Strengthening relationships: amyloids create adhesion nanodomains in yeasts

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Abstract

Budding yeasts adhere to biotic or abiotic surfaces and aggregate to form biofilms, using wall-anchored glycoprotein adhesins. The process is paradoxical: adhesins often show weak binding to specific ligands, yet mediate remarkably strong adherence. Single-molecule atomic force microscopy, genomics, biochemistry, and cell biology have recently explained the puzzle, with *Candida albicans* Als adhesins as the paradigm. The strength of adhesion results partly from force-activated amyloid-like clustering of hundreds of adhesin molecules to form arrays of ordered multimeric binding sites. The various protein domains of eukaryotic adhesins cooperate to facilitate this fascinating new mechanism of activation.

Human and microbial amyloids

Protein amyloids (see Glossary) are justly infamous as markers of neurodegenerative diseases such as Alzheimer's, Parkinson's, and vCJD (mad cow disease), and so have been considered an abnormal state of proteins. However, it is clear that many sequences in the proteome can form amyloids [1], and that the ability to form ordered amyloid arrays can have positive functional consequences as well. In mammalian cells, functional amyloids include high-density storage granules of peptide hormones in exocytotic vesicles [2], protein templates for melanin deposition [3], and perhaps as factors in synaptic remodeling and

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Adhesin: a microbial cell adhesion protein or glycoprotein. The yeast adhesins are 600-1600 amino acids in length, are highly *N*- and *O*-glycosylated, and are covalently crosslinked to cell wall polysaccharides

Amyloid: an insoluble fibrous aggregate of proteins characterized by assembly of identical or near-identical short sequences in many molecules to form ordered regions of β-sheets orthogonal to the fiber axis. Amyloid-forming sequences are common, but usually buried within protein domains and inaccessible for amyloid interactions. Amyloids are associated with cytotoxicity in neurodegenerative diseases of humans.

GPI anchor: a glycosyl phosphatydylinositol phospholipid covalently attached to the C- terminal carboxylate group of an extracellular protein. In most eukaryotes, the lipid fatty acid chains are intercalated into the plasma membranes. In fungi, the GPIs of some proteins are cleaved in the glycan moiety, leaving the lipid portion in the membrane. The reducing end of the protein-associated carbohydrate moiety is covalently added to a hydroxyl group in the cell wall glucan.

Protein domain: in the sense used here, a compactly and independently folded region within a protein sequence. Such folded domains are typically about 100-300 residues. Long proteins such as yeast adhesins are composed of many sequential domains. This is a more restricted definition than common usage of 'domain' as any sequence fragment with an identifiable function.

learning [4]. In microbes, the list of functional amyloids is growing fast. Gram-negative adhesive curli and chaplins are discussed in an accompanying article by Blanco *et al.* [5]. Amyloids are also widely found in biofilms, although we are not yet sure what their role is [6]. In yeasts, heritable amyloid prions consisting of transcription regulators regulate gene expression and have been characterized both as disease states and as sources of evolutionary variability [7-10]. Amyloid interactions also mediate assembly of fungal hydrophobin cell coats, and are responsible for heterokaryon incompatibility and meoiotic drive in *Podospora anserina* [11, 12].

The recent discovery of functional amyloids in yeast cell adhesion proteins (adhesins) adds a new way of thinking about the roles of amyloids [13-16]. Cell surface clusters of amyloid-interacting adhesins can explain a long-standing paradox: there is very strong cell-cell and cell-substrate adhesion of fungi, but on the molecular level the assayed adhesins have weak ligand binding together with broad ligand specificity. Amyloid formation also helps to explain the unexpectedly complex domain structure of yeast adhesins.

Yeast cell adhesion proteins

Yeast adhesins mediate flocculation, invasive growth, biofilm, mat, and flor formation, and host-pathogen binding; and they share a basic architecture [17-20]. These adhesins bind carbohydrate ligands through N-terminal lectin domains or peptide ligands through tandem immunoglobulin (Ig)-like domains. C-terminal to these domains are variable numbers of β-sheet tandem repeat domains, long highly glycosylated extensions of up to 1000 amino acids, and at the C terminus, modified glycosylphosphatidylinisotol (GPI) anchors form covalent bonds to cell wall glucan fibers [17, 21]. Potential amyloid-forming sequences are found in various regions in the different adhesins; we are just beginning to identify those that are functional *in vivo* [14-16].

Candida albicans *Als adhesins as a paradigm*

The *Candida albicans* Als proteins are typical yeast adhesins. There are multiple isoforms encoded at 8 diploid loci in most strains [20]. Als adhesins share the common design features, shown in cartoon form in Figure 1a. Each Als protein has two tandem Ig-like adhesion domains [20, 22, 23]. This is followed by an evolutionarily conserved Thr-rich β-sheet domain with a 7-residue functional amyloid sequence, which mediates the molecular interactions described in this article. The Thr-rich domain is followed by highly glycosylated β-sheet tandem repeats (TR), and a long Ser-Thr-rich highly glycosylated stalk regions preceding the modified GPI anchor [17, 20, 24]. Als proteins bind to a wide variety of ligands with low affinity, but can mediate tight cell adhesion to both biotic and abiotic substrates [20, 25-28].

A remarkable trait of Als adhesins is that their different protein domains synergize to bind an extremely broad range of ligands. The N-terminal Ig-like regions bind to broad consensus "τφ+" peptides (τ, a residue common in turns; φ, a bulky hydrophobic residue; +, Lys or Arg), which constitute 2-5% of tripeptide sequences in the proteome [25]. Because this motif is usually buried in native proteins, they become much better ligands after denaturation, consistent with demonstrations that peptide flexibility is important, and that *C. albicans* preferentially binds to damaged regions of tissues and denatured proteins [22, 25, 29]. The list of peptide and protein ligands of Als proteins is impressively long, currently including dozens of different sequences in proteins and synthetic peptides [23, 25, 26, 29-32]. Als proteins bind to other Als proteins, as well as to non-related proteins on the surfaces of other yeasts and bacteria to form aggregates and co-aggregates, and may well be a factor in the high level of co-infection of *C. albicans* with other yeasts and bacteria [30, 33-37]. At least one Als protein can also bind to fucose-containing glycans, a finding that

further expands the range of potential ligands in host tissues [27]. The peptide and glycan interactions have K_d values near 10^{-4}-10^{-3} M, with rapid dissociation rates [27]. Binding to macromolecules is somewhat stronger, with K_d values ranging from 10^{-5}-10^{-6} M, values considered to be 'weak' [27].

The TR domains of Als adhesins mediate hydrophobic effect interactions that are even weaker, and may have little or no specificity [24, 38]. Each TR domain is a 36 residue β-sheet fold with large surface-exposed regions of aliphatic and aromatic amino acid side chains [24, 38-40]. These regions mediate adhesion to hydrophobic surfaces to initiate biofilms, and also interact with each other to promote Als protein homotypic binding (Figure 1) [13, 16, 19, 24, 37, 38]. Therefore, the Als adhesins bind many ligands, but with binding energies of a few kcal/mol. Weak ligand binding affinities and hydrophobic-effect interactions of tandem repeat domains are shared by other adhesin families, including other *C. albicans* adhesins, the *Saccharomyces* Flo family mannose-specific lectins and the *Candida glabrata* Epa galectins [17, 41, 42].

Cellular activation

Als adhesins mediate binding of yeast to ligand-coated beads. About 5 min after the cells bind to beads, other yeasts begin to bind to the yeast already bound to beads as an increased 'stickiness' propagates around the cell surface of the bound cells [39, 43, 44]. Cell-cell binding is strong enough to deform the cells and is resistant to high shear forces in vortex mixing. This activation is not affected by inhibitors of gene expression or signal transduction, or even by heat-killing the cells. Instead, the process is perturbed by agents that perturb protein secondary structural changes or amyloid formation [13, 43, 44].

Amyloid formation

Recent work demonstrates that Als activation is a consequence of the formation of cell surface arrays of hundreds of closely spaced molecules. The close packing results in the physical equivalent of multivalency. Because the binding sites are close to each other, a ligand which dissociates from one adhesin molecule is much more likely to rebind to another [45]. This local concentration effect mirrors the interaction of pentavalent IgM and its ligands, a phenomenon known as antibody avidity [46]. The formation of amyloid adhesion domains leads to a large gain in free energy of cell binding, with a concomitant decrease in macroscopic K_d value, but retains the extremely broad range of effective ligands. Depending on the degree of ordering and the local concentration of ligands and adhesins, the effective K_d value and strength of adhesion for the cell surface as a whole increase exponentially with the number of interacting pairs of molecules [46, 47]. For a system where there are several hundred adhesin-ligand pairs, the chances of dissociation become vanishingly small. Indeed, physicians sampling oral and vaginal *Candida* colonies may need to scrape hard enough to dislodge attached epithelial cells. Thus, the formation of amyloid regions in Als-expressing yeast cells generates strong interactions from adhesins with weak ligand binding.

We propose that a 5 to 7-residue core sequence forms the amyloids through repeating interactions between identical sequences in Als molecules [1, 7, 48]. The Als core sequences are unusual in their amino acid composition: about 70% Ile, Val, and non-glycosylated Thr, all β-branched aliphatic residues. (These sequences score highly positively in the β-aggregate predictor TANGO [49] and a 'steric zipper' geometric screen [1], but not in the sequence-based predictor, WALTZ [50].) This composition is conserved in diverse adhesins from different yeasts and different gene families [14-16]. The hydrophobicity of Ile and Val probably promotes rapid amyloid formation, and the lack of ionizable groups is consistent with the observed pH and ionic insensitivity of adhesion activation [39]. Thus, the amyloid-

like properties are complemented by high hydrophobicity, and are different from other amyloid sequences that are rich in Asn, Gln, and other polar residues [1, 50].

Als-mediated adhesion is activated by force-induced amyloids

The key discovery of amyloid nanodomains owes much to recent progress in atomic force microscopy (AFM) techniques, which can detect, localize, and force-probe single adhesin molecules on the surface of live cells [51, 52]. Such single-molecule AFM experiments directly demonstrate Als protein clusters on the cell surface following application of extension force to single molecules (Figure 2)[53], through a process modeled in Figure 1b.

An AFM probe is derivatized with an antibody to an epitope tag in Als5p, or with homologous Als5p protein itself, then tapped over the surface to find regions where Als molecules bind to the tip. As the AFM probe is withdrawn, force-extension curves for single Als molecules show successive unfolding of domains, and the extension lengths are consistent with initial unfolding of the Thr-rich domain, then the tandem repeats, and finally the Ig-like domains [40].

In an initial mapping of a $1\mu m^2$ area of the cell surface, there was a random pattern of Als5p molecules (Figure 2, second column) [13, 53]. However, upon re-mapping the same area, the Als5p molecules were clustered on the surface of the wall (Figure 2, third column). The adhesins are densely packed on the cell surface (Figure 1b), and we estimate about 5 molecules per pixel [45][17]. However, we propose that most of the molecules are cryptic, because they are bound to other adhesin molecules on the cell surface.

How do amyloids form on the cell surface?

The clustering is remarkable since the adhesins are anchored to the wall matrix. Amyloid interactions are possible because the long stalk regions allow each Als molecule to swivel around its attachment point to explore an area at least 240 nm in diameter [54][[17, 40, 53]. Therefore, each tethered molecule can interact with hundreds of others.

A model in Figure 1b shows how homotypic interactions between Als molecules on the surface of a cell could lead to amyloid nanodomain formation after stimulation by extension forces in the AFM or through cell-cell interactions. In the initial unactivated state, Als proteins would bind to each other through rapidly dissociable hydrophobic effect interactions of the TR domains, and probably ligand binding of Ig domains [22, 25, 28, 55]. Extension of single adhesin molecules by AFM should lead to partial unfolding of the Thr-rich domains, exposing the amyloid sequences, which sequentially immediately follow the Ig-like domains [22, 39]. This exposure makes a thermodynamic trap: a nearby molecule that has a transient exposure of the amyloid domain is likely to interact through its amyloid sequences, beginning to nucleate formation of the cell surface nanodomain [1, 48].

Importantly, both AFM and confocal microscopy demonstrate that the observed Als nanodomains result from amyloid interactions since the nanodomains do not form in *Saccharomyces cerevisiae* cells expressing Als5p with the non-amyloid-forming V326N single-site mutation (Figure 2 and Figure 3)[13, 40, 53]. Anti-amyloid dyes and a specific anti-amyloid peptide inhibit cellular aggregation as well as formation of nanoadhesion domains [13, 14]. Cells expressing non-amyloid Als5p^{V326N} are rescued for adhesion activation and for nanodomain formation by inclusion of a specific amyloid-forming peptide. Thus single-molecule, genetic and chemical experiments show that the amyloid-forming region is important and functional in formation of nanoadhesion domains and subsequent formation of strong adhesive bonds between cells.

Two lines of evidence suggest that the trigger for amyloid formation results from partial unfolding of an Als Thr-rich domain to expose the Als5p amyloid core sequence $I^{325}VIVATT^{331}$ and its homologs in other Als proteins (Table 1) [15, 16]. First, the Thr-rich domain appears to be the first to unfold in single molecule AFM [40, 53]. Second, soluble full-length Als5p forms amyloids *in vitro* under native-like conditions, suggesting that the core sequence can be exposed in these same conditions [14, 15]. These ideas support the model of Figure 1b, in which the Thr-rich domain is initially folded, but can partially unfold to expose the amyloid-forming sequence and initiate of amyloid nanodomains formation on the cell surface.

Another fascinating discovery is the ability of the nanodomains to grow and propagate across the entire cell. Using AFM and fluorescence microscopy, nanodomains were formed within ~30 min after force activation and migrated at a speed of ~20 nm·min^{-1}, indicating that domain formation and propagation are slow, time-dependent processes [13, 53]. The amyloid nanodomains recruit nearby Als molecules to activate more nanodomains around the entire cell surface, and might even spread from cell to cell (Figures 1b, Figure 2 last column, and Figure 3)[13, 43, 53], so the yeast cells aggregate homotypically. There is circumstantial evidence that amyloid formation strengthens cell-to-cell bonding (Figure 3d) [13, 14, 16], because anti-amyloid small molecules or peptides causes disaggregation, consistent with amyloid bonds between cells.

Synergistic roles of the different Als domains in amyloid adhesion

Formation of amyloid adhesion nanodomains is an emergent property, based on the activities of the various domains in Als (and also in other yeast adhesins) [13-16]. The initial adhesion to a surface is through Ig-domain-mediated binding to peptide ligands or hydrophobic effect surface binding of Als Tandem repeats [23]. In the C-terminal region of each adhesin, the long, glycosylated 'stalk' elevates the N-terminal and mid-regions away from the cell surface and allows the flexibility needed for adhesin molecules to interact and form amyloids *in vivo* on the cell surface [21, 28]. These essential domain properties are somewhat variable, so that different Als adhesins are specialized to mediate diverse biological functions including adhesion, aggregation, biofilm formation, Fe acquisition, and cell wall architecture [18-20].

Amyloids: a general and versatile mechanism for activating cell adhesion?

Other yeast adhesins also show activation by amyloid formation [14]. In surveys, most adhesins had predicted amyloid forming regions, with amino acid composition rich in β-branched aliphatic amino acids, similar to the Als sequences [14]. Several of these sequences were tested as peptides or as secreted proteins, including the *S. cerevisiae* flocculins Flo1p and Muc1p/Flo11p. All tested sequences formed amyloids. Furthermore, similar to Als aggregation, Flo1p-mediated flocculation and Muc1p-mediated flocculation were inhibited by anti-amyloid dyes [14]. Thus, in limited tests, amyloid formation is a common strategy for formation of robust adhesions in yeast cell adhesion proteins.

A metazoan connection?

The mosaic modular structure of metazoan cell adhesion molecules (CAMs) and the presence of potential amyloid-forming TANGO-positive sequences are tantalizing clues that mammalian cell adhesion molecules might have the ability to form amyloids as well. Additionally, yeast surface amyloid nanodomains are densely packed cell adhesion molecules reminiscent of metazoan desmosomes and spot junctions, as well as clusters of integrins or cadherins [56]. Many mammalian CAMs have analogous or homologous domains which occur in a similar order from the N-terminus towards the C-terminus: Ig or

lectin domains, Thr-rich mucin domains, small β-sheet-rich TRs (e.g. EGF domains) and poorly structured extracellular juxtamembrane 'stalk-like' regions [23]. Indeed, many of these CAMs have predicted amyloid forming sequences with composition similar to the Ile, Val, Thr-rich sequences of the fungal adhesins (Table 1). Thus, these cell adhesion proteins might be activated in a similar manner (Box 1), but this hypothesis is so far untested.

Conclusions

The complex domain structure of *C. albicans* Als adhesins is typical of fungal adhesins. Complementary single-molecule, genetic and chemical experiments have revealed that there is a synergistic relationship between the activities of the ligand binding domains, amyloid forming regions, and the less specific hydrophobic effect TR domains. The TR domains are responsible for initial fast, weak binding with hydrophobic surface, including homotypic binding to other TR domains. The peptide-binding Ig domains form bonds with diverse peptide ligands, with intermediate strengths of adhesion. Finally, the cells might be 'cemented' together and 'glued' to the substrate through strong homotypic amyloid bonds, which make nanodomain arrays of hundreds to thousands of activated interacting cell adhesion proteins. The formation of amyloid domains allows formation of strong cell-cell and cell-substrate bonds to an astounding variety of adhesive ligands to mediate biofilm formation as well as fungal-host and fungal-fungal interactions.

Acknowledgments

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Box 1. Outstanding questions

1. What is the structure of the amyloid-like domains on the cell surface?

2. What is the influence of the amino acid composition of the amyloid core sequences? What is the effect of length and flexibility of the stalk regions?

3. Can we design anti-amyloid treatments to disrupt adhesion in host-pathogen interactions and ameliorate disease?

4. What are the roles of adhesin amyoids in biofilm, flor, and mat formation?

5. Can we integrate the tools of biology and nanotechnology, as discussed here, to discover amyloid-like adhesion domains in other pathogens?
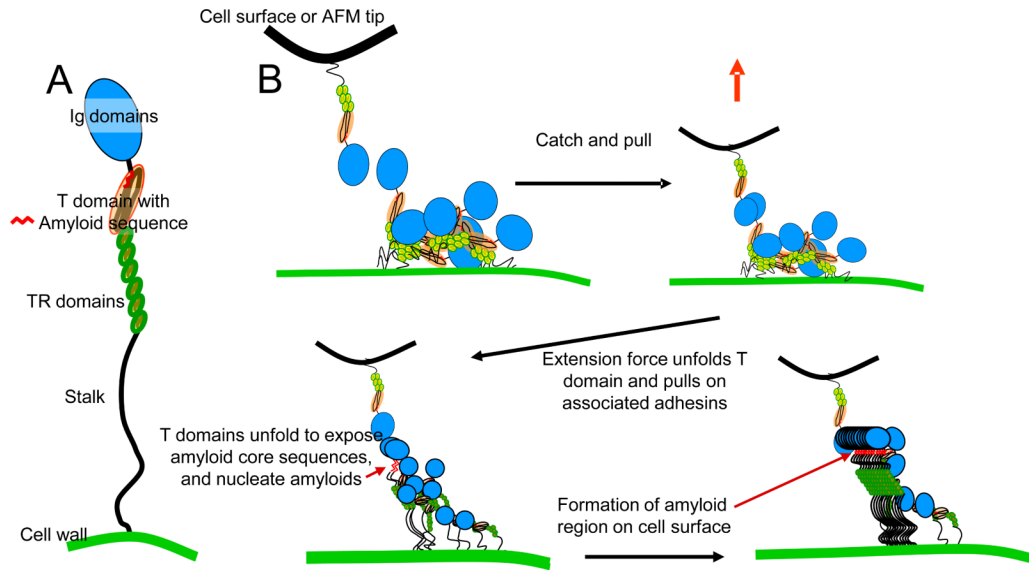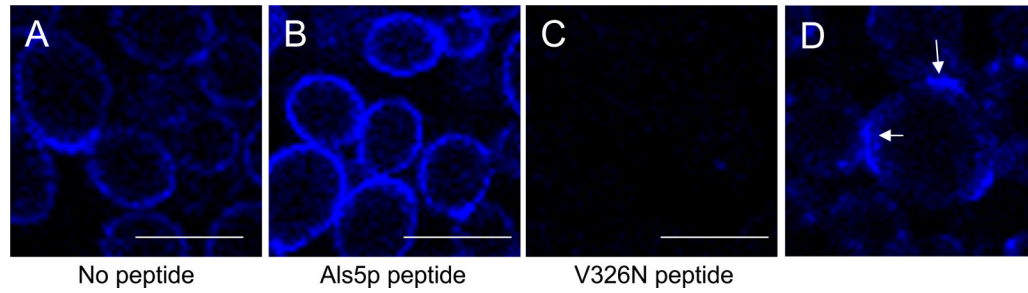
Figure 1.
Als proteins: multidomain adhesion proteins that form amyloids. **(a)** Model illustrating the domains of Als protein. **(b)** Model showing how homotypic binding of Als adhesins and mechanical stimulus lead to formation of cell surface amyloid nanodomains activating cell adhesion.
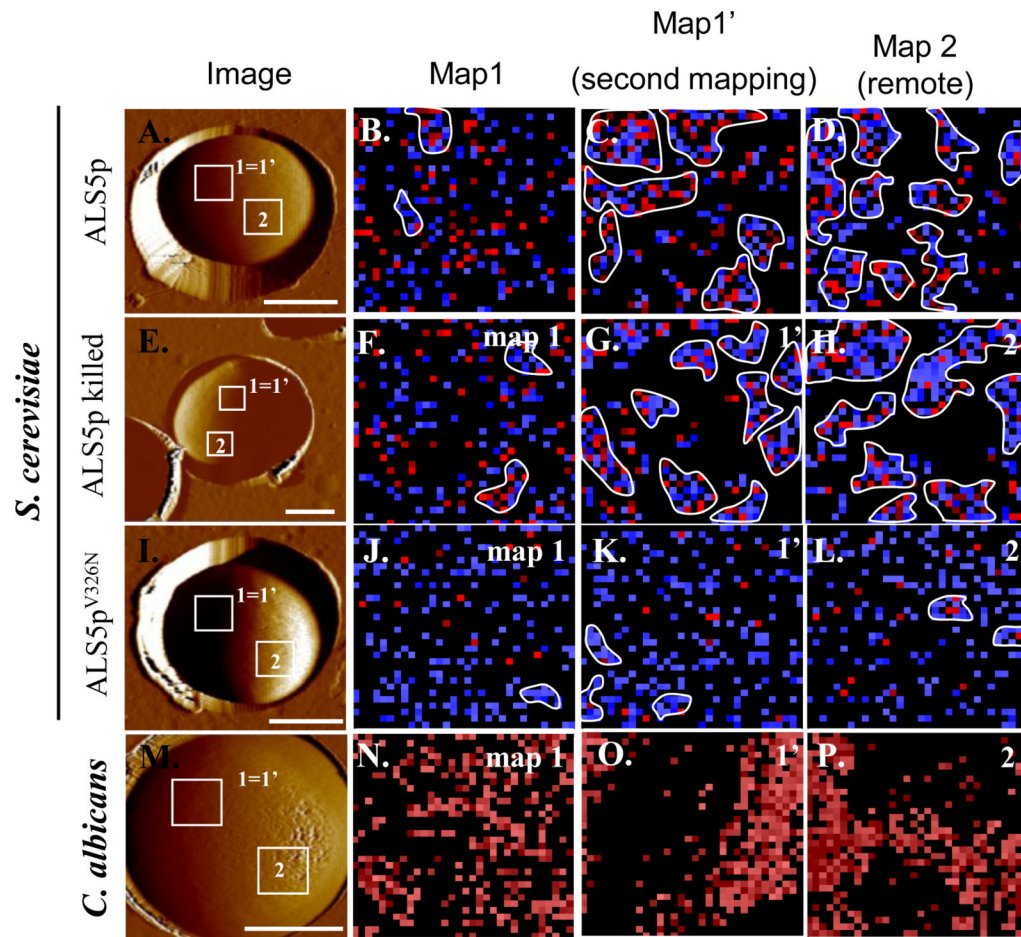
Figure 2.
Induction of surface nanodomains follows application of extending force to individual Als molecules on the surface of yeasts. On the left are AFM images of yeast trapped in a microporous membrane. The second column shows maps of position of Als proteins within the 1 μm square marked "1=1'." Blue pixels show interactions of <150 pN and red pixels show interactions resistant to forces >150 pN. Clusters of 10 or more contiguous colored pixels are outlined in white [13]. The third column shows clustering of the Als molecules in a second mapping of the same region of the cell walls, and the last column shows similar clustering in a remote region (Map 2). Clustering is a result of surface amyloid formation, as shown by lack of clustering in cells expressing the non-amyloid V326N form of Als5p. Reprinted with permission from Garcia *et al.* [13].

A	B	C	D
No peptide	Als5p peptide	V326N peptide	

Figure 3.
Confocal imaging of amyloid patches on aggregated *C. albicans* Day 286. These are activated in a cell adhesion assay and stained with thioflavin T, 100 nM. **(a)** and **(d)** aggregated cells. **(b)** Fluorescence was enhanced after incubation with an amyloid-promoting peptide from Als5p. **(c)** Anti-amyloid V326N peptide inhibits aggregation and extinguishes fluorescence [13]. **(d)** Regions of enhanced fluorescence are marked where cells are tightly apposed. Bars represent 8 μm. Reprinted with permission from Garcia *et al.* [13].

Table 1

Ile-, Val-, and Thr-rich potential amyloid sequences from yeast and mammalian CAMs [a]

	Accession number	Sequence	Position in ORF[b]	TANGO β-aggregate %[c]	%IVT[d]
Yeast proteins					
C. albicans Als1 Als3, Als5	EAK99144, EAK93472, EAK99139	IVIVATT	325	93	83
S. cerevisiae Flo1	AAC09499	TVIVI	308, 1547	43	100
S. cerevisiae Flo11	NP_012284	VVSTTV	1036, 1367	75	83
Mammalian proteins					
Integrin α4	NP 000876	VFVYI	340	94	60
		YIVLFY	502	69	33
N-CAM	AAB31836	IVTIVGL	660	98	71
e-Cadherin	CAA78353.1	VGVFII	202	98	67
		VISVVTT	325	83	87
		VVITTL	391	62	83
Galectin 4	AAP35679.1	LVFIVLA	103	99	43
Galectin 12	EAW74155.1	YVTTIF	49	99	67
SigLec	AAF34702.1	SVFVTAL	146	87	43

[a] More extensive lists of such sequences for other Als proteins and other yeast adhesins have been published [14-16].

[b] ORF denotes open reading frame.

[c] β-Aggregate potential of the designated sequence according to the predictor TANGO [15, 49].

[d] IVT indicates percent of Ile + Val + Thr in the designated sequence.