Heparin Binding Motifs and Biofilm Formation by *Candida albicans*

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ABSTRACT

Candida albicans is a leading pathogen in infections of central venous catheters, which are frequently infused with heparin. Binding of C. albicans to medically relevant concentrations of soluble and plate-bound heparin was demonstrable by confocal microscopy and ELISA assay. A sequence-based search identified 34 C. albicans surface proteins containing at least one match to linear heparin binding motifs (HBM). The virulence factor Int1 contained the largest number (five) of putative HBMs; peptides encompassing two of five motifs bound to heparin-Sepharose. Alanine substitution of lysine residues 805/806 in 804QKKHQIH (Motif 1 of Int1) markedly attenuated biofilm formation in central venous catheters in rats, whereas alanine substitution of lysine 1595/arginine 1596 in 1593FKKRFFKL (Motif 4 of Int1) did not impair biofilm formation. Affinity-purified IgG recognizing Motif 1 abolished biofilm formation in central venous catheters; pre-immune IgG had no effect. Soluble peptides from multiple C. albicans surface proteins such as Eno1, Pgk1, Tdh3, and Ssa1/2 but not Int1 were detected after heparin treatment of C. albicans, suggesting that heparin changes candidal surface structures and may modify some antigens critical for immune recognition. These studies define a new mechanism of biofilm formation for C. albicans and a novel strategy for inhibiting catheter-associated biofilms.
BACKGROUND

Heparin is a highly sulfated, non-branched, anionic polysaccharide composed of uronic acid (predominantly iduronic acid) in 1,4 linkage with glucosamine [1]. Doses of heparin up to 1000 units/ml are frequently used for anti-coagulation in central venous catheters [2]; hence, the interactions between heparin and microorganisms may have important implications for catheter-associated infections.

HIV-1 gp120 and Tat are known to bind to heparin [3, 4], and many more microbial proteins bind to heparan sulfate, an identical polysaccharide with reduced sulfation [for review see 1]. In one study, four intracellular DNA-binding proteins from C. albicans (Gcf1, Nhp6, Htb1, Hta1) bound to heparin-Sepharose [5]. However, the amino acids that mediate microbial binding to heparin have not been identified.

Eukaryotic proteins that bind heparin express conformational or linear heparin binding motifs. Conformational motifs include the recently described CPC clip motif, a structural signature in which two cationic residues surround one polar residue [6, 7]. Linear heparin binding motifs (HBMs) are conserved sequences of basic (B) and hydropathic (X) amino acids in specific patterns identified by Cardin (XBBXBX), Weintraub (XBBBXXBX), and Sobel (XBBBXXBBXBBX) [1, 8, 9]. Basic amino acids such as lysine, arginine, and rarely histidine are critical for interaction with anionic sulfate or carboxylate groups in heparin through electrostatic and hydrogen bonds. For example, substitution of alanine for basic amino acids in linear HBMs in the morphogen Sonic Hedgehog abolished binding to heparin [10]. Because of the widespread use of heparin in central venous catheters, we identified linear HBMs in C. albicans surface proteins and characterized their functions in heparin binding and biofilm formation.
METHODS

Sequence-based search for determination of linear heparin binding motifs

Non-redundant proteins were identified from Gene Ontology functional annotations in Candida Genome Database (www.candidagenome.org; Assembly 21) and relevant references [11, 12] and were mapped to Assembly 21. In the search for HBMs, basic amino acids were H, K, and R. Hydropathic amino acids were W, F, Y, L, I, C, M, G, V, S, T, A, N, P, and Q. The 159 proteins that met the search criteria were manually curated to confirm surface localization by prediction of a glycosylphosphatidylinositol anchoring sequence [13-16] or by assignment to the cell surface in the Candida Genome Database.

Strains, media, and chemicals

C. albicans laboratory strains used in this study were BWP17wt [17] and derivatives as described in Supplemental Table 1. All strains were maintained at -80°C in 20% glycerol. Working cultures were plated on Yeast/Peptone/Dextrose plates (10g yeast extract, 20g peptone, 20g glucose, 1.5% agar per liter) at 30°C for 48 hours then stored at 4°C.

Buffers including RPMI-1%HEPES and RPMI-5%MOPS were obtained from Life Technologies. Formaldehyde was purchased from Fisher; DMSO and calcofluor white from Sigma; filipin from Polysciences Inc.; and Fluoromount G from SouthernBiotech.

Commercial-grade heparin was purchased from Sigma; pharmaceutical heparin (1000 units/ml preservative free or 20,000 units/ml with 1.5 mg/ml methylparaben and 0.15 mg/ml propylparaben) from AAP Pharmaceuticals; and desulfated heparin analogs from Neoparin, Inc.
Binding of heparin-Alexa Fluor 488 to C. albicans

Heparin was labeled with Alexa Fluor 488 by a method modified from Osmond [18]. After purification on a PD-10 desalting column (GE Healthcare) equilibrated in autoclaved nanopure water, fractions containing the labeled material detected at 490 nm were combined and dried overnight on a SpeedVac concentrator (Savant) with heating. The resulting solid was redissolved in autoclaved nanopure water to 10 mg/ml and stored at 4°C.

After overnight growth at 30°C in YPD, 2 X 10^7 C. albicans cells (BWP17wt) were labeled with a PKH26 Red Fluorescent Cell staining kit (Sigma), according to manufacturer’s instructions, followed by Heparin-Alexa Fluor 488 (0.1 ml of 10 mg/ml solution). Organisms were incubated at 30°C with shaking (225 rpm) for 30 min, at which time a 0.4 ml aliquot was removed, pelleted (10,000 rpm for 3 min), and washed twice with PBS. After reconstitution with 0.5 ml PBS, DAPI (4’,6-Diamidino-2-phenylindole dihydrochloride, Sigma, stock solution of 5 mg/ml) was added to a final concentration of 1 µg/ml and the solution allowed to stand at RT for 10 min. Cells were pelleted and washed twice with PBS, then mounted on a microscope slide using Fluoromount G.

ELISA assay

Sigma heparin (fresh solution made daily), diluted to 25 units/ml in autoclaved, sterile-filtered PBS, was added as 0.1 ml aliquots (2.5 units) to each well of an allyl amine-coated 96-well heparin binding microtiter plate (BD Biosciences). In indicated experiments, equimolar amounts of desulfated heparins were added in lieu of heparin. The plate was incubated at room temperature overnight in the dark [19]. In the morning,
the plate was washed with acetate buffer (100 mM NaCl, 50 µM NaOAc, 0.2% Tween 20, pH 7.2), incubated with 3% bovine serum albumin (BSA) in PBS at 30°C for 1 hour, then washed with PBS. Overnight cultures of C. albicans BWP17wt grown at 30°C in YPD with shaking at 225 rpm were diluted to an OD₆₀₀ of 0.2 in 25 ml of YPD and grown at 30°C to mid-log phase (OD₆₀₀ 0.6-0.7). Cells were pelleted (3,000 rpm for 7 minutes), washed twice with PBS and diluted in RPMI-HEPES to 4X10⁵, 2X10⁵, and 1X10⁵ CFU/ml, respectively, before application of 100 µl per well. After incubation at 30°C for 1 hour and washing with PBS, 0.1 ml of biotinylated rabbit anti-C. albicans IgG recognizing soluble proteins in a C. albicans lysate (Meridian Life Science), diluted 1:2500 in FACS-Tween (0.3% BSA in PBS with 0.05% Tween 20), was added to each well. After incubation at 30°C for 1 hour and washing with PBS-Tween (0.05% Tween 20 in PBS), 0.1 ml of streptavidin alkaline phosphatase (Biolegend) diluted 1:10,000 in FACS-Tween was added, and the plate was incubated at 30°C for 30 minutes. After washing with PBS-Tween and AKP buffer (100 mM Tris base, 50 µM MgCl₂, 100 mM NaCl, pH 9.5.), 0.1 ml of alkaline phosphatase substrate (KPL) was added to each well for 45 minutes, then absorbance at 595 nM was read on a Beckman Coulter DTX 880. Experiments were performed in quadruplicate.

Construction of mutants

C.albicans genomic DNA was isolated from saturated overnight cultures using glass beads as described [20]. A lithium acetate method was used to transform C. albicans [17]. Plasmids and PCR products were purified using kits (Fermentas/ThermoFisher, Pittsburg, PA) or established methods [21]. Pfu enzyme (New England Biolabs) with High Fidelity buffer was employed for all amplifications. Products were
sequenced to affirm fidelity prior to use. Primers are described in Supplemental Table 2. A single copy of \textit{INT1}, including 1450 bp upstream and 548 bp downstream from the \textit{INT1} open reading frame (www.candidagenome.org, Assembly 21), was integrated into the hisG locus of the \textit{int1}^{-/} strain VBIDM2 [22] to produce the reconstituted strain KO509. Briefly, a copy of \textit{INT1} was generated by PCR, using primers 1 and 2 with BWP17wt DNA as template and cloned into the \textit{SacI}/\textit{MluI} sites of pGEMHIS [17] to create pKO509. pKO509 was digested with Swal and transformed into VBIDM2 to create the reconstituted strain KO509. PCR-mediated overlap extension mutagenesis [23] was used to produce copies of \textit{INT1} mutated at putative heparin binding domains. Briefly, primer pairs 1+3 and 2+4 (or 1+5 and 2+6) were used to produce two overlapping fragments of \textit{INT1} in which putative HBMs were mutated (FKKRFFKL $\rightarrow$ FKAAFFKL or KQKKHQ $\rightarrow$ KQAAHQ), and a full length mutated sequence generated in a third PCR using primers 1+2 with the fragments as template. The mutated sequences were cloned into the \textit{SacI}/\textit{MluI} sites of pGEMHIS to create pKO503 and pKO507, respectively. A construct mutated at both sites (pKO508) was produced using primers 1+5 and 2+6 with plasmid DNA from pKO503 as template. Full length mutated products cloned into pGEMHIS were used to transform VBIDM2 as above, producing strains KO503, KO507, and KO508. The correct insertion and orientation of all constructs were confirmed by PCR. In growth curves performed with and without 100 units/ml heparin at 30° C in RPMI-HEPES, there was no difference in the doubling times of the wild type, double disruptant, \textit{INT1} reintegration, or the reintegrants containing alanine substitutions in Motif 1, Motif 4, or Motifs 1&4.
Identification of peptides from *C. albicans* by mass spectrometry

Ten million (1 x 10⁷) *C. albicans* were incubated with 250 units of pharmaceutical heparin (Hep+) or without heparin (Hep-) in RPMI-HEPES for one hour at 37°C on a rotator. Organisms were pelleted and discarded, and supernatants were removed. 300 µl of Hep+ and Hep- supernatants were incubated with 100 µl avidin agarose beads (Thermo Scientific) for 60 min at room temperature on a rotator in the presence of 50 units heparin (Hep+); an equal volume of RPMI-HEPES was substituted for heparin in the Hep- supernatants. Beads were pelleted; 100 µl of beads were incubated with 100 µl 3.0 M NaCl for 30 mins at room temperature on a rotator. Beads were pelleted and the supernatants were withdrawn, precipitated with an equal volume of TCA, incubated on ice overnight at 4°C, and stored at -80°C until analysis by mass spectroscopy.

Six biological replicates of TCA precipitated proteins from equal cellular equivalence of heparin-treated (Hep+) and untreated (Hep-) conditioned medium from cultures of *C. albicans* were solubilized in 50 µL of Laemmli buffer. Samples were subjected to buffer exchange and concentration using an Amicon Ultra 3 kDa microfuge filtration cartridge at 14,000 x g for 15 min with 5 subsequent additions of 50 µL of 1X Laemmli buffer between spins. The resulting retained proteins (6 Hep+ and 6 Hep-) were subsequently prepared for SDS-PAGE by combining into 2 pools of 3 samples for the Hep+ and Hep- conditioned medium. The replicate sample pools were loaded onto two 4-12% mini gels and separated using the MOPS buffer system followed by silver staining (Sigma Proteosilver). Gel lanes from the replicates of Hep+ and Hep- samples were gridded into 11 equal regions followed by in gel trypsin digestion and extraction of peptides as described previously [24].
The recovered peptides from the gridded gel sections were analyzed by liquid chromatography coupled nano-electrospray mass spectrometry (nLC-MSMS) on a TripleTOFTM 5600 (AB Sciex, Toronto, On, Canada) attached to an Eksigent (Dublin, CA) nanoLC ultra nanoflow system. Only proteins with a minimum of 2 peptides with Mascot peptide score indicating a peptide identity and a false discovery rate (FDR) against an inverse database at less than 1% were reported. Semi-quantitative measurements between the Hep+ and Hep– proteins were generated by using a minimum of 2 tryptic peptides from each protein as surrogates for the amount of proteins from the two groups. This was accomplished by capturing extracted ion profiles for each peptide and comparing the mono-isotopic peak intensity at the apex of the signal for the M+2H of M+3H signal for each peptide.

**Antibody production**

A peptide corresponding to amino acids 799HKQEKQHHQIHKV812 (Motif 1 of Int1 underlined) was conjugated to KLH via an N-terminal cysteine residue (Pacific Immunology, Ramona, CA; www.pacificimmunology.com; NIH Animal Welfare Assurance Number A41820-01; USDA License 93-R-283). Two NZW rabbits were immunized once with conjugated peptide in a proprietary formulation of Freund’s complete adjuvant and boosted 3 times with conjugated peptide in Freund’s incomplete adjuvant. The same peptide was conjugated to CNBr-Sepharose and used for affinity purification of epitope-specific IgG. The final serum titer for both animals was >1:100,000 by ELISA. Pre-immune serum and serum from bleed 3 were chromatographed on a 2 ml Protein A column (Thermo Scientific) to yield pre- and post-immune IgG.
Rat model of biofilm formation

Polyethylene catheters were inserted into the jugular veins of female Sprague-Dawley rats, heparinized with 100 units heparin/ml, and clamped for 24 hours as previously described [25]. After removal of 0.5 ml for culture, the desired \textit{C. albicans} strain was instilled and allowed to dwell for 24 hours before animals were sacrificed. Catheters were removed aseptically as described [25] and prepared for scanning electron microscopy at 100X and 2000X to assess biofilm formation on the intra-luminal surface of the catheter. For antibody experiments, $1 \times 10^7$ \textit{C. albicans} yeast cells (BWP17wt) were pre-incubated with a 1:10 dilution of pre-immune IgG or affinity-purified IgG (both at 1.0 mg/ml) for one hour at 30°C, prior to injection into the catheter and retention for 24 hours.

Ethics statement

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin according to the guidelines of the Animal Welfare Act, The Institute of Laboratory Animal Resources Guide for the Care and Use of Laboratory Animals, and Public Health Service Policy.

Statistics

Statistics were performed using GraphPad Prism 6 statistical software package. The Bonferroni correction was applied whenever multiple comparisons were made.
RESULTS

Linear heparin binding motifs in Candida albicans surface proteins

Approximately 400 C. albicans proteins have been identified in cell wall fractions from yeast or hyphae, depending upon the extraction technique [11]. The 34 proteins in Table 1 include only those that have the attribution “cell surface”, “fungal cell wall”, “yeast cell wall”, or “hyphal cell wall” as the cellular component in the Candida Genome Database. Three of these proteins---Als7, Pga4, and Rbt1---have glycosylphosphatidylinositol anchors. The genes encoding eleven of 34 proteins---Als7, Cat1, Dot4, Eno1, Gph1, Ino1, Rbt1, Sam2, Srb1, Ssa2, and Ugp1---are located in newly defined transcriptionally active regions that are involved in biofilm formation [26].

No effects of heparin on C. albicans growth and morphology

One hundred units/ml of heparin---the concentration recommended to prevent clotting of central venous catheters [2]---had no effect on doubling times of planktonic yeast cells grown in YPD, RPMI-HEPES, RPMI-MOPS or CSM. There were no differences between heparin-treated and untreated organisms in formation of hyphae, integrity of membrane sterols, and location of septin rings (Supplemental Figure).

Binding of heparin by C. albicans in vitro

Deposition of soluble heparin-Alexa Fluor 488 was seen at the cell surface (Figure 1A iii) and at the interface of adjoining yeast cells (Figure 1A iv). In an ELISA assay, binding of C. albicans to solid-phase heparin remained stable from 1.25
units/well (6.3 µg/well) to 20 units/well (100 µg/well) (Figure 1B). Absorbance increased linearly as input of C. albicans increased from 1x10^4 to 4x10^4 CFU/well (Figure 1C).

Heparin’s extremely high anionic charge is contributed by sulfated residues at position 2-O of iduronic acid and the 2-N and 6-O positions of glucosamine. With equimolar amounts of desulfated heparins, binding of 4x10^4 C. albicans was decreased by 11% when heparin was desulfated at the 2-O position (p=0.003), and by 21% with desulfation at the 2-N position (p=0.002) (Figure 1D). Desulfation at the 6-O position did not significantly reduce binding. The 2-O and 2-N sulfate residues are adjacent in the helical wheel structure of heparin, potentially indicating a preferential “face” for heparin binding [27].

Int1, the surface protein that encodes the largest number of putative heparin binding motifs (Table 1), is a serodominant antigen involved in adhesion, filamentation, and virulence [28, 29]. To understand whether predicted heparin binding motifs in Int1 were functional, overlapping His-tagged polypeptides encompassing aa51-385, 385-659, 656-1193, 1188-1551, and 1548-1711 of Int1 were expressed in Saccharomyces cerevisiae. Polypeptides spanning aa656-1193 and aa1548-1711 bound to a heparin-Sepharose column and were eluted with 0.5-1 M NaCl. Polypeptides spanning aa51-385, 385-659, and 1188-1551 failed to bind.

Amino acids 656-1193 encompass one potential heparin binding site, 804QKKHQLHK (basic residues underlined; Motif 1 in Table 1). Amino acids 1548-1711 encompass a canonical Weintraub motif 1593FKRFFKL (Motif 4 in Table 1) and a canonical Cardin motif 1612SHKTRA (Motif 5). The three lysine residues and single arginine residue in Motif 4 are located on the rim of a positively charged pocket that
might facilitate binding to a strong anion such as heparin via electrostatic interaction. Motif 5 did not share this conformation.

Standard PCR-mediated mutagenesis [17] was used to derive a set of isogenic mutants with alanine substitutions in basic residues in heparin binding motifs 1 and 4. In addition to the wild type strain BWP17wt and the \( \Delta int1 \) double disruptant, four reintegrants were made by re-insertion of a single copy of the \( INT1 \) gene into the double disruptant. The reintigrant contained one wild type copy of \( INT1 \). In the remaining single-copy reintegrants, alanine substitutions were made in two basic residues in Motif 1 \((804QAAHQIHK)\), in Motif 4 \((1593FKAAFFKL)\), or in both Motifs 1 and 4.

Influence of heparin binding motifs on biofilm production in central venous catheters

In a rat model of biofilm formation in heparinized jugular venous catheters [25], the wild type strain BWP17wt generated a profuse biofilm (Figure 2A). Biofilm formation by the \( \Delta int1 \) double disruptant was much reduced (Figure 2B). Reintegration of one wild type copy of \( INT1 \) restored a profuse biofilm (Figure 2C). However, alanine substitution of lysines\(_{805/806}\) in Motif 1 greatly impaired biofilm formation (Figure 2D). Although alanine substitution of lysine\(_{1595}\) and arginine\(_{1596}\) in Motif 4 did not reduce biofilm formation, the Motif 1&4 mutant again produced sparse biofilm (Figure 2E,F). Preincubation of \textit{C. albicans} with an affinity-purified IgG antibody recognizing Motif 1 also markedly inhibited biofilm formation, although pre-immune IgG had no effect (Figure 3 A,B). Post-immune IgG antibody recognizing Motif 1 bound 10-fold more effectively to \textit{C. albicans} compared to pre-immune IgG, as assessed by flow cytometry.
(Figure 3C). These results show that lysines\textsubscript{805/806} in Motif 1 are essential for biofilm formation \textit{in vivo} and that an antibody against Motif 1 effectively inhibits biofilm formation.

\textbf{Elution of C. albicans surface proteins}

In other systems, heparin has been reported to cleave surface proteins [30] and change protein conformation [31]. After incubation with 250 units/ml heparin at 37° C for one hour, soluble peptides from more than 50 \textit{C. albicans} surface proteins were detected in the supernatant, as determined by mass spectrometry. Peptides representing 12 proteins were found in supernatants of untreated organisms. Heparin treatment led to a 7-fold increase in intensity of IDVVDQAK (Eno1), a 10-fold increase in intensity of SLLDAAVK (Pgk1), and a 5-fold increase in VPTTDVSVDLTVR (Tdh3) peptides (Figure 4A-C), compared to untreated organisms. The 10 proteins whose peptides were found in highest concentration in the supernatant (Table 2) are known to be localized to the cell surface. Six of 10--- Eno1, Tdh3, Pgk1, Ssb1, and Ssa1/2--- contain putative heparin binding motifs (Table 1) and are considered important antigens for innate and adaptive immune responses against \textit{C. albicans} [32-35]. Whether the presence of these antigens in the supernatant represents protein secretion, cleavage, or another form of removal is not known; however, their displacement from the candida cell surface may impair host recognition. Because soluble Int1 peptides were not identified after heparin treatment, Int1 remains a viable surface-expressed target for antibody therapy.
CONCLUSIONS

One of a plethora of virulence mechanisms, biofilm formation in *Candida albicans* involves numerous genes that affect adhesion, hyphal production, and secretion of extracellular matrix [36, 37]. Many of these genes are controlled by a network of six master transcriptional regulators [26]. In evaluating a possible relationship between *C. albicans*, heparin, and biofilm formation, attention to heparin concentrations used in humans is critical. Although in one study heparin concentrations (2,000 to 10,000 units/ml) inhibited *C. albicans* growth and biofilm formation *in vitro* [38], these concentrations of heparin are 2- to 100-fold greater than those routinely prescribed for use in central venous catheters [2]. Moreover, because *in vitro* assays of biofilm do not necessarily predict *in vivo* phenotypes [39], definitive testing is best performed *in vivo*.

Our results show that *C. albicans* binds both soluble and plate-bound heparin. Sulfation at the 2-O residue of iduronic acid and the 2-N residue of glucosamine correlates positively with binding. *In vivo*, lysines$_{805/806}$ of Motif 1 in Int1 are essential for biofilm production in central venous catheters. An IgG antibody directed against Motif 1 inhibits biofilm formation. As shown by the flow cytometry experiment (Figure 3, inset), the antibody to Motif 1 does not lyse the organisms. Although we do not yet know the mechanism of biofilm formation that is targeted by the antibody, there are at least two possibilities. First, heparin may serve as a bridge between heparin binding motifs on individual *Candida* cells, thereby facilitating cell-to-cell adhesion, an important early step in biofilm formation [36]. Alternatively, heparin may also link *Candida* cells and heparin binding proteins of the endothelial matrix such as fibronectin and thereby serve to tether the *Candida* cells to the endothelialized surface of the catheter.
These results have important clinical implications because of the use of heparin in central venous catheters, in which setting Candida spp. are the fourth most common cause of infections [40]. Indeed, the presence of central venous catheters is considered a major risk factor in patients at highest risk of candidemia [41]. In a comparison of chlorhexidine- and heparin-coated catheters, all candidal colonization and candidemias were found in patients who received heparin-coated catheters [42]. From this standpoint, monthly infusion of a humanized antibody directed against Motif 1 might be an effective preventative for patients requiring long-term central venous catheters.

Putative linear heparin binding motifs are also present in Staphylococcus epidermidis and Staphylococcus aureus (Jason Lu Long and Margaret Hostetter, unpublished data), two organisms that are even more prominent causes of catheter-associated infection [40]. Both clinical and laboratory studies have implicated heparin in biofilm formation by S. aureus [43, 44]. The presence of putative linear heparin binding motifs in several organisms that figure prominently in central line-associated bloodstream infections suggests that these motifs may serve as biomarkers for organisms able to form biofilms in the presence of heparin or may, as in the case of C. albicans, directly mediate this complication. In characterizing the mechanisms underlying the interaction between C. albicans surface proteins and heparin, the studies reported here define both a new mechanism of biofilm formation and a novel strategy for inhibiting catheter-associated biofilms.
REFERENCES


Figure Legends

Figure 1. *C. albicans* binds heparin *in vitro*. (A) Confocal microscopy of *C. albicans* after staining with PKH26 (i), DAPI (ii), heparin-Alexa Fluor 488 (iii). Arrows (iv) show co-localization of heparin with *C. albicans* cell surface. (B) Binding of 10,000 CFU *C. albicans* (OD_{595}) to increasing concentrations of heparin immobilized on an allyl amine-coated 96-well microtiter plate. Values are ± SD of duplicate wells. (C) Heparin binding ELISA assay with 2.5 units/well heparin and increasing *C. albicans* input. Graph represents mean ± SD of four experiments; *p < 0.007 for all inputs. (D) Binding of *C. albicans* (40,000 CFU/well) to equimolar amounts of heparin analogs desulfated at the 2-O, 2-N, or 6-O positions versus heparin control (normalized to 100%). Graph represents mean ± SD of three experiments, performed in triplicate *p < 0.003 vs. heparin control.

Figure 2. Lysine residues{805/806} in Motif 1 are essential for biofilm formation *in vivo*. Intra-luminal biofilm formation was assessed by scanning electron microscopy (SEM) in heparinized central venous catheters in rats after injection of (A) Wild type (WT), (B) *INT1* double disruptant, (C) *INT1* re integrant, (D) Motif 1 mutant, (E) Motif 4 mutant, (F) Motif 1&4 mutant. In each pair, SEM shows the intra-luminal biofilm at 100X (left panel) and 2000X (right panel). SEMs are representative of 4 animals infected with each strain.
Figure 3. IgG antibody recognizing Motif 1 abolishes biofilm. Intra-luminal biofilm after pre-incubation of *C. albicans* with pre-immune IgG (A) or post-immune IgG recognizing the peptide 799HKQEKKKHQ1HKV812 (B), which encompasses Motif 1 (underlined). SEM at 100X (left panel) and 2000X (right panel). SEMs are representative of observations in 3 animals. (C) Histograms representing binding of pre-immune IgG (purple curve) and post-immune IgG (blue curve).

Figure 4. Elution of candidal peptides after heparin treatment. Semi-quantitative analysis of soluble peptide intensity after incubation of *C. albicans* with (Hep+) or without (Hep-) at 100 units/ml heparin (A) Eno1 peptide; (B) Pgk1 peptide; (C) Tdh3 peptide.
FOOTNOTES

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Poster: Gordon Conference, January 2013, Galveston, TX
Platform presentation: St. Jude/PIDS, February 2013, Memphis, TN

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Optical Density (OD) at 10,000 CFU/well C. albicans

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C

D

Heparin: □ 2-O-Desulph □ 2-N-Desulph □ 6-O-Desulph

C. albicans (CFU/well)
Table 1. Putative linear heparin binding motifs in *C. albicans* surface proteins

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* = Weintraub motif  # = Cardin motif

A = "cell wall" in protein description (as of Jan 2011); obtained from Candida Genome Database (CGD)
B = "cell wall" in Gene Ontology (GO) annotation (as of Jan 2011); obtained from CGD
C = cell wall proteins as reviewed by Alberti-Segui [12]
D = cell wall proteins as reviewed by Chaffin [11]
Table 2. Proteins with the largest number of peptides recovered from supernatant after heparin treatment of *C. albicans*

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*Proteins with putative linear heparin binding motifs

#Surface-associated and cell wall proteins by GO