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Characterization of Binding of *Candida albicans* to Small Intestinal Mucin and Its Role in Adherence to Mucosal Epithelial Cells

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In order to approximate and adhere to mucosal epithelial cells, *Candida* must traverse the overlying mucus layer. Interactions of *Candida* species with mucin and human buccal epithelial cells (BECs) were thus investigated in vitro. Binding of the *Candida* species to purified small intestinal mucin showed a close correlation with their hierarchy of virulence. Significant differences ($P < 0.05$) were found among three categories of *Candida* species adhering highly (*C. dubliniensis*, *C. tropicalis*, and *C. albicans*), moderately (*C. parapsilosis* and *C. lusitaniae*) or weakly (*C. krusei* and *C. glabrata*) to mucin. Adherence of *C. albicans* to BECs was quantitatively inhibited by graded concentrations of mucin. However, inhibition of adherence was reversed by pretreatment of mucin with pronase or *C. albicans* secretory aspartyl proteinase Sap2p but not with sodium periodate. Saturable concentration- and time-dependent binding of mucin to *C. albicans* was abrogated by pronase or Sap2p treatment of mucin but was unaffected by β-mercaptoethanol, sodium periodate, neuraminidase, lectins, or potentially inhibitory sugars. Probing of membrane blots of the mucin with *C. albicans* revealed binding of the yeast to the 66-kDa cleavage product of the 118-kDa C-terminal glycopeptide of mucin. Although no evidence was found for the participation of *C. albicans* cell surface mannoproteins in specific receptor-ligand binding to mucin, inhibition of binding by *p*-nitrophenol (1 mM) and tetramethylurea (0.36 M) revealed that hydrophobic interactions are involved in adherence of *C. albicans* to mucin. These results suggest that *C. albicans* may both adhere to and enzymatically degrade mucins by the action of Saps, and that both properties may act to modulate *Candida* populations in the oral cavity and gastrointestinal tract.

Candida species are the most frequent cause of life-threatening invasive fungal infections in the immunocompromised host and are responsible for 10% of all nosocomial bloodstream infections (3). The leading cause of candidiasis, *Candida albicans*, resides as a commensal of the mucosae and the gastrointestinal tract (1). However, colonization often leads to opportunistic mucosal or deep organ infection in the immunocompromised host (9, 10, 16, 19, 27, 58). Invasion of the human gastrointestinal mucosa by *C. albicans* and its passage across the bowel wall into the bloodstream is an important portal of entry, leading to systemic or disseminated candidiasis (9, 10, 16, 27). The risk of invasive candidiasis is especially prominent in patients with acute leukemia (35). Factors responsible for gastrointestinal colonization and systemic dissemination, however, are not well understood.

Mucus O-linked glycoproteins (mucins) are produced and secreted from the mucus cells of salivary glands, esophagus, stomach, and small and large intestines, as well as the gallbladder and pancreatic ducts (reviewed in reference 26). Mucins have a high intrinsic viscosity due to their large size (2×10^6 Da) and extreme hydrophilicity, and they form a gel-like material which plays an important role in lubrication of epithelial surfaces and host defense. Many species of commensal (13) and pathogenic (2, 15, 29, 46, 53, 56, 57, 59–61, 64) bacteria have the ability to bind to and/or degrade mucins. The fate of pathogenic bacteria that bind to mucins can include (i) removal with mucus flow, (ii) colonization within the mucus layer, and (iii) penetration of the mucus and adherence to epithelial cells (26). Binding sites on mucins may compete with receptors on underlying epithelial cells, thereby retarding access of microorganisms to the mucosal surface and favoring their removal (26). However, degradation of mucins by bacterial proteinases or glycosidases has the opposite effect by facilitating penetration of the mucus barrier (26).

In contrast to the substantial body of information that exists regarding the interactions of bacteria with mucin, relatively little is known of the role of mucin in the pathogenesis of mucosal and invasive candidiasis. In intact hamsters inoculated intragastrically with *C. albicans*, indigenous bacteria colonizing the mucus gel act as a resistance barrier to mucosal association and systemic dissemination. In contrast, *C. albicans* is found embedded within mucus and on the surface of intestinal villi in antibiotic-treated animals (36). In addition to treatment with antibacterial antibiotics, penetration of the mucin layer may also be facilitated by the *C. albicans* secretory aspartyl proteinase Sap2p, which has been shown to degrade murine small intestinal mucin in vitro (11). Edgerton et al. (22, 23) showed that high- and low-molecular-weight mucins are involved in *C. albicans* adhesion to saliva-coated dental acrylic surfaces and that protein and carbohydrate moieties on the candidal surface appear to participate in the association. However, Hoffman and Haidaris (33, 34) could not confirm these findings and found that *C. albicans* binds to the heparan sulfate side chains of a nonmucin proteoglycan secreted by rat submandibular glands. Taken together, these studies raise the possibility that *C. albicans* may both associate with and degrade mucins and that both properties may act to modulate *C. albicans* populations in the oral cavity and gastrointestinal tract. Although the association of *C. albicans* with mucin has been documented, the mechanism of binding remains unknown.

The objectives of the present study were to (i) quantitate the differential binding of *C. albicans* and other *Candida* species to

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mucin, (ii) investigate the effects of mucin and Sap2p on the binding of *C. albicans* to buccal epithelial cells (BECs), and (iii) determine the potential receptors and ligands involved in binding of *C. albicans* to mucin. Our results showed that (i) binding of the *Candida* species to purified rabbit intestinal mucin is directly correlated with their hierarchy of virulence, (ii) mucin blocks the adherence of *C. albicans* to BECs, but Sap2p treatment of mucin can partially restore adherence, and (iii) *C. albicans* binds to the protein backbone of the 118-kDa C-terminal glycopeptide of mucin through hydrophobic interactions.

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MATERIALS AND METHODS

Strains and culture conditions. Strains used include *C. albicans* LAM-1 (N. Deslauriers [41]), LGH 1095 (K. C. Hazen [31]), CA30 (G. T. Cole [25, 43]), MYCO177 (Laboratoire de Santé Publique du Québec [LSPQ]), and 4918 (R. A. Calderone [6, 44]); *C. dubliniensis* MYCO1027 (LSPQ [18]), MYCO1181 (LSPQ [18]), MYCO1255 (LSPQ [18]), and MYCO1278 (LSPQ [4, 18]); *C. tropicalis* MYCO117 (LSPQ), MYCO774 (LSPQ), 93C1177 (Sainte-Justine Hospital [SJH]), and 94C234 (SJH); *C. parapsilosis* 90C5686A (SJH), 92P682 (SJH), 93C227 (SJH), and 94C213 (SJH); *C. krusei* MYCO152 (LSPQ), MYCO216 (LSPQ), LSPQ 68 (LSPQ), and 6258 (American Type Culture Collection [ATCC]); *C. lusitaniae* MYCO175 (LSPQ), MYCO735 (LSPQ), LSPQ 36 (LSPQ), and 66035 (ATCC); and *C. glabrata* MYCO576 (LSPQ), LSPQ 26 (LSPQ), 66032 (ATCC), and 90030 (ATCC).

Blastoconidia were grown in Sabouraud dextrose broth (Difco Laboratories, Detroit, Mich.) at 25°C for 24 h, or at 30 or 37°C for 18 h, with rotary agitation. The cells were collected by centrifugation and washed twice with 10 mM phosphate-buffered saline (PBS), pH 7.2.

CSH. Cell surface hydrophobicity (CSH) of *C. albicans* was measured using the microsphere assay as described by Hazen and Hazen (31). Microsphere attachment was assessed by bright-field microscopy at magnification of ×400. The percentage of cells with three or more attached microspheres was recorded as the percent hydrophobicity. *C. albicans* LGH 1095 was used as an internal control (31).

Purification of rabbit intestinal mucin. Rabbit intestinal mucin was purified essentially as described by Khatri et al. (37). Briefly, scrapings from the small intestinal epithelium of a male New Zealand White rabbit (weight, 1.5 kg) were quickly added to 75 volumes of 10 mM sodium phosphate buffer (pH 6.5) containing an inhibitor mixture of Na_2EDTA (5 mM), phenylmethylsulfonyl fluoride (1 mM), and *N*-ethylmaleimide (5 mM), plus 6 M guanidium hydrochloride. The scrapings were homogenized in a Waring blender for 25 s. Insoluble material was removed by centrifugation at 30,000 × *g* for 30 min at 4°C. The supernatant was adjusted to a concentration of 4 M guanidium hydrochloride, and CsCl was added to give a buoyant density of 1.40 g/ml. Density gradient ultracentrifugation was performed at 150,000 × *g* for 48 h at 4°C. Carbohydrate-rich mucin fractions were estimated by the periodic acid-Schiff procedure (62), conducted using nitrocellulose as solid support in a microsample filtration manifold (Minifold I; Schleicher & Schuell, Keene, N.H.). Graded amounts (0.4 to 50 μg) of porcine stomach mucin (type III; Sigma Chemical Co., St. Louis, Mo.) were used as a positive control. Mucin-rich fractions (fractions 3 to 7, ρ = 1.32 to 1.42 g/ml) were pooled, and a second identical density gradient ultracentrifugation was performed. The mucin-rich fractions (fractions 2 to 6, ρ = 1.34 to 1.46 g/ml) were dialyzed against 10 mM sodium phosphate buffer (pH 6.5) containing 0.2 M guanidine hydrochloride and 5 mM Na_2 EDTA. CsCl was added to give a density of 1.45 g/ml, and a third density gradient ultracentrifugation was performed. Carbohydrate-rich fractions (fractions 4 to 7, ρ = 1.39 to 1.49) were pooled, dialyzed against 4 M guanidium hydrochloride, and stored at −20°C. Immediately before further study, the mucin was thawed and dialyzed against PBS to remove guanidium hydrochloride.

Adherence of *Candida* to BECs. BECs were harvested from healthy adult male and female volunteers by gently scraping the mucosal surface of the cheeks with a cotton-tipped applicator and suspending the cells in PBS. The epithelial cells were washed twice in PBS, counted in a hemacytometer, and diluted to a density of 10^5 cells per ml of PBS. Adherence of *Candida* to the epithelial cells was conducted in Immulon 1 microtiter plates (Dynex Technologies Inc., Chantilly, Va.), treated with 100 μl of 0.01% poly-L-lysine (Sigma) per well, for 18 h at 4°C. The plates were washed three times with distilled water, and 100 μl of 1.25% glutaraldehyde was added to each well and kept for 5 min at 22°C. After two additional washes with distilled water, at least 3×10^4 of epithelial cells were distributed in each well to achieve confluence. The plates were centrifuged for 10 min at 4°C, and the wells were dried by incubating at 37°C for 18 h. *C. albicans* LAM-1 was grown in Sabouraud dextrose broth at 25°C for 24 h with rotary agitation, washed twice in PBS, and biotinylated by a modification of the method of Casanova et al. (8). Yeast cells were washed twice in 0.1 M PBS (pH 8.5) and suspended in the same buffer at 10^8 cells/ml. Aliquots of 50 μl of biotinamidocaproate *N*-hydroxysuccinimide ester (BNHS; 20 mg/ml of dimethyl sulfoxide [DMSO]; Sigma) were added to 2 ml of resuspended *C. albicans*, and the mixture was incubated with shaking for 1 h at 22°C. The cells were recovered and washed twice in 10 mM Tris-buffered saline (TBS; pH 7.5) containing 1% bovine serum albumin (BSA). *Candida* cells were freshly biotinylated prior to each adherence assay. Prior to use, plates containing BECs were washed twice with PBS, and the cells were fixed with a 0.2% solution of glutaraldehyde for 10 min at 22°C. The plates were washed twice with PBS, treated with PBS containing 5% BSA to block nonspecific binding, and washed five times with PBS. Graded concentrations of mucin in 100 μl of PBS, or PBS alone, were added to the wells, as well as mucin treated with pronase, Sap2p, or sodium periodate. Then 10^7 biotinylated *C. albicans* LAM-1 or 100 μl of PBS was added to the wells, and the plates were incubated at 37°C for 1 h with rotary agitation, washed five times with PBS, and fixed with 0.5% formaldehyde in PBS for 48 h at 4°C. The plates were washed three times with PBS containing 0.05% BSA, and streptavidin-horseradish peroxidase (Boehringer Mannheim, Mannheim, Germany) was diluted 1:2,000 in PBS containing 0.05% BSA and added to the wells (100 μl/well). The plates were incubated for 1 h at 37°C, washed three times with PBS containing 0.05% BSA, and washed twice with PBS. A substrate solution of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Boehringer Mannheim) was added to the wells (150 μl/well), and A_{410} readings were recorded after the appearance of color during incubation at 22°C. Wells containing BECs alone served as negative controls.

Adherence of *Candida* to mucin. Binding of *Candida* to mucin was quantitated by a microplate assay and by flow cytometry.

In the microplate assay, *C. albicans* LAM-1 was grown in Sabouraud dextrose broth at 25°C for 24 h with rotary agitation, washed twice in PBS, and suspended at 10^8 cells/ml. Then 10^7 cells in 100 μl of PBS was added to the wells of MaxiSorp microtiter plates (Nalge Nunc International Corp., Naperville, Ill.) and incubated for 1 h at 37°C. The wells were aspirated using a pipette, and the cells were fixed by adding 100 μl of 0.2% glutaraldehyde in PBS to each well for 10 min at 22°C. The plates were washed twice with PBS, treated with PBS containing 5% BSA to block nonspecific binding, and again washed twice with PBS. Then 100 μl of control or treated mucin (50 μg/ml), labeled on the peptidic portion with BNHS or on the glycosidic portion (12) with D-biotinyl-ε-amidocaproic acid hydrazide, was added to each well. The plates were incubated at 37°C for 1 h with rotary agitation, washed five times with PBS, and fixed with 0.5% formaldehyde in PBS for 48 h at 4°C. Subsequent steps were identical to those used in the assay of adherence of *Candida* to BECs.

Various sugars were included in the microplate assay in order to block potential specific receptor-ligand interactions. All sugars (α-L-fucose and D-mannose [Sigma], D-galactose [Difco], D-glucose [BDH Inc., Toronto, Ontario, Canada], and *N*-acetyl-D-glucosamine [Nutritional Biochemicals Corp., Cleveland, Ohio]) were used at a concentration of 25 mg/ml in PBS (14), and PBS alone served as a negative control.

Adherence of *Candida* to mucin was also quantitated by flow cytometry. For this purpose, mucin was labeled with fluorescein on the peptidic or glycosidic portion. Labeling on the peptidic portion was done by adding 1 mg of fluorescein-5-EX succinimidyl ester (Molecular Probes, Eugene, Oreg.) in 100 μl of DMSO to 150 mg of mucin in 2 ml of 0.1 M sodium carbonate buffer (pH 8.3) and then incubating the mixture for 1 h at 22°C in the dark. The labeled mucin was dialyzed against PBS containing 0.02% NaN_3 and kept at 4°C in the dark. The glycosidic portion of mucin was labeled by a modification of the method of Duijndam et al. (21). Mucin (150 mg) was first treated with 50 mM sodium periodate in 2.2 ml of 55 mM sodium acetate (pH 6.0) containing 3 mM EDTA. After incubation at 22°C for 1 h in the dark, the reaction was stopped with 63 μl of ethylene glycol, and the mucin was dialyzed against PBS. Then the mucin was labeled by adding 200 μl of fluorescein-5-thiosemicarbazide (1 mg/ml in DMSO; Molecular Probes) and incubating the mixture for 3 h at 22°C in the dark. The labeled mucin was dialyzed against PBS containing 0.02% NaN_3 and stored at 4°C in the dark. To compare binding of various *Candida* species to mucin, yeast cells were grown in Sabouraud dextrose broth at 25°C for 24 h with rotary agitation, washed twice in PBS, and suspended at 2×10^6 cells in 100 μl of PBS. Labeled mucin or 100 μl of PBS was added to the yeast cells, and the mixture was incubated for 30 min at 37°C. After two washes in PBS, the cells were resuspended and fixed in 400 μl of 2% paraformaldehyde in PBS. Analysis by flow cytometry was performed as previously described (17).

Treatments of mucin. To delineate the site(s) on mucin involved in binding to *C. albicans*, adherence assays were conducted with mucin treated with pronase, Sap2p, β-mercaptoethanol, sodium periodate, neuraminidase, or lectins.

A 5% (wt/vol) solution of mucin, sterilized by autoclaving, was incubated with various concentrations of pronase (type XIV, from *Streptomyces griseus*; Sigma) for 24 h at 37°C with rotary agitation, and the enzyme was inactivated by boiling for 10 min.

Recombinant *C. albicans* Sap2p was produced by *Saccharomyces cerevisiae* S86 plasmid transformant, grown in 1 liter of CAT medium at 30°C for 18 h, as previously described (20). The culture supernatant was passed through a filter (pore size, 0.22 μm), the pH was raised from 3.5 to 6.0 with NaOH to limit autodegradation of Sap2p, and the filtrate was concentrated in a stirred ultrafiltration cell (Amicon Inc., Beverly, Mass.) equipped with a YM10 membrane (10-kDa exclusion limit). The concentrate was diluted in 0.1 M sodium acetate

(pH 3.5), concentrated in a Centricon-10 tube (Amicon), and lyophilized. In a typical experiment, the Sap2p concentrate contained 660 μg of protein/ml, compared to 6.9 μg/ml in the unconcentrated culture supernatant. Proteolytic activity of the concentrate was demonstrated by degradation of casein in a petri dish assay (12) and quantitated by the following procedure. Sap2p concentrate (150 μl) was added to 600 μl of BSA (1% [wt/vol]) in 0.1 M sodium citrate buffer (pH 3.5) and incubated for 60 min at 37°C. Four hundred microliters of cold 10% trichloroacetic acid was added to precipitate proteins, and the samples were centrifuged at 1,500 × *g* for 10 min. Peptide breakdown products in the supernatant were estimated by measuring the A_{280} and protein concentration (Bio-Rad, Hercules, Calif.). Expressed in arbitrary units, the activity was 0.4 μg of released peptides/ml of supernatant/min/μg of Sap2p concentrate. No measurable Sap2p activity was found when the concentrate was boiled for 10 min or treated with pepstatin A (2 μg/ml) or when culture supernatants of *S. cerevisiae* antisense orientation constructs (20) were assayed. A 5% (wt/vol) solution of mucin in 500 μl of 0.1 M sodium acetate buffer (pH 3.5) was incubated with 2.5 mg of lyophilized Sap2p preparation for 24 h at 37°C and boiled for 10 min to inactivate the enzyme.

Treatment with β-mercaptoethanol was done by adding 25 μl to 475 μl of mucin (7.5 mg/ml), followed by dialysis against PBS.

Mucin (5% [wt/vol]) was treated with 50 mM sodium periodate in 0.1 M sodium acetate (pH 5.5) for 20 min at 22°C in the dark, with agitation. The reaction was stopped with glycerol, and the treated mucin was dialyzed against PBS.

For treatment with neuraminidase (sialidase, from *Vibrio cholerae* [E.C. 3.2.1.18]; Boehringer Mannheim), 120 μl of the enzyme (0.1 U/ml) was added to 1 ml of mucin (100 μg/ml) in 0.05 M sodium acetate (pH 5.5) containing 4 mM $CaCl_2$ and 100 μg of BSA/ml. After incubation at 37°C for 24 h, the enzyme was inactivated by boiling for 10 min. Positive and negative controls for neuraminidase activity were done using the substrate 2-(3′-methoxyphenyl)-*N*-acetyl-α-neuraminic acid and the assay method described by Palese et al. (52), performed in the presence or absence of the enzyme.

Biotinylated mucin was treated with various lectins of known sugar specificity in an attempt to block possible glycoside receptors for *C. albicans*. The lectins (Sigma) and their respective buffers were BSII (from *Bandeiraea simplicifolia*; specific for *N*-acetyl-D-glucosamine) in PBS supplemented with 0.1 mM each $CaCl_2$, $MnCl_2$ and $MgCl_2$; *Limulus polyphemus* agglutinin (specific for sialic acid) in 0.05 M TBS (pH 7.2) containing 0.1 M $CaCl_2$; winged pea lectin (from *Tetragonolobus purpureas*; specific for α-L-fucosyl residues) in PBS; Roman snail lectin (from *Helix pomatia*; specific for terminal *N*-acetyl-α-D-galactosaminyl residues) in PBS; and jequirity bean lectin (from *Abrus precatorius*; specific for D-galactose) in PBS. Lectins (100 μg/ml) were incubated with an equal volume of BNHS-labeled mucin (50 μg/ml) at 37°C for 30 min with gentle agitation (14). The mucin was pelleted three times by centrifugation for 10 min at 2,000 × *g* and resuspended in the appropriate buffer, and adherence to *C. albicans* was quantitated in the microplate assay. In experiments using concanavalin A (ConA; Sigma), the lectin (1 to 100 μg/ml in PBS containing 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$) was incubated with an equal volume of mucin (50 μg/ml) labeled on the peptidic portion with fluorescein, at 37°C for 30 min with gentle agitation. Binding of *C. albicans* to mucin was then quantitated by flow cytometry.

Control mucin was exposed to the same procedures described above, but including buffer instead of the active agents.

SDS-PAGE and probing of membrane blots. Samples of mucin were boiled in sodium dodecyl sulfate (SDS)-sample buffer with or without β-mercaptoethanol and loaded in a 7% polyacrylamide separating gel to perform SDS-polyacrylamide gel electrophoresis (PAGE) (42) as previously described (11). Following electrophoresis, protein bands were visualized by silver staining or transferred onto polyvinylidene difluoride (PVDF) membranes (Boehringer Mannheim) by the method of Towbin et al. (63) and dried at room temperature. The membrane blots were then probed with BNHS-labeled *C. albicans* LAM-1, grown in Sabouraud dextrose broth at 30°C for 18 h. Briefly, the PVDF membranes, cut into vertical strips, were placed in a small incubation tray (Bio-Rad, Hercules, Calif.) containing 2 ml of 1% (wt/vol) skim milk and 1% (wt/vol) BSA in PBS (PBS-SB) per lane. Then, 2×10^8 biotinylated *C. albicans* cells were added to each lane, and the tray was incubated for 1 h at 37°C with gentle agitation. The strips were washed twice with TBS-T (TBS containing 0.05% [vol/vol] Tween 20) and twice with PBS-SB, 1 ml of streptavidin-horseradish peroxidase diluted 1:1,500 in PBS-SB was added, and the tray was incubated for 1 h at 22°C with gentle shaking. The strips were washed twice with TBS-T, the luminescent substrate was added as recommended by the manufacturer (BM Chemiluminescence Blotting Substrate; Boehringer Mannheim), and the strips were then exposed to X-Omat AR imaging film (Eastman Kodak Co., Rochester, N.Y.). PVDF strips not containing mucin served as a negative control, while strips obtained after transfer of porcine stomach mucin resolved by SDS-PAGE in reducing conditions was used as a positive control.

Statistical analysis. The one-way analysis of variance and the Mann-Whitney *U* test were used for statistical analyses (24). Differences were considered significant at $P < 0.05$.

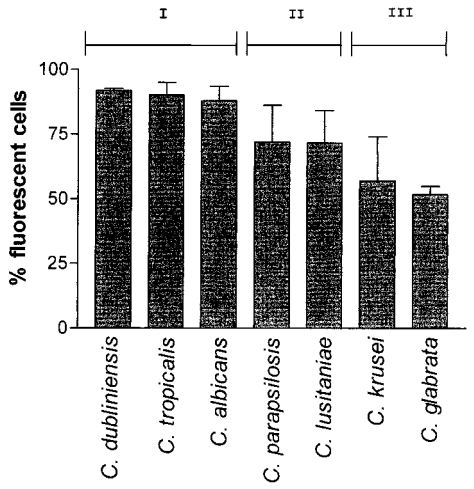

FIG. 1. Flow cytometric binding assay of fluorescein-labeled mucin (3.5 mg/ml) to *Candida* spp. after incubation for 30 min at 37°C. Each value represents the mean ± standard deviation of four strains per species, each tested in duplicate within the same experiment. Means of groups I to III were significantly different from each other ($P < 0.05$, one-way analysis of variance).

RESULTS

Analysis of *Candida* species binding to mucin. Because adherence of *Candida* species to vaginal and buccal epithelial cells (38) and to endothelial cells (55) is closely correlated with their hierarchy of virulence, four strains of each of seven *Candida* species were evaluated in duplicate for their capacity to bind to mucin (Fig. 1). A one-way analysis of variance demonstrated significant differences ($P < 0.05$) among means of three categories of *Candida* species, classified as I (highly adhering to mucin; *C. dubliniensis*, *C. tropicalis*, and *C. albicans*), II (moderately adhering to mucin; *C. parapsilosis* and *C. lusitaniae*), and III (weakly adhering to mucin; *C. krusei* and *C. glabrata*). Overall, the rank order for binding was in close correlation with adhesion to BECs (38) and endothelial cells (55). *S. cerevisiae* 894 (20), representative of a yeast species with low virulence, bound less mucin (22% fluorescent yeasts) than any of the *Candida* species.

Effect of mucin on *C. albicans* adherence to BECs. A concentration-dependent reduction in adhesion of biotinylated *C. albicans* LAM-1 to BECs was observed in the presence of mucin (Fig. 2A). However, when mucin was treated with pronase, the blocking effect of mucin was reversed, allowing the yeasts to adhere again to BECs (Fig. 2B). Additionally, Sap2p treatment of mucin partially restored adhesion of *C. albicans* to BECs (Table 1). In contrast, adhesion was not significantly altered when sugars of mucin were oxidized with sodium periodate (Table 1). These results suggested that the 118-kDa C-terminal glycopeptide of mucin (37), known to be susceptible to proteolytic degradation by pronase (45), may be involved in *Candida*-mucin interactions and as a substrate for *C. albicans* Sap2p.

Analysis of binding of *C. albicans* to mucin. Concentration- and time-dependent binding of mucin to *C. albicans* was observed by flow cytometry and the microplate assay (Fig. 3). Interestingly, mean fluorescence was 2.6-fold greater when *C. albicans* was grown at 25°C compared to 37°C, although proportions of fluorescent yeasts were nearly identical (97 and 96%, respectively) at a saturating concentration of mucin (7.5 mg/ml). However, no effect of growth phase of *C. albicans* on binding to mucin was observed by comparing cultures grown for 1, 4, 16, or 24 h at 30°C (data not shown). In addition,

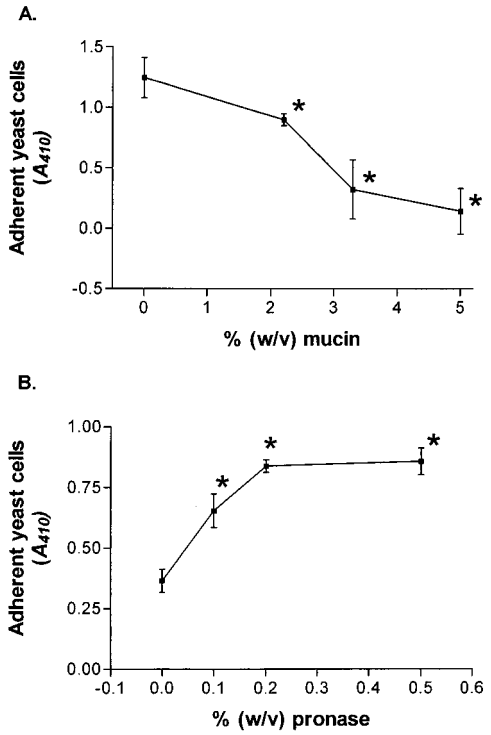

FIG. 2. (A) Effect of mucin on adherence of *C. albicans* LAM-1 to BECs after incubation for 1 h at 37°C. Each value is the mean ± standard deviation of triplicate observations within the same experiment. *, significantly different from control without mucin as well as values at the other concentrations of mucin ($P < 0.05$, Mann-Whitney *U* test). (B) Reversal by pronase treatment of the blocking effect of 2.5% (wt/vol) mucin on adherence of *C. albicans* to BECs. Each value is the mean ± standard deviation of triplicate observations within the same experiment. *, significantly different from control without pronase ($P < 0.05$, Mann-Whitney *U* test).

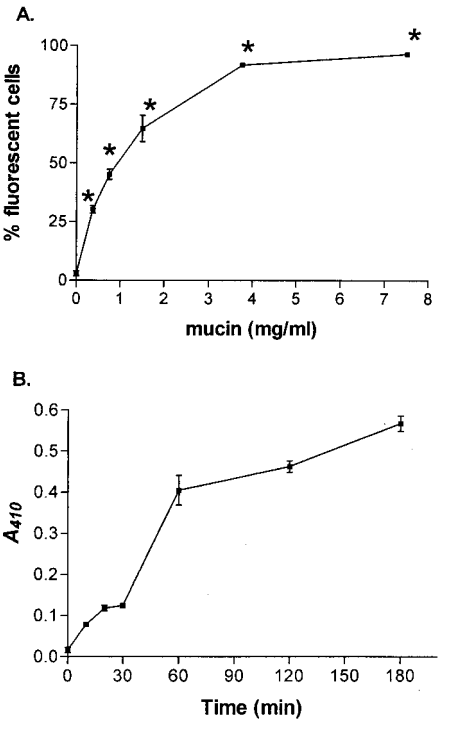

FIG. 3. (A) Flow cytometric analysis showing saturable concentration-dependent binding of fluorescein-labeled mucin to *C. albicans* LAM-1 after incubation for 30 min at 37°C. Each value is the mean ± standard deviation of triplicate observations within the same experiment. *, significantly different from control without mucin ($P < 0.05$, Mann-Whitney *U* test). Similar results were obtained using the microplate assay (data not shown). (B) Microplate assay showing time-dependent binding of mucin (50 μg/ml) to *C. albicans*. Each value is the mean ± standard deviation of triplicate observations within the same experiment.

binding was unaltered by variation in pH (3.5 to 8.5), except in very acidic conditions (pH 2.2), which resulted in a decrease in binding of 34%.

When the mucin was treated with β-mercaptoethanol, sodium periodate, or neuraminidase, no effect was observed on the binding of *C. albicans* to mucin (Fig. 4). In addition, no effect was observed when lectins and potentially inhibitory sugars were incubated with mucin prior to and during the adhesion assay, respectively (data not shown). However, pronase and Sap2p reduced the binding of the yeast to mucin (Fig. 4), in accordance with the results obtained with the assay utilizing BECs (Fig. 2 and Table 1). Addition of the aspartyl proteinase inhibitor pepstatin A (2 μg/ml) to the Sap2p concentrate resulted in a reversal of inhibition of binding (83% fluorescent cells).

To further define the site on mucin involved in binding, purified intestinal mucin was resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with biotinylated *C.*

TABLE 1. Effect of treatments of mucin on adhesion of *C. albicans* LAM-1 to BECs

Treatment and assay components	A_{410}[a]
Sap2p	
C. albicans + BECs	0.721 ± 0.142
C. albicans + 5.0% (wt/vol) mucin + BECs	0.265 ± 0.023[b]
C. albicans + Sap2p (5 mg/ml)-treated mucin + BECs	0.382 ± 0.094[c]
C. albicans + sham-treated mucin + BECs	0.267 ± 0.033
Sodium periodate	
C. albicans + BECs	1.095 ± 0.064
C. albicans + 5.0% (wt/vol) mucin + BECs	0.402 ± 0.035[b]
C. albicans + periodate-treated mucin + BECs	0.400 ± 0.057[d]
C. albicans + sham-treated mucin + BECs	0.419 ± 0.064

[a] Mean ± standard deviation ($n = 3$).
[b] Significantly different from control in the absence of mucin ($P < 0.05$, Mann-Whitney *U* test).
[c] Significantly different from control containing sham-treated mucin ($P < 0.05$, Mann-Whitney *U* test).
[d] Not significantly different from control containing sham-treated mucin ($P > 0.05$, Mann-Whitney *U* test).

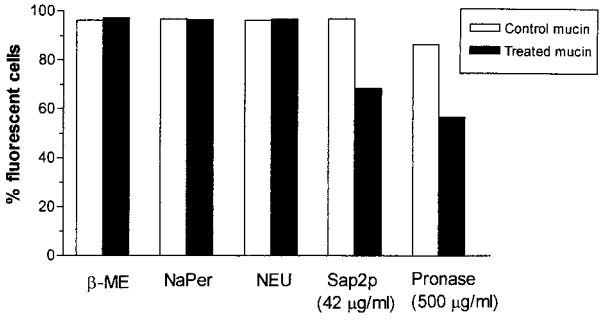

FIG. 4. Flow cytometric binding assay of treated fluorescein-labeled mucin (3.5 mg/ml) to *C. albicans* LAM-1 after incubation for 30 min at 37°C. Each value is the mean of duplicate observations within the same experiment. β-ME, β-mercaptoethanol; NaPer, sodium periodate; NEU, neuraminidase.

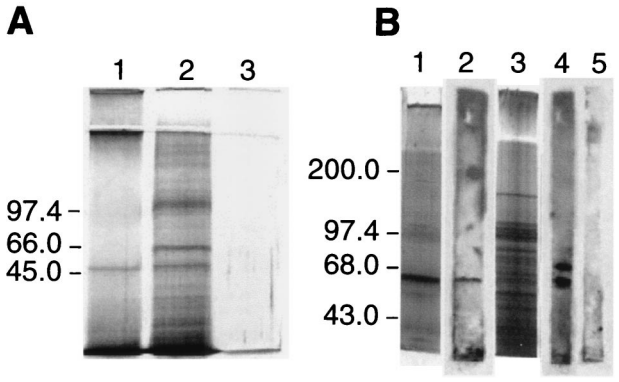

FIG. 5. SDS-PAGE and probing of membrane blots of purified rabbit intestinal mucin with *C. albicans* LAM-1. (A) SDS-PAGE of purified rabbit intestinal mucin without (lane 1) or with (lane 2) β-mercaptoethanol. Without thiol reduction (lane 1), the mucin gave only the expected high-M_r bands at the top of the gel, whereas after reduction (lane 2) this material decreased with the appearance of the major 118-kDa C-terminal glycoprotein, and a 66-kDa breakdown product. Lane 3, negative control containing β-mercaptoethanol alone. (B) Evidence that *C. albicans* binds to the C-terminal glycoprotein of mucin. Purified rabbit intestinal mucin was resolved by SDS-PAGE in reducing conditions with β-mercaptoethanol (lane 1), transferred to a PVDF membrane, and probed with BNHS-labeled *C. albicans* (lane 2). Binding of *C. albicans* was also demonstrated by a positive control employing porcine stomach mucin resolved by SDS-PAGE in reducing conditions (lane 3) and probed with *C. albicans* after transfer to a PVDF membrane (lane 4). Membrane blots not containing mucin did not bind *C. albicans* (lane 5). Positions of molecular mass standards (kilodaltons) are shown at the left.

albicans. When examined by SDS-PAGE, the purified rabbit intestinal mucin contained, in denaturing conditions only, the expected major 118-kDa C-terminal glycopeptide as well as a 66-kDa polypeptide, corresponding most likely to a previously characterized mucin breakdown product (37) (Fig. 5A). A negative control containing β-mercaptoethanol alone showed an absence of confounding artifacts. Probing of membrane blots of the mucin with *C. albicans* revealed binding of the yeast to the 66-kDa species, easily released by cleavage of the 118-kDa glycopeptide (37) (Fig. 5B). The C-terminal glycopeptide of mucin is thus likely responsible for binding to *C. albicans. C. albicans* also bound to two components of commercial porcine stomach mucin, which most likely represent breakdown products resulting from proteolytic degradation (Fig. 5B).

Three approaches were used to examine the potential participation of *C. albicans* cell surface mannoproteins in specific receptor-ligand binding to mucin. First, *C. albicans* LAM-1 was grown in yeast nitrogen base supplemented with 500 mM glucose, galactose, or mannose at 30°C for 18 h as described by McCourtie and Douglas (48). Using flow cytometric analysis, binding to mucin was not enhanced by the galactose supplement, which promotes expression of the outer fibrillar mannoprotein layer of *C. albicans* (48). Second, ConA (1 to 100 μg/ml in PBS containing 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$) incubated with *C. albicans* for 30 min at 37°C, or *C. albicans* 20A cell walls, *C. tropicalis* 83-48062 mannan, and *C. albicans* 526B mannan (generously provided by E. Reiss, Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention) incubated at 1 to 100 μg/ml with mucin, was without effect on binding of *C. albicans* to mucin by flow cytometry. Third, heterozygous NGY21 and NGY22 and homozygous NGY23 and NGY24 mannosyltransferase null mutants of *C. albicans* (5), which lack the addition of the second α-(1,2)-linked mannose residue of O-linked mannan, did not differ among themselves or with *C. albicans* LAM-1 in binding to mucin, measured both as percent fluorescent yeasts and mean fluorescence. Taken together, these results make it unlikely that *C. albicans* mannoproteins are the sole components involved in specific receptor-ligand binding of the yeast to mucin.

To determine whether hydrophobic interactions are involved in binding of *C. albicans* to mucin, the inhibitors *p*-nitrophenol (0.001 to 0.01 M) and tetramethylurea (0.36 to 1.0 M) were included in the flow cytometric binding assay. No inhibition of binding was observed with *C. albicans* grown at 25 or 30°C. However, *p*-nitrophenol (0.001 M) and tetramethylurea (0.36 M) decreased binding by 43 and 37%, respectively, when *C. albicans* was cultured at 37°C, indicating the participation of hydrophobic bonding in adherence of *C. albicans* to mucin. No further inhibition of binding was found at the higher concentrations of both inhibitors. *C. albicans* remained viable after exposure to *p*-nitrophenol but was killed in the presence of tetramethylurea.

C. albicans LAM-1 was markedly more hydrophobic at 25°C than at 37°C during the stationary phase of growth in yeast nitrogen base containing 100 mM glucose (CSH values [mean ± standard error; four independent observations] of 93.0 ± 4.5 and 8.2 ± 6.1, respectively), as was the LGH 1095 control strain (88.7 ± 2.6 and 11.2 ± 9.8). *C. albicans* LAM-1 thus has properties similar to those of most *C. albicans* strains, including the control isolate, being strikingly more hydrophobic at 25°C than at 37°C (30). The CSH values for *C. albicans* LAM-1 and LGH 1095 obtained at stationary phase after growth at 30°C in yeast nitrogen base containing 500 mM of glucose, galactose, or mannose were not significantly different ([mean ± standard error; three or four independent observations] 60.1 ± 16.2, 38.9 ± 17.6, and 45.5 ± 18.8 [LAM-1] and 82.2 ± 11.0, 54.4 ± 12.9, and 79.8 ± 2.6 [LGH 1095]).

Finally, the participation of electrostatic forces in binding was examined by conducting the flow cytometric binding assay in the presence of increasing concentrations of NaCl (0.2 to 2.6 M), but this possibility was excluded by unaltered adherence of *C. albicans* to mucin (data not shown).

DISCUSSION

The ability of microbial pathogens to bind to and penetrate the mucin layer which overlies mucosal surfaces may facilitate their colonization capabilities and access to host epithelial cells. The results of the present study show that (i) binding of the *Candida* species to purified rabbit intestinal mucin is directly correlated with their hierarchy of virulence, (ii) mucin blocks the adherence of *C. albicans* to BECs but Sap2p treatment of mucin can partially restore adherence, and (iii) *C. albicans* binds to the protein backbone of the 118-kDa C-terminal glycopeptide of mucin through hydrophobic interactions. The results of the present in vitro study concur with in vivo evidence for binding of *C. albicans* to intestinal mucin, observed by scanning electron microscopy in experimentally infected hamsters (36), as well as apparent clearance of the mucin layer surrounding *C. albicans* yeast cells within 1 h of oral-intragastric inoculation of infant mice (10). The observed restoration of adherence of *C. albicans* to BECs by Sap2p treatment of mucin is supported by our earlier observations that the enzyme is responsible for proteolysis of mucin by *C. albicans* in vitro (11). In addition, *C. albicans SAP2* is consistently expressed in vivo in humans with oral candidiasis (49). The findings thus support the hypothesis that the enhanced virulence capabilities of *C. albicans* compared to the non-*albicans* species at mucosal sites may be at least partially explained by increased binding to the overlying mucus layer, followed by facilitated penetration of the mucus barrier by the selective expression of *SAP* genes (49). Binding of *C. albicans* to mucin

was unaltered over a wide range of pH values (3.5 to 8.5), consistent with attachment to mucin in the oral cavity (pH 7), vagina (pH 5), and small intestine (pH 8). After successfully binding to and penetrating the mucus layer, *C. albicans* has a further selective advantage by adhering to a greater degree to mucosal epithelial cells compared to the other, less virulent *Candida* species (38).

Understanding the molecular mechanism(s) of binding of *C. albicans* to mucin is clearly important if its effect is to prevent adherence of *Candida* to host mucosal surfaces. Such knowledge may suggest novel approaches for the prevention of *Candida* colonization and infection, by interfering with the adherence process. Our results indicate that *C. albicans* binds to the protein backbone of the 118-kDa C-terminal glycopeptide of mucin (37, 65). This interpretation is supported by binding of the yeast to the C-terminal 66-kDa portion of the 118-kDa glycopeptide of purified rabbit intestinal mucin (37). The 66-kDa product is released by cleavage of the aspartylproline bond located at residue 374 of the C-terminal end of rat intestinal mucin Muc2 (37, 65). Dialysis of the mucin may have resulted in limited proteolysis, which has been shown to occur after the required removal of the guanidium hydrochloride (37). Although not directly sequenced, the rabbit homologue of rat intestinal Muc2 also contains the 118-kDa glycopeptide (54) and would be expected to yield a similar cleavage product (65). The results also demonstrate that *C. albicans* binds to the protein backbone of the C-terminal glycopeptide, which contains N-linked oligomannoside side chains (26), including six potential N-glycosylation sites within the 66-kDa C-terminal product (65). Indeed, binding of *C. albicans* to mucin was unaltered by its treatment with ConA or sodium periodate or by the presence of mannose, but binding was abrogated by treatment of mucin with the proteolytic enzymes pronase and Sap2p. The 118-kDa C-terminal glycopeptide is known to be susceptible to proteolytic degradation by pronase (45). Inhibition studies using synthetic peptides representing defined portions of the C-terminal glycopeptide (37) will be required to further define the site of binding of *C. albicans*.

We next investigated whether specific receptors and ligands or nonspecific attractive forces are involved in binding of *C. albicans* to mucin. Among the specific adhesin-receptor interactions described for *C. albicans*, different domains of the protein component of mannoprotein have been shown to specifically recognize L-fucose of the H blood group antigen (7) or the disaccharide component βGalNac(1,4)βGal of asialo-GM1 (66) on human BECs. Such a mechanism could be postulated since mucin contains galactose, GalNAc, fucose, and sialic acid as sugar moieties of the O-linked glycan (26). However, such a mechanism was excluded by an absence of inhibition of binding after treatment of mucin with lectins specific for each of these sugars, treatment with sodium periodate or neuraminidase, or incubation with potentially inhibitory sugars, *C. albicans* cell walls, or mannan. A second specific interaction involves the recognition of the tripeptide sequence arginine-glycine-aspartic acid (RGD), found in extracellular matrix proteins, by a *C. albicans* integrin analog (40). This possibility was excluded by the absence of RGD sequences in both rat (65) and human (28) *MUC2* genes, which encode small intestinal mucin. However, inhibition of binding of *C. albicans* to mucin by *p*-nitrophenol and tetramethylurea indicated the participation of hydrophobic (London-van der Waals) interactions. The inhibitory effect could not be ascribed to a loss of viability of *C. albicans*, since the yeast cells were killed by tetramethylurea but remained viable in the presence of *p*-nitrophenol. Interestingly, the C-terminal glycopeptide of intestinal mucin which we have shown to bind *C. albicans* is enriched in hydrophobic amino acids (56). The fact that inhibition of binding was observed with *C. albicans* grown at 37°C, but not at 25 or 30°C, accords with the enhanced hydrophobicity of the yeast at 25°C compared to 37°C (32) and suggests that the hydrophobic attractive forces after growth at 25 or 30°C were too strong for inhibition of binding to mucin to take place. The enhanced mean fluorescence of binding of *C. albicans* to mucin which we observed after growth at 25°C compared to 37°C may also be explained by the greater hydrophobicity of *C. albicans* grown at 25°C. The similar CSH values of *C. albicans* measured after growth in yeast nitrogen base containing 500 mM galactose, compared to 500 mM glucose or mannose, are consistent with the absence of enhancement of binding of *C. albicans* to mucin under these growth conditions. Finally, the unaltered binding of both heterozygous and homozygous *Camnt1* null mutants of *C. albicans* to mucin supports studies which suggested that CSH of *C. albicans* is related to the abundance of phosphodiester-linked, acid-labile β-(1,2)-linked oligomannosyl chains (47). *CaMNT1* encodes an α-(1,2)-mannosyltransferase which adds the second mannose residue in the acid-stable region, which despite being implicated in adherence to human BECs and rat vaginal epithelial cells (5) does not appear involved in the hydrophobicity of *C. albicans* (47). Electrostatic interactions may coexist with hydrophobic forces in adherence of *Candida* species (39), but this possibility was excluded by adding NaCl (0.2 to 2.6 M), which did not alter the binding of *C. albicans* to mucin.

Previous attempts to characterize the interactions of *C. albicans* with mucin have been focused on salivary mucins. Edgerton et al. (22, 23) showed that human submandibular-sublingual saliva containing high- and low- molecular-weight mucins (MG1 and MG2, respectively) promotes the adhesion of *C. albicans* to dental acrylic, suggesting a role for mucins in adhesion. Protease and glycosidase treatment of *C. albicans* or preincubation with mannose or galactose inhibited adhesion of the yeast to saliva-coated dental acrylic, suggesting that *C. albicans* mannoproteins may be involved in binding to salivary mucins. In accordance with these results, Nikawa et al. (50, 51) showed that *C. albicans* binds to commercial bovine submaxillary gland mucin and that binding is inhibited by deglycosylation of mucin or treatment of *C. albicans* with proteases or α-mannosidase. In contrast, Hoffman and Haidaris (33, 34) were unable to confirm these findings and concluded that *C. albicans* does not bind to mucins but rather binds to pronase-resistant heparan sulfate side chains of a novel proteoglycan secreted by rat submandibular glands. These apparently contradictory results may have been caused by the use of commercial mucin (50, 51), which is known to be degraded and to contain contaminants, or may have resulted from the susceptibility of mucin preparations and particularly the 118-kDa C-terminal glycopeptide to proteolytic damage (37).

Binding of pathogenic microorganisms to mucin is recognized as a critical initial step in successful colonization of host mucosal surfaces (26). The study reported here describes the first example of binding of a microbial pathogen to the protein backbone of the 118-kDa C-terminal glycopeptide of mucin. *Escherichia coli* O157:H7 also binds to the glycopeptide, but to the N-linked oligomannoside side chains (57). O-Glycans in the tandem repeats of mucin represent a far more ubiquitous recognition site for pathogenic bacteria, either through specific receptor-ligand interactions or by hydrophobic bonding (26). The production of mucin-degrading proteases has been implicated as a virulence factor in the breakdown of mucus and penetration of the mucin barrier by a number of enteropathogenic bacteria (15, 29, 46, 53, 59, 61). Although taxonomically and structurally distinct, the results demonstrate that in a man-

ner analogous to pathogenic bacteria, the fungus *C. albicans* may both adhere to and degrade mucins in the oral cavity and small intestine, and both properties may act to modulate *C. albicans* populations in the gastrointestinal tract. Mucins may thus play a role both in host mucosal defense and enhanced susceptibility to infection by *C. albicans*.

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