as targets for adherence when immobilized on CM magnetic beads, whereas other immobilized peptides are

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Fig. 1. Comparison of the stability of adherence of C. albicans yeast cells to 10-mer peptides of threonine and alanine immobilized on CM magnetic beads (approx. diameter 0.7 μm). Photomicrographs were taken at 0 h and after incubating the slide for 24 h. Note that after 24 h of incubation Thr10-coated beads remained attached to yeast cell aggregates, whereas Ala10-coated beads dissociated from yeast cells in significant numbers. At this magnification the beads are barely visible. Bar, 500 μm.
not targets for adherence (Gaur et al., 2002). Specifically, 10-mer homopolymers of threonine (Thr<sub>10</sub>), serine (Ser<sub>10</sub>) and alanine (Ala<sub>10</sub>) served as targets for adherence but a collagen peptide, COL (Klotz & Smith, 1995; Gaur et al., 2002), was not a target for adherence. We used these same peptides in a free form (i.e. not immobilized) in a competitive adherence assay with C. albicans and S. cerevisiae expressing Als5p mixed with FN-coated TA magnetic beads. As shown in Fig. 2(a), Thr<sub>10</sub> and Ser<sub>10</sub> peptides were partially effective in inhibiting C. albicans adherence to FN-coated TA magnetic beads. No significant inhibition was observed with Ala<sub>10</sub> or the COL peptide. Almost total inhibition of adherence of S. cerevisiae expressing Als5p occurred with Thr<sub>10</sub> and Ser<sub>10</sub> peptides and partial inhibition with Ala<sub>10</sub> peptide (Fig. 2b). No inhibition of adherence of S. cerevisiae expressing Als5p was obtained with the COL peptide. These results extend previous observations demonstrating that many peptides that serve as targets of adherence also inhibit adherence of yeast cells to FN-coated TA magnetic beads. The magnitude of inhibition caused by peptides is more pronounced in S. cerevisiae expressing Als5p than in C. albicans. The partial inhibition observed in C. albicans may be related to the simultaneous presence of multiple Als proteins that may differ in their preference to peptide ligands.

### Stimulation of adherence by denatured proteins

We have observed previously that some proteins are capable of serving as targets for adherence when immobilized; however, many of these same proteins when in solution fail to competitively inhibit adherence of yeast cells to FN-coated TA magnetic beads (Gaur et al., 1999). For example, free plasma FN does not inhibit SRS adherence to FN-coated TA magnetic beads. In contrast, many small synthetic peptides that serve as targets for SRS adherence when immobilized are capable of inhibiting adherence when added exogenously to the mix of yeast cells and FN-coated TA magnetic beads (see results in previous section). Soluble native proteins have secondary and tertiary structures that may prevent interaction with C. albicans adhesins, whereas the small synthetic peptides may lack...
this feature. We therefore denatured the native proteins by boiling and immediately added them into the assay to determine if they would have an effect on adherence of *C. albicans* and *S. cerevisiae* expressing Als5p. Results obtained from these experiments using FN-coated TA magnetic beads as the adherence target mixed with *C. albicans* and *S. cerevisiae* expressing Als5p are shown in Figs 3(a) and 3(b), respectively. Addition of denatured laminin (dLM), fibronectin (dFN) and BSA (dBSA) resulted in a stimulation of adherence of *C. albicans* and *S. cerevisiae* expressing Als5p. In contrast, addition of native proteins had no effect on adherence (FN and BSA) or was inhibitory (LM). The timing of the addition of denatured proteins did not appear to be important, as stimulation of adherence occurred even when the denatured protein was added after completion of adherence (results not shown). These results show that native and denatured proteins have different interactions with *C. albicans* and *S. cerevisiae* expressing Als5p (see Discussion for explanation).

**Aggregation of yeast cells caused by denatured proteins**

In order to understand the mechanism of stimulation of adherence by denatured proteins, we compared the phenotypes of *C. albicans* and *S. cerevisiae* expressing Als5p after incubating yeast cells with native and denatured FN. *C. albicans* yeast cells showed significant aggregation when incubated with denatured FN as compared to native FN (Fig. 4). A preparation of *S. cerevisiae* expressing Als5p had a few small aggregates, which did not change significantly when incubated with native FN. However, enhanced aggregation of yeast cells was observed upon incubation with dFN. Similar results were obtained when denatured LM and BSA were used. The yeast cell aggregation induced by denatured proteins was stable, as aggregates could not be dissociated by vortexing or extensive washing with TE buffer. Incubation of *S. cerevisiae* (not expressing ALS5) with either native or denatured FN, LM and BSA did not cause aggregation.

**Adherence by live as well as dead *C. albicans* yeast cells**

The above results suggest a preferential interaction of *C. albicans* with denatured proteins or peptides lacking secondary structures. This suggested to us that Als proteins involved in SRS adherence might act in a similar way to the molecular chaperones that specifically interact with non-native proteins. One of the molecular chaperones, Hsp70, is resistant to thermal denaturation and can bind to denatured proteins and prevent their aggregation at elevated temperature. Therefore, we investigated the effect of temperature on stability of Als proteins by preincubating *C. albicans* yeast cells at different temperatures (4, 25, 37, 45, 55 and 70 °C) and then checking adherence of these cells at room temperature. Interestingly, *C. albicans* yeast cells that had been preincubated at all temperatures including...
70°C were fully competent in SRS adherence, although preincubation of the cells at 70°C resulted in a total loss of viability (Fig. 5). As heat-killed *C. albicans* yeast cells are fully competent in SRS adherence, we determined the adherence of *C. albicans* yeast cells killed by other means such as strong acid. As shown in Fig. 6, preincubation of *C. albicans* yeast cells in 0.1 M HCl for up to 6 h did not have any significant effect on SRS adherence. Preincubation of *C. albicans* yeast cells in 1 M HCl consistently had an initial stimulatory effect on SRS adherence followed by a gradual decline. Total loss of cell viability was observed within 30 min of incubation in 0.1 M or 1 M HCl. We have observed loss of *C. albicans* cell viability by incubation in 50% formamide or upon long storage, yet the SRS adherence activity of the yeast cells treated in this manner is unaffected. These results suggest that *C. albicans* adhesins for SRS adherence are resistant to denaturation and are fully functional in dead cells.

**DISCUSSION**

**SRS adherence involves formation of hydrogen bonds with the peptide backbone and amino acid side chains**

It has been previously demonstrated that SRS adherence is observed when proteins and peptides are covalently coupled to magnetic beads and primarily involves the formation of hydrogen bonds. This conclusion is based on the observation that formamide and urea, which can disrupt hydrogen bonds, specifically inhibit the interaction of *C. albicans* with the ligand and do not cause denaturation of adhesins (Gaur & Klotz, 1997; Gaur *et al.*, 1999). There are two places in a target peptide where the formation of hydrogen bonds can occur: the amino acid side chains with appropriate functional groups and the peptide backbone. Our results suggest that the primary interaction occurs to the peptide backbone and the secondary interaction occurs with groups capable of forming hydrogen bonds in the side chain of an amino acid.

Using peptides containing varying numbers of threonine or alanine residues, we have shown that peptides of a minimum of six amino acid residues must be present to serve as adherence targets. The side chain of threonine has a hydroxyl group capable of forming a hydrogen bond, whereas the alanine side chain contains a methyl group, which cannot form a hydrogen bond. Since six residues are required for both threonine and alanine peptides for maximal adherence to occur, we propose that the primary interaction occurs to the backbone of a minimum five peptide bonds. As demonstrated, adherence to threonine peptides is more stable than to alanine peptides, presumably because the threonine side chain has the potential to form a hydrogen bond that would provide stability to this interaction, whereas the side chain of alanine cannot form a hydrogen bond.

The secondary and tertiary structures of protein ligands prevent interactions with *C. albicans* and *S. cerevisiae* expressing Als5p. The discussion above suggesting the initial interaction of *C. albicans* Als proteins with the peptide backbone of a specific amino acid sequence is further supported by results obtained from the competition of adherence experiments using free peptides and proteins. Peptides that serve as targets of adherence when immobilized compete with the adherence of *C. albicans* and *S. cerevisiae* expressing Als5p to FN-coated TA magnetic beads when added exogenously to the assay. In contrast, when soluble native proteins are added to the assay, these proteins rarely have any measurable effect on adherence of *C. albicans* or *S. cerevisiae* expressing Als5p. However, if these same proteins are denatured and added to the assay, they paradoxically stimulate adherence by promoting yeast cell aggregation. Denaturation of proteins breaks the...
secondary and tertiary structures and, as shown in this work, only denatured proteins and not native proteins bind to *C. albicans* and *S. cerevisiae* expressing Als5p. Many peptide groups in native proteins are involved in the formation of hydrogen bonds that maintain the secondary and tertiary structure and thus are not available to interact with *C. albicans* Als proteins. These peptide groups of protein ligands probably become accessible for the interaction with Als proteins following denaturation.

**The mechanism of interaction in SRS adherence is similar to binding of the molecular chaperone Hsp70 to nascent polypeptides and small peptides**

Molecular chaperones are a group of proteins involved in protecting nascent polypeptides from misfolding and aggregation (Hartl & Hayer-Hartl, 2002). There are different classes of molecular chaperones; however, all of them function by their unique ability to interact with non-native proteins (nascent and unfolded polypeptides) and not with native proteins. One of these classes is the Hsp70 family, which is found in all living organisms. Hsp70 or DnaK has two functional domains: the N-terminus is an ATP-dependent regulatory domain that controls the activity of the C-terminal peptide-binding domain. It is estimated that in an average protein the Hsp70 binding sites occur approximately every 40 residues and typically are seven residues long with hydrophobic amino acids in their central region. The cocrystal structure of DnaK with a 7-mer peptide substrate has been solved and the primary interactions determined (Zhu et al., 1996). The structure which has been determined at 2.0 Å resolution clearly identifies the hydrogen bond interactions with the peptide backbone and hydrophobic interactions with the amino acid side chain. These interactions are strikingly similar to what we are proposing in SRS adherence, in which primary interactions are with the peptide backbone and secondary interactions are with the side chain through hydrogen bonds. The peptide backbone in the DnaK–peptide cocrystal structure has an extended conformation. Our results support a similar extended polypeptide structure of protein ligands in which the peptide backbone is accessible. The peptide-binding domain of DnaK is folded into a compact β-sandwich of two sheets with four antiparallel strands in each. Circular dichroism analysis of the N-terminal portion (Ig-like domain) of Als5p suggested an abundance of β-strands and very low helical content (Hoyer, 2001). As it is expected that the Ig-like domain of Als proteins is involved in the interaction with SRS adherence targets, these adhesins may also have structural similarities with Hsp70. Furthermore, *C. albicans* Als proteins are resistant to denaturation as is Hsp70. Thus, Als proteins may have similarities with Hsp70 in the way they interact with protein ligands as well as similarities in physical properties. To our knowledge this is the first example of receptor–ligand interactions in which the specificity is achieved by the lack of secondary structure in the protein ligands.

This work is the continuation of our efforts to characterize molecular features of protein ligands in defining SRS adherence targets that may be generated in the susceptible host during candidiasis. We have demonstrated in this work that the accessibility of the peptide backbone is an important specificity determinant in the interaction of *C. albicans* Als adhesins with protein ligands. In the susceptible host, SRS adherence targets with their accessible peptide backbone might be generated in response to the host inflammatory reaction or actions of the micro-organism. One such mechanism could involve *C. albicans*-secreted hydrolytic enzymes such as aspartic proteases and lipases. Both classes of enzymes are encoded by genes belonging to large gene families (Hube et al., 2000; Hube & Naglik, 2001; Naglik et al., 2003). The hydrolytic activity of these enzymes has the potential to modify host surfaces in ways that may make them more adhesive for *C. albicans*. The complete understanding of these mechanisms may provide an opportunity to design strategies to develop novel therapeutic approaches for treating candidiasis.

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