Heparin-Binding Motifs and Biofilm Formation by *Candida albicans*

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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 enzyme-linked immunosorbent assay (ELISA). A sequence-based search identified 34 *C. albicans* surface proteins containing ≥1 match to linear heparin-binding motifs. The virulence factor Int1 contained the most putative heparin-binding motifs (n = 5); peptides encompassing 2 of 5 motifs bound to heparin-Sepharose. Alanine substitution of lysine residues K805/K806 in 804QKKHQIHK811 (motif 1 of Int1) markedly attenuated biofilm formation in central venous catheters in rats, whereas alanine substitution of K1595/R1596 in 1593FKKRFFKL1600 (motif 4 of Int1) did not impair biofilm formation. Affinity-purified immunoglobulin G (IgG) recognizing motif 1 abolished biofilm formation in central venous catheters; preimmune IgG had no effect. After heparin treatment of *C. albicans*, soluble peptides from multiple *C. albicans* surface proteins were detected, such as Eno1, Pgk1, Tdh3, and Ssa1/2 but not Int1, 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.**

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

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Human immunodeficiency virus type 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 [reviewed in 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 (HBMs). Conformational motifs include the recently described CPC clip motif, a structural signature in which 2 cationic residues surround 1 polar residue [6, 7]. Linear HBMs are conserved sequences of basic (B) and hydropathic (X) amino acids in specific patterns identified by Cardin (XBBXBX), Weintraub (XBBBXXBX), and Sobel (XBBBXXBBBXXBBX) [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 HBMs

Nonredundant proteins were identified from Gene Ontology functional annotations in the 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 Supplementary Table 1. All strains were maintained at −80°C in 20% glycerol. Working cultures were plated on yeast peptone dextrose (YPD) plates (10 g of yeast extract, 20 g of peptone, 20 g of glucose, and 1.5% agar per liter) at 30°C for 48 hours then stored at 4°C.

Buffers, including Roswell Park Memorial Institute medium (RPMI)–1% HEPES and RPMI–5% MOPS (3-morpholinopropane-1-sulfonic acid), were obtained from Life Technologies. Formaldehyde was purchased from Fisher, dimethyl sulfoxide and calcofluor white from Sigma, filipin from Polysciences, and Fluoromount G from SouthernBiotech. Commercial-grade heparin was purchased from Sigma, pharmaceutical heparin (1000 U/mL preservative free or 20 000 U/mL with 1.5 mg/mL methylparaben and 0.15 mg/mL propylparaben) from AAP Pharmaceuticals, and desulfated heparin analogs from Neoparin.

Binding of Heparin-Alexa Fluor 488 to *C. albicans*

Heparin was labeled with Alexa Fluor 488 by a method modified from Osmond et al [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, 2×10^7 *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 minutes, at which time a 0.4-mL aliquot was removed, pelleted (10 000 rpm for 3 minutes), and washed twice with phosphate-buffered saline (PBS). After reconstitution with 0.5 mL of PBS, 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 room temperature for 10 minutes. Cells were pelleted, washed twice with PBS, and then mounted on a microscope slide using Fluoromount G.

ELISA Techniques

Sigma heparin (fresh solution made daily), diluted to 25 U/mL in autoclaved, sterile-filtered PBS, was added as 0.1-mL aliquots (2.5 U) to each well of an allylamine-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 mmol/L sodium chloride [NaCl], 50 µmol/L sodium acetate, 0.2% Tween 20; pH 7.2), incubated with 3% bovine serum albumin in PBS at 30°C for 1 hour, and 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 optical density at 600 nm (OD_{600}) of 0.2 in 25 mL of YPD and grown at 30°C to midlog phase (OD_{600}, 0.6–0.7). Cells were pelleted (3000 rpm for 7 minutes), washed twice with PBS, and diluted in RPMI-HEPES to 4×10^5, 2×10^5, and 1×10^5 colony-forming units (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 assay buffer (0.3% bovine serum albumin 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 assay buffer was added, and the plate was incubated at 30°C for 30 minutes. After washing with PBS-Tween and alkaline phosphatase buffer (100 mmol/L Tris base, 50 µmol/L magnesium chloride, 100 mmol/L NaCl; pH 9.5.), 0.1 mL of alkaline phosphatase substrate (KPL) was added to each well for 45 minutes, and then absorbance at 595 nmol/L was read on a Beckman Coulter DTX 880. Experiments were performed in quadruplicate.

Construction of Mutants

Candida albicans genomic DNA was isolated from saturated overnight cultures using glass beads, as described elsewhere [20]. A lithium acetate method was used to transform *C. albicans* [17]. Plasmids and polymerase chain reaction (PCR) products were purified using kits (Fermentas) or established

methods [21]. Pfu enzyme (New England Biolabs) with High Fidelity buffer was employed for all amplifications. Products were sequenced to affirm fidelity before use. Primers are described in Supplementary Table 2. A single copy of *INT1*, including 1450 base pairs upstream and 548 base pairs downstream from the *INT1* open reading frame (www.candidagenome.org; Assembly 21), was integrated into the hisG locus of the *int1*$^{-/-}$ strain VBIDM2 [22] to produce the reconstituted strain KO509. Briefly, a copy of *INT1* was generated by PCR, using primers 1 and 2 with BWP17wt DNA as template and cloned into the *Sac*I/*Mlu*I sites of pGEMHIS [17] to create pKO509. pKO509 was digested with *Swa*I and transformed into VBIDM2 to create the reconstituted strain KO509.

PCR-mediated overlap extension mutagenesis [23] was used to produce copies of *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 2 overlapping fragments of *INT1* in which putative HBMs were mutated (FKKRFFKL → FKAAFFKL or KQKKHQ →KQAAHQ), and a full-length mutated sequence was generated in a third PCR using primers 1 + 2 with the fragments as template. The mutated sequences were cloned into the *SacI/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 pKO503 as template. Full-length mutated products cloned into pGEMHIS were used to transform VBIDM2 as described 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 U/mL heparin at 30° C in RPMI-HEPES, there was no difference in doubling times between the wild type, the double disruptant, the *INT1* reintegrant, and the reintegrants containing alanine substitutions in motif 1, motif 4, or motif 1&4.

Identification of Peptides From *C. albicans* by Mass Spectrometry

Ten million (1×10^7) *C. albicans* were incubated with 250 U of pharmaceutical heparin (Hep^+) or without heparin (Hep^-) in RPMI-HEPES for 1 hour at 37°C on a rotator. Organisms were pelleted and discarded, and supernatants were removed. Next, 300 µL of Hep^+ and Hep^- supernatants were incubated with 100 µL of avidin agarose beads (Thermo Scientific) for 60 minutes at room temperature on a rotator in the presence of 50 U 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 of 3.0 mol/L NaCl for 30 minutes at room temperature on a rotator. Beads were pelleted, and the supernatants were withdrawn, precipitated with an equal volume of trichloroacetic acid (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 *g* for 15 minutes with 5 subsequent additions of ×1 Laemmli buffer (50 µL) between spins. The resulting retained proteins (6 Hep^+ and 6 Hep^-) were subsequently prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis by combining into 2 pools of 3 samples for the medium conditioned with Hep^+ and Hep^-. The replicate sample pools were loaded onto 2 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 elsewhere [24].

The recovered peptides from the gridded gel sections were analyzed by liquid chromatography coupled nanoelectrospray mass spectrometry on a TripleTOF 5600 (AB Sciex) attached to an Eksigent 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 against an inverse database at <1% were reported. Semiquantitative 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 2 groups. This was accomplished by capturing extracted ion profiles for each peptide and comparing the monoisotopic peak intensity at the apex of the signal for the M + 2H or M + 3H signal for each peptide.

Antibody Production

A peptide corresponding to amino acids 799HKQEKQKKHQIHKV812 (motif 1 of Int1 underlined) was conjugated to KLH via an N-terminal cysteine residue (Pacific Immunology; www.pacificimmunology.com; National Institutes of Health animal welfare assurance No. A41820-01; US Department of Agriculture 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 cyanogen bromide–Sepharose and used for affinity purification of epitope-specific IgG. The final serum titer for both animals was >1:100 000 by ELISA. Preimmune serum and serum from bleed 3 were chromatographed on a 2-mL Protein A column (Thermo Scientific) to yield pre- and postimmune IgG.

Rat Model of Biofilm Formation

Polyethylene catheters were inserted into the jugular veins of female Sprague-Dawley rats, heparinized with 100 U/mL heparin, and clamped for 24 hours, as described elsewhere [25]. After removal of 0.5 mL for culture, the desired *C. albicans* strain was instilled and allowed to dwell for 24 hours before

animals were euthanized. Catheters were removed aseptically, as described elsewhere [25], and prepared for scanning electron microscopy at ×100 and ×2000 to assess biofilm formation on the intraluminal surface of the catheter. For antibody experiments, 1×10^7 *C. albicans* yeast cells (BWP17wt) were preincubated with a 1:10 dilution of preimmune IgG or affinity-purified IgG (both at 1.0 mg/mL) for 1 hour at 30°C, before 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.

Statistical Analyses

Statistical analyses were performed using the GraphPad Prism 6 statistical software package. The Bonferroni correction was applied whenever multiple comparisons were made.

RESULTS

Linear HBMs in *C. 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 with one of the following listed as the cellular component in the Candida Genome Database: cell surface, fungal cell wall, yeast cell wall, or hyphal cell wall. Three of these proteins—Als7, Pga4, and Rbt1—have glycosylphosphatidylinositol anchors. The genes encoding 11 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

Heparin at 100 U/mL—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 Complete Supplemental Medium (CSM). There were no differences between heparin-treated and untreated organisms in formation of hyphae, integrity of membrane sterols, or location of septin rings (Supplementary Figure).

Binding of Heparin by *C. albicans* In Vitro

Deposition of soluble heparin–Alexa Fluor 488 was seen at the cell surface (Figure 1*A* iii) and at the interface of adjoining yeast cells (Figure 1*A* iv). In an ELISA, binding of *C. albicans* to solid-phase heparin remained stable from 1.25 U (6.3 μg) to 20 U (100 μg) per well (Figure 1*B*). Absorbance increased linearly as the input of *C. albicans* increased from 1×10^4 to 4×10^4 CFU per well (Figure 1*C*).

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 4×10^4 *C. albicans* was decreased by 11% when heparin was desulfated at the 2-O position ($P = .003$), and by 21% with desulfation at the 2-N position ($P = .002$; Figure 1*D*). 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 HBMs (Table 1), is a serodominant antigen involved in adhesion, filamentation, and virulence [28, 29]. To understand whether predicted HBMs in Int1 were functional, overlapping His-tagged polypeptides encompassing amino acids 51–385, 385–659, 656–1193, 1188–1551, and 1548–1711 of Int1 were expressed in *Saccharomyces cerevisiae*. Polypeptides spanning amino acids 656–1193 and amino acids 1548–1711 bound to a heparin-Sepharose column and were eluted with 0.5–1 mol/L NaCl. Polypeptides spanning amino acids 51–385, 385–659, and 1188–1551 failed to bind.

Amino acids 656–1193 encompass one potential heparin-binding site, 804QKKHQIHK (basic residues underlined; motif 1 in Table 1). Amino acids 1548–1711 encompass a canonical Weintraub motif 1593FKKRFFKL (motif 4 in Table 1) and a canonical Cardin motif 1612SHKTRA (motif 5). The 3 lysine residues and single arginine residue in motif 4 are located on the rim of a positively charged pocket that might facilitate binding via electrostatic interaction to a strong anion, such as heparin. 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 HBMs 1 and 4. In addition to the wild-type strain BWP17wt and the *int1* double disruptant, 4 reintegrants were made by reinsertion of a single copy of the *INT1* gene into the double disruptant. The reintegrant contained 1 wild-type copy of *INT1*. In the remaining single-copy reintegrants, alanine substitutions were made in 2 basic residues in motif 1 (804QAAAHQIHK), motif 4 (1593FKAAFFKL), or both.

Influence of HBMs 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 2*A*). Biofilm formation by the Δ*int1* double disruptant was much reduced (Figure 2*B*). Reintegration of 1 wild-type copy of *INT1* restored a profuse biofilm (Figure 2*C*). However, alanine substitution of lysines K805/K806 in motif 1 greatly impaired biofilm formation (Figure 2*D*). Although alanine substitution of lysine K1595 and arginine

Table 1. Putative Linear Heparin-Binding Motifs in *Candida albicans* Surface Proteins

Gene ID (Assembly 21)	Protein	Reference[a]	No. of Motifs	Motif 1	Motif 2	Motif 3	Motif 4	Motif 5
orf19.4257	Int1	C	5	$_{804}$QKKHQIHK[b]	$_{1383}$THKGRF[c]	$_{1530}$MKRGKP[c]	$_{1593}$FKKRFFKL[b]	$_{1612}$SHKTRA[c]
orf19.1738	Ugp1	B, D	3	$_{176}$SHRIRV[c]	$_{310}$IKKFKY[c]	$_{368}$IRHFKG[c]	. . .	. . .
orf19.3370	Dot4	B	3	$_{442}$NKKGKS[c]	$_{519}$CHKCHN[c]	$_{635}$FKRFKF[c]	. . .	. . .
orf19.3651	Pgk1	A, B, D	3	$_{136}$GKKVKA[c]	$_{146}$VKKFRQ[c]	$_{168}$AHRAHS[c]	. . .	. . .
orf19.4660	Rps6A	B, D	2	$_{185}$QRKRALKA[b]	$_{192}$AKKVKN[c]	. . .	. . .	. . .
orf19.5107	Not5	B	2	$_{114}$QKRSRF[c]	$_{333}$VKKLKP[c]	. . .	. . .	. . .
orf19.5130	Pdi1	D	2	$_{210}$NKKFKN[c]	$_{301}$GKKYRG[c]	. . .	. . .	. . .
orf19.6387	Hsp104	D	2	$_{53}$VKRARY[c]	$_{199}$ARRSKS[c]	. . .	. . .	. . .
orf19.1065	Ssa2	B, D	1	$_{258}$LRRLRT[c]	. . .	. . .	. . .	. . .
orf19.1067	Gpm2	B	1	$_{45}$IKKNHL[c]	. . .	. . .	. . .	. . .
orf19.1327	Rbt1	A, B, C	1	$_{121}$GKKVKQ[c]	. . .	. . .	. . .	. . .
orf19.2762	Ahp1	B, D	1	$_{171}$LKRIHN[c]	. . .	. . .	. . .	. . .
orf19.2803	Hem13	B, D	1	$_{257}$IRRGRY[c]	. . .	. . .	. . .	. . .
orf19.3590	Ipp1	B, D	1	$_{74}$TKKGKL[c]	. . .	. . .	. . .	. . .
orf19.377	Phr3	B, C	1	$_{115}$PHHHLNRY[b]	. . .	. . .	. . .	. . .
orf19.395	Eno1	B, D	1	$_{141}$AKKGKF[c]	. . .	. . .	. . .	. . .
orf19.3966	Crh12	A, B, C	1	$_{420}$TKHIHN[c]	. . .	. . .	. . .	. . .
orf19.4035	Pga4	B, C, D	1	$_{259}$AKRPRP[c]	. . .	. . .	. . .	. . .
orf19.4152	Cef3	B	1	$_{623}$LRKYKG[c]	. . .	. . .	. . .	. . .
orf19.4304	Gap1	B	1	$_{72}$QRKLKT[c]	. . .	. . .	. . .	. . .
orf19.4980	Ssa1	B, D	1	$_{259}$LRRLRT[c]	. . .	. . .	. . .	. . .
orf19.5188	Chs1	B, C	1	$_{126}$PKRQKT[c]	. . .	. . .	. . .	. . .
orf19.5788	Eft2	B, D	1	$_{581}$NKHNRI[c]	. . .	. . .	. . .	. . .
orf19.6190	Srb1	B, D	1	$_{123}$FHKAHG[c]	. . .	. . .	. . .	. . .
orf19.6214	Atc1	A, B	1	$_{959}$PKRVKV[c]	. . .	. . .	. . .	. . .
orf19.6229	Cat1	B	1	$_{82}$GKKTRI[c]	. . .	. . .	. . .	. . .
orf19.6367	Ssb1	B, D	1	$_{263}$LRRLRT[c]	. . .	. . .	. . .	. . .
orf19.657	Sam2	B, D	1	$_{380}$PKKLKF[c]	. . .	. . .	. . .	. . .
orf19.6814	Tdh3	B, D	1	$_{70}$GHKIKV[c]	. . .	. . .	. . .	. . .
orf19.7021	Gph1	B, D	1	$_{656}$TKHHIPKA[b]	. . .	. . .	. . .	. . .
orf19.7400	Als7	B, C	1	$_{1482}$SKRNKN[c]	. . .	. . .	. . .	. . .
orf19.7585	Ino1	B, D	1	$_{151}$MKRAKV[c]	. . .	. . .	. . .	. . .
orf19.7676	Xyl2	A, B, D	1	$_{330}$THRFKF[c]	. . .	. . .	. . .	. . .
orf19.940	Bud2	B	1	$_{716}$LRKGKS[c]	. . .	. . .	. . .	. . .

[a] Letter designations refer to descriptors found in the following sources: A, "cell wall" in the protein description obtained from the Candida Genome Database (as of January 2011); B, "cell wall" in the Gene Ontology annotation (as of January 2011) obtained from the Candida Genome Database; C, "cell wall proteins" in the review by Alberti-Segui et al [12]; and D, "cell wall proteins" in the review by Chaffin [11].

[b] Weintraub motif.

[c] Cardin motif.

K1596 in motif 4 did not reduce biofilm formation, the motif 1&4 mutant again produced sparse biofilm (Figure 2*E* and 2*F*). Preincubation of *C. albicans* with an affinity-purified IgG antibody recognizing motif 1 also markedly inhibited biofilm formation, although preimmune IgG had no effect (Figure 3*A* and 3*B*). Postimmune IgG antibody recognizing motif 1 bound 10-fold more effectively to *C. albicans* compared to preimmune IgG, as assessed by flow cytometry (Figure 3*C*). These results show that lysines K805/K806 in motif 1 are essential for biofilm formation in vivo and that an antibody against motif 1 effectively inhibits biofilm formation.

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 U/mL heparin at 37°C for 1 hour, soluble peptides from >50 *C. albicans* surface proteins were detected in the supernatant, as determined by mass spectrometry. Peptides

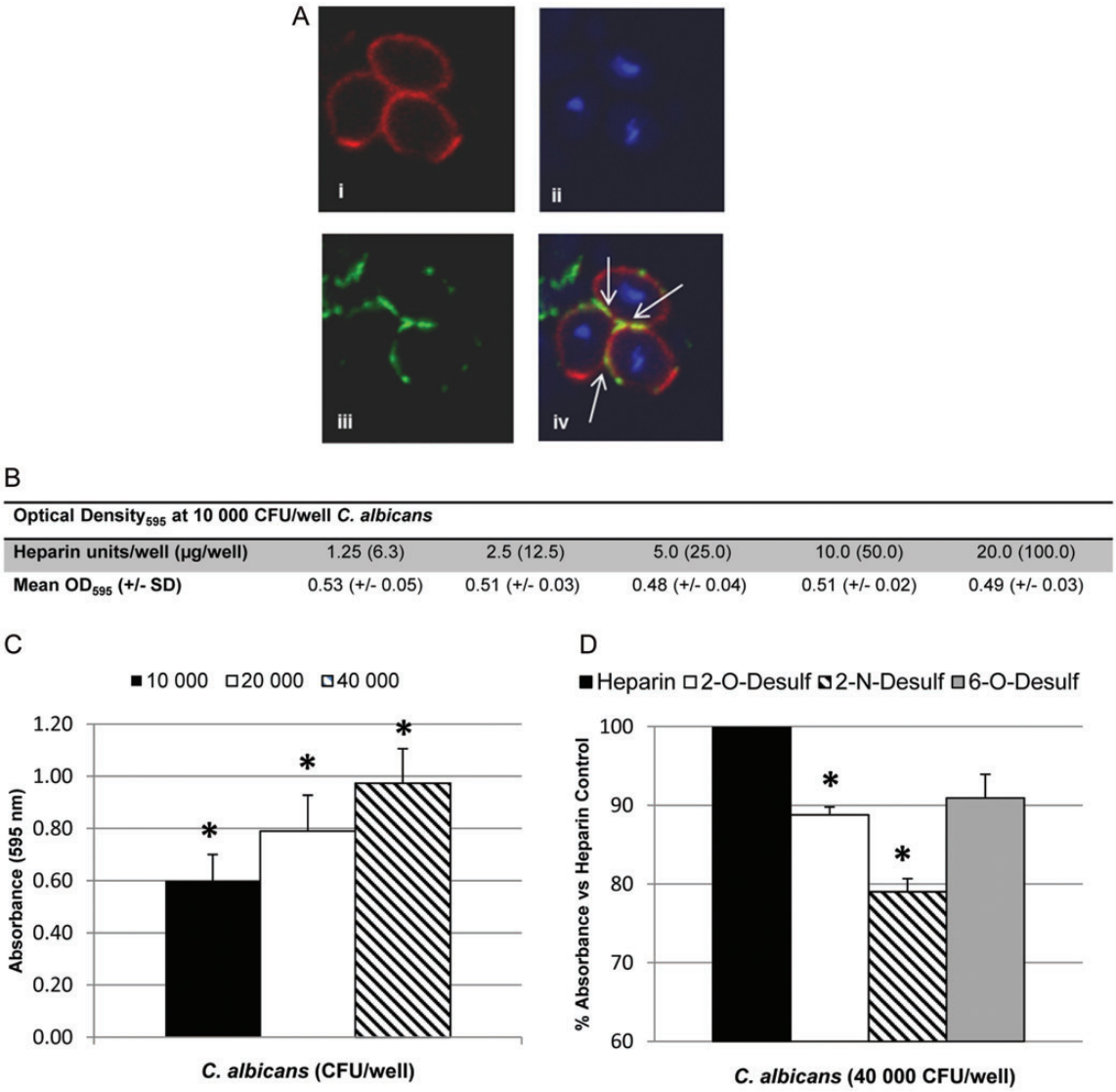

Figure 1. *Candida albicans* binds heparin in vitro. *A*, Confocal microscopy of *C. albicans* after staining with PKH26 *(top left),* 4′,6-diamidino-2-phenylindole dihydrochloride (*top right*), or heparin–Alexa Fluor 488 (*bottom left*). Arrows (*bottom right*) show colocalization of heparin with *C. albicans* cell surface. *B*, Binding of 10 000 colony-forming units (CFU) of *C. albicans* (optical density at 595 nm [OD_{595}]) to increasing concentrations of heparin immobilized on an allylamine-coated 96-well microtiter plate. Values represent means±standard deviations (SDs) of duplicate wells. *C*, Heparin-binding enzyme-linked immunosorbent assay with 2.5 U of heparin per well and increasing *C. albicans* input. Graph represents means ± SDs of 4 experiments; $^*P < .007$ for all inputs. *D*, Binding of *C. albicans* (40 000 CFU per 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 means ± SDs of 3 experiments, performed in triplicate $^*P \leq .003$ vs heparin control.

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 VPTTDVS VVDLTVR (Tdh3) peptides (Figure 4*A*–*C*), compared with 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 HBMs (Table 1) and are considered important antigens for innate and adaptive immune responses against *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 candidal 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.

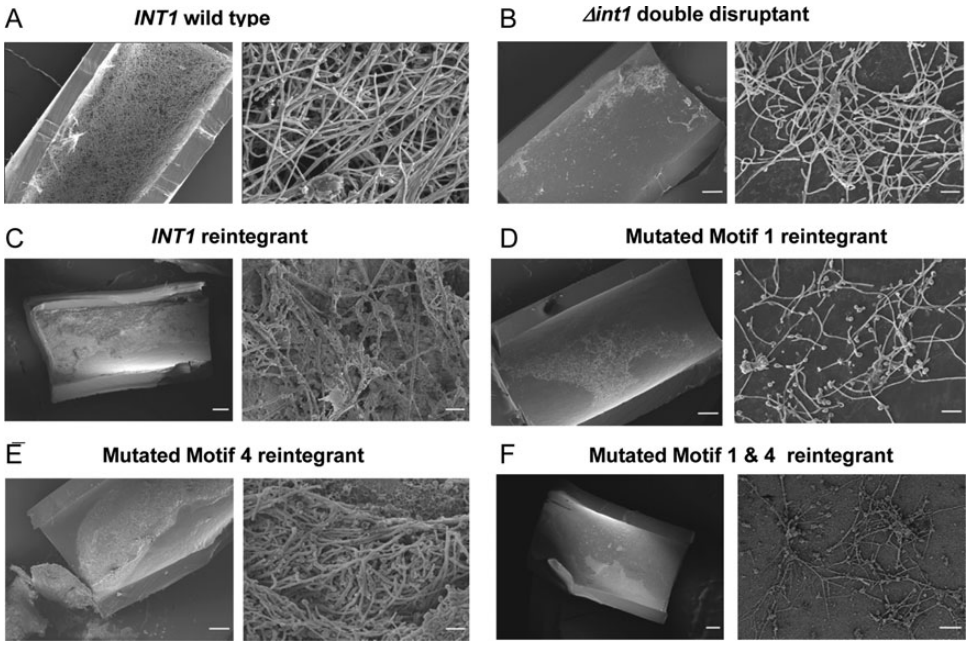

Figure 2. Lysine residues K805/K806 in motif 1 are essential for biofilm formation in vivo. Intraluminal biofilm formation was assessed with scanning electron microscopy in heparinized central venous catheters in rats after injection of wild type (*A*), *INT1* double disruptant (*B*), *INT1* reintegrant (*C*), motif 1 mutant (*D*), motif 4 mutant (*E*), or motif 1&4 mutant (*F*). In each pair, scanning electron microscopy shows the intraluminal biofilm at ×100 (*left panel*) and ×2000 (*right panel*). Micrographs are representative of 4 animals infected with each strain.

DISCUSSION

One of a plethora of virulence mechanisms, biofilm formation in *C. 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 6 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 (2000 to 10 000 U/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 K805/K806 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

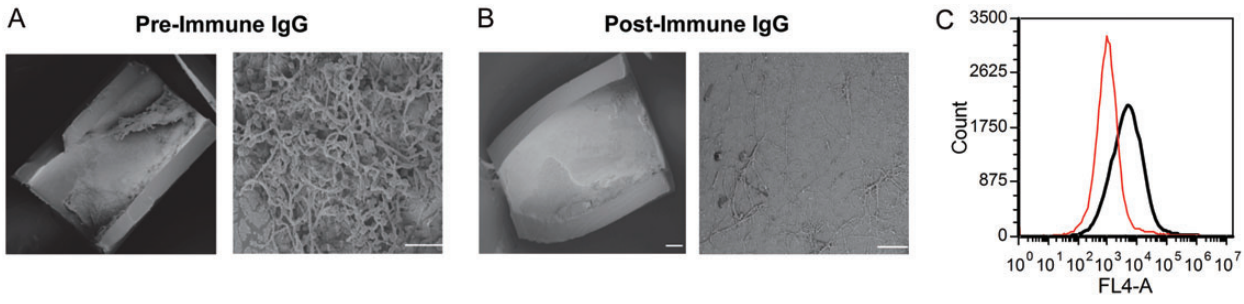

Figure 3. Immunoglobulin G (IgG) antibody recognizing motif 1 abolishes biofilm. Intraluminal biofilm after preincubation of *Candida albicans* with preimmune IgG (*A*) or postimmune IgG recognizing the peptide 799HKQEKQKKHQIHKV812 (*B*), which encompasses motif 1 (*underlined*). Scanning electron microscopy at ×100 (*left panel*) and ×2000 (*right panel*). Micrographs are representative of observations in 3 animals. *C*, Histograms representing binding of preimmune (*red curve*) and postimmune (*black curve*) IgG.

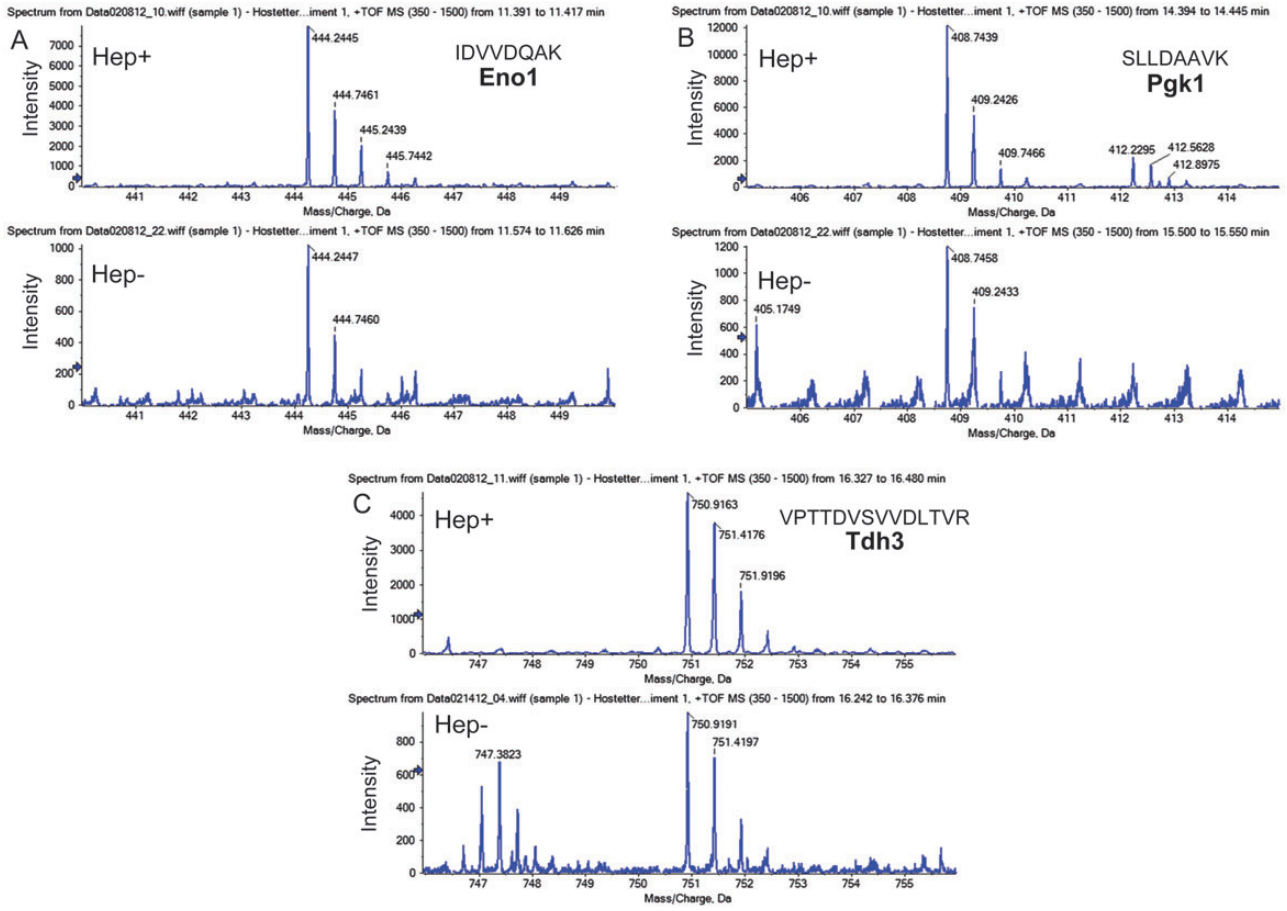

Figure 4. Elution of candidal peptides after heparin treatment. Semiquantitative analysis of soluble peptide intensity after incubation of *Candida albicans* with (Hep$^+$) or without (Hep$^-$) 100 U/mL heparin. *A,* Eno1 peptide. *B,* Pgk1 peptide. *C,* Tdh3 peptide. Abbreviation: TOF MS, time-of-flight mass spectrometry.

shown by the flow cytometry experiment (Figure 3C), 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 possibilities. First, heparin may serve as a bridge between HBMs 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.

Table 2. Proteins With the Most Peptides Recovered From Supernatant After Heparin Treatment of *Candida albicans*

ID	Protein Score	Protein Mass (Da)	Peptide Matches, No.
Eno1[a,b]	1751	47 202	53
Tdh3[a,b]	1154	35 925	57
Pgk1[a,b]	886	45 266	30
Ssb1[a,b]	849	66 610	25
Met6[b]	830	85 763	25
Ssa2[a,b]	827	70 199	24
Ssa1[a,b]	772	70 452	20
Atp2[b]	704	54 035	20
Pdc1[b]	594	62 744	17
Hsp90[b]	581	80 773	13

[a] Proteins with putative linear heparin-binding motifs.

[b] Surface-associated and cell wall proteins according to Gene Ontology annotations (Candida Genome Database, January 2011).

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 HBMs are also present in *Staphylococcus epidermidis* and *Staphylococcus aureus* (Jason Long Lu and Margaret Hostetter, unpublished data), 2 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 HBMs 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.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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