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Evidence for Degradation of Gastrointestinal Mucin by *Candida albicans* Secretory Aspartyl Proteinase

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A zone of extracellular digestion of the mucin layer around *Candida albicans* blastoconidia was observed by transmission electron microscopy in the jejunum of mice inoculated intragastrically (G. T. Cole, K. R. Seshan, L. M. Pope, and R. J. Yancey, J. Med. Vet. Mycol. 26:173–185, 1988). This observation prompted the hypothesis that a putative mucinolytic enzyme(s) may contribute to the virulence of *C. albicans* by facilitating penetration of the mucus barrier and subsequent adherence to and invasion of epithelial cells. Mucinolytic activity was observed as zones of clearing around colonies of *C. albicans* LAM-1 grown on agarose containing yeast nitrogen base, glucose, and hog gastric mucin. In addition, concentrated culture filtrate obtained after growth for 24 h in yeast nitrogen base, supplemented with glucose and mucin as the sole nitrogen source, contained proteolytic activity against biotin-labelled mucin which was inhibited by pepstatin A. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the culture filtrate revealed two components of 42 and 45 kDa, with pIs of 4.1 and 5.3, respectively. A zymogram showed that mucin was degraded only by the 42-kDa component, which was also recognized by immunoblotting with an anti-secretory aspartyl proteinase (anti-Sap) 2p monoclonal antibody. The N-terminal sequence of the first 20 amino acids matched that reported for Sap2p. These results demonstrate that Sap2p is responsible for proteolysis of mucin by *C. albicans* in vitro and may be involved as a virulence factor in the breakdown of mucus and penetration of the mucin barrier by *C. albicans*.

Invasive candidiasis is the most frequent opportunistic fungal infection in immunocompromised patients and is identified at autopsy in 15 to 30% of those with acute leukemia (1, 37). Penetration of the gastrointestinal mucosa by *Candida* species is thought to be the most frequent portal of entry, leading to systemic dissemination (51, 54). The mechanisms involved in transcytosis, however, are not well understood (7, 8).

Candida albicans possesses a multiplicity of factors that could be involved in the invasive process. Putative virulence attributes include adhesins, dimorphism, phenotypic switching, and secretion of hydrolytic enzymes such as proteinases and phospholipases (11, 24, 47). Extracellular proteolytic activity of *C. albicans* has been extensively studied and is due to secreted aspartyl proteinases (Saps) (EC 3.4.23.6), which can break down a number of host substrates, including epithelial keratin (38), dermal collagen (26), albumin (48), hemoglobin (41), and immunoglobulin A (45).

Sap isoenzymes are now known to be the products of at least seven distinct genes, which are expressed and regulated differentially. Northern (RNA) analysis has shown that expression of SAP1 (23) and SAP3 (57) is regulated during phenotypic switching between the white and opaque forms of strain WO-1 (36), while SAP2 (60) is expressed by yeast cells in media containing protein as the sole nitrogen source (22, 55). The expression of SAP4 to SAP6 is detected at neutral pH during serum-induced yeast-to-hyphal-phase transition, but expression of the SAP7 gene has not been detected to date under any in vitro conditions (22, 56). Although genes SAP1 to SAP7 have been cloned and sequenced (22, 33, 34, 56, 57), only the products of genes SAP1, SAP2, and SAP3 have been isolated and characterized (56). An additional Sap isoenzyme, tentatively referred to as Sap8p, has been identified by N-terminal sequencing, but the corresponding gene has not yet been cloned (35, 56). Several lines of evidence suggest that Saps may be directly involved in fungal colonization and invasion of host tissues. First, the expression of Saps was detected on the surface of blastoconidia adhering to human nonkeratinized buccal epithelium and on invading germ tubes in vitro (2), in vaginal secretions of patients suffering from *Candida* vaginitis (12), and in experimental vaginitis (13). Second, *C. albicans* isolates from human immunodeficiency virus-infected patients expressed higher levels of Sap activity than isolates from control patients (14, 40). Third, a specific inhibitor of aspartyl proteinases, pepstatin A, blocked adherence of *C. albicans* to cultured human epithelial keratinocytes (39) and to human oral mucosa (2). Fourth, Sap-deficient mutants obtained by chemical mutagenesis were less virulent in mice than their parental strains (28, 44). Among *Candida* species other than *C. albicans*, *Candida tropicalis* secretes one acid proteinase (Sapt1) (52), whereas *Candida parapsilosis* secretes two isoenzymes (Sapp1 and Sapp2) (16, 46). However, virtually nothing is known of the impact of *Candida* virulence factors on the ability of the fungus to invade epithelial cells in the gastrointestinal tract and disseminate to deep organs.

The infant mouse model has been used for examination of various aspects of gastrointestinal and systemic candidiasis (5–8, 15). In 5-day-old mice, passage of *C. albicans* across the gastrointestinal wall occurs after oral-intragastric inoculation and results either in mortality or persistent colonization. While the absence of systemic spread of the fungus in persistently colonized mice indicates that normal host defense mechanisms effectively prevent invasion by *C. albicans*, subsequent immu-

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nosuppression with cortisone and cyclophosphamide results in invasion by hyphae in the cardial-atrium fold of the stomach as well as in systemic dissemination. Histological examination using transmission electron microscopy showed progressive extracellular digestion of the mucin layer around *C. albicans* CA30 yeast cells located in the jejunum of mice inoculated intragastrically (8). This observation prompted the hypothesis that a putative mucinolytic enzyme(s) contributes to the virulence of *C. albicans* by facilitating penetration of the mucus barrier and subsequent adherence to and invasion of epithelial cells.

We examined the extracellular mucinolytic activity of *C. albicans* by (i) studying the ability of yeast cells to use mucin as a nitrogen or carbon source and (ii) characterizing the enzyme(s) involved in mucin degradation. Our results showed that at least one member of the Sap family, Sap2p, is involved in degradation of highly glycosylated mucin in vitro.

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MATERIALS AND METHODS

Microorganisms. *C. albicans* LAM-1 (serotype A) was originally isolated from the blood of a patient with systemic candidiasis (29). Sap-producing *C. albicans* C9 was kindly provided by B. B. Magee, University of Minnesota, St. Paul, and *C. albicans* CA30, isolated from the kidneys of a leukemic patient, was obtained from G. T. Cole, Medical College of Ohio, Toledo. Cultures were maintained at 4°C on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.).

Culture conditions. Blastoconidia were grown in Sabouraud liquid broth (BBL, Cockeysville, Md.) for 18 h at 37°C with rotary agitation (240 rpm). The cells were collected by centrifugation (1,500 × *g*, 10 min) and suspended at 1.0×10^3 cells per ml in 1.7% (wt/vol) yeast nitrogen base (YNB) medium without amino acids and ammonium sulfate (Difco) supplemented with 0.35% (wt/vol) porcine stomach mucin (type III; Sigma Chemical Co., St. Louis, Mo.), and, in selected experiments, 1% (wt/vol) glucose and/or 0.5% (wt/vol) ammonium sulfate. For induction of aspartyl proteinase under known conditions (22), blastoconidia were grown in YNB supplemented with 1% (wt/vol) glucose and 0.2% (wt/vol) bovine serum albumin (BSA) (fraction V; Sigma).

Preparation of culture supernatant. After incubation at 37°C with rotary agitation, cells were removed by centrifugation (1,500 × *g*, 10 min). The pH of the supernatants was raised from 3.5 to 6.0 with 1 M KH_2PO_4 to limit autodegradation of enzymes (44), and the supernatants were kept on ice throughout the concentration process. High-molecular-weight mucin was removed by filtration through a cross-flow Sartocon-Micro unit (Sartorius AG, Göttingen, Germany) with a 100-kDa exclusion limit. The filtrate was first concentrated through a Sartocon-Micro unit with a 10-kDa exclusion limit and/or in a stirred-cell device containing an ultrafiltration membrane with a 10-kDa exclusion limit (Amicon Inc., Beverly, Mass.) and finally through a Centriplus-10 tube (Amicon). This gave a minimum of 100X concentrate, which was stored at −80°C. Protein content was measured by the method of Bradford using the Bio-Rad (Hercules, Calif.) protein assay and BSA as a standard (3).

Detection and quantitation of mucinolytic activity. The presence of mucinolytic activity was determined by two methods. In the first, cultures were incubated for 3 days at 37°C on a medium containing 1.5% (wt/vol) agarose, 1.7% (wt/vol) YNB, 0.5% (wt/vol) mucin, and 1% (wt/vol) glucose. Plates were subsequently stained with 0.1% (wt/vol) amido black in 3.5 M acetic acid for 30 min and destained with 1.2 M acetic acid. Zones of mucin lysis were observed as discolored halos around colonies.

In the second method, mucinolytic activity was quantitated in a microplate assay using immobilized biotin-labelled mucin as substrate, as described previously (9). Briefly, microplate wells were coated with biotin-labelled mucin, and after washing to remove nonadsorbed mucin, concentrated culture supernatants were added and incubated for 60 min at 37°C in 100 mM sodium acetate buffer (pH 3.5). After incubation, the undigested labelled mucin was detected with streptavidin-peroxidase. Enzymatic activity was determined by reporting absorbance readings on a plot obtained with a standard curve of labelled mucin. Activity was expressed as the percent decrease in absorbance compared with that of control wells devoid of enzyme or containing uninoculated culture medium (9).

SDS-PAGE and Western blot (immunoblot). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (30) with 10% (wt/vol) polyacrylamide separating gels (80 by 70 by 1.5 mm) in a Mini-Protean II Cell (Bio-Rad). Samples (2 to 5 μg) and broad-molecular-weight size standards (Gibco BRL, Canadian Life Technologies Inc., Burlington, Ontario, Canada) were boiled at 100°C for 5 min in an SDS-sample buffer containing 60 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, and 0.025% (wt/vol) bromophenol blue. Electrophoresis was performed at room temperature at a constant current of 150 V until the bromophenol blue tracking dye reached the bottom of the gel. Protein bands were visualized by silver staining. For immunoblotting, protein bands on SDS-PAGE gels were transferred electrophoretically by the method of Towbin et al. (53) in a buffer containing 250 mM Tris-HCl (pH 8.8), 192 mM glycine, and 20% (vol/vol) methanol to a nitrocellulose membrane (pore size, 0.2 μm). After electrophoretic transfer (100 V for 2 h at 4°C) in a Mini Trans-Blot cell (Bio-Rad), the nitrocellulose membrane was blocked overnight (4°C) by incubation in 2% (wt/vol) skim milk in Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 7.5], 60 mM NaCl). The next day, the blocked membrane was incubated for 4 h with mouse anti-Sap2p monoclonal antibody IFG_3, kindly provided by T. L. Ray, University of Iowa, diluted 1:100 in TBS–2% (wt/vol) skim milk. After three 10-min washes with TBS-T (TBS containing 0.1% [vol/vol] Tween 20), the blot was incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Gibco BRL), diluted 1:5,000 in TBS–2% (wt/vol) skim milk. After 1 h of incubation at room temperature, the membrane was washed as described above and developed with alkaline phosphatase color development reagent (10 ml of a 100 mM Tris-HCl [pH 9.6]–100 mM NaCl–5 mM $MgCl_2$ buffer containing 33 μl of nitroblue tetrazolium solution and 44 μl of BCIP solution [5-bromo-4-chloro-3-indolylphosphate]) (Gibco BRL).

IEF and two-dimensional electrophoresis. Native isoelectric focusing (IEF) was carried out in a vertical minigel system by the procedure of Robertson et al. (43), with some modifications. Gels (80 by 70 by 1.5 mm) were cast from a mixture of the following ingredients: 7 ml of water, 2 ml of acrylamide mixture (30% [wt/vol] acrylamide, 1% [vol/vol] bisacrylamide), 2.4 ml of 50% (vol/vol) glycerol, and 0.6 ml of Pharmalyte (pH range, 4 to 6.5; Pharmacia Biotech Inc., Baie d'Urfé, Québec, Canada). After deaeration, 50 μl of freshly prepared 10% [wt/vol] ammonium persulfate and 20 μl of TEMED (*N*, *N*, *N*′, *N*′-tetramethylenediamine) were added. Sample wells were formed at the top of the gel with a five-well comb. After polymerization was completed, the comb was removed and the wells were rinsed with distilled water. The wells and upper chamber were filled with the anode solution (20 mM acetic acid), and the lower chamber was filled with the cathode solution (25 mM sodium hydroxide). pI markers (Pharmacia Biotech) and samples (2 to 5 μg) were mixed with an equal volume of IEF sample buffer containing 60% (vol/vol) glycerol and 4% (vol/vol) Pharmalyte (pH range, 4 to 6.5). The electrodes were connected so that the polarity was reversed, consistent with the electrode solutions. Electrophoresis was performed at 4°C for 1.5 h at 200-V constant voltage and then at 400-V constant voltage for an additional 1.5 h. After focusing was completed, the gel was removed and either silver stained, subjected to a second dimension in SDS-PAGE, immunoblotted, or prepared for a zymogram evaluation. For the two-dimensional analysis, the IEF gel lane was equilibrated for 30 min in a solution containing 62.5 mM Tris-HCl (pH 6.8), 2.3% (wt/vol) SDS, 10% (vol/vol) glycerol, and 5% (vol/vol) β-mercaptoethanol. Subsequently, the gel was placed in direct contact with the stacking gel of the SDS-PAGE system and run in a 10% (wt/vol) polyacrylamide separating gel under the conditions described above. Molecular weight standards were run simultaneously, and protein bands were silver stained.

Western blot from IEF gel. Following the isoelectric focusing, the gel was washed four times (10 min) in a buffer containing 5 mM Tris-HCl (pH 8.8)–1% (wt/vol) SDS to remove the carrier ampholytes. The proteins were electrotransferred to nitrocellulose paper and probed with the mouse anti-Sap2p monoclonal antibody.

Zymogram from IEF gel. The mucinolytic activity of the proteins separated by native IEF was evaluated by overlaying the gel on mucin-coated paper. One hundred microliters of biotin-labelled mucin (400 μg/ml) was copolymerized in 12% (wt/vol) polyacrylamide gel and electrotransferred to nitrocellulose paper as described earlier. When blotting was complete, the mucin-coated paper was washed three times (10 min) in 100 mM sodium acetate buffer (pH 3.5) and placed on several filter papers (Whatman 3MM) underlaid with soft absorbent tissue paper. The IEF gel, which was immersed in 100 mM sodium acetate buffer (pH 3.5) for 30 min, was then carefully overlaid on the nitrocellulose paper and incubated for 14 to 16 h at 37°C in a moist chamber. After removal of the gel from the paper surface, the paper was washed three times (10 min) in TBS and blocked in TBS–2% (wt/vol) skimmed milk (1 h, 22°C). After three washes with TBS-T, the paper was incubated for 60 min with streptavidin-POD diluted 1:1,500 with TBS-T. Three additional washes in TBS-T and two in TBS were employed before the paper was exposed to a chromogenic peroxidase substrate solution containing 10 ml of methanol, 1 ml of 4-chloro-1-naphthol solution (30 mg/ml in methanol), 39 ml of TBS, and 30 μl of 30% (vol/vol) hydrogen peroxide. The reaction was developed in darkness and stopped by washing the membrane with distilled water.

Metabolic labelling. Blastoconidia of *C. albicans* (3 ml, 1.0×10^4 cells per ml) were cultured for 24 h in YNB containing 0.35% (wt/vol) mucin, glucose, and 40 μCi of Trans^{35}S-label (specific activity, > 1,000 Ci/mmol; ICN Pharmaceuticals Inc., Costa Mesa, Calif.) per ml. The culture supernatant was ultrafiltered through a Centriplus-100 tube (Amicon), and the filtrate was concentrated by precipitation with acetone. The proteins were resuspended in 20 μl of IEF sample buffer and subjected to IEF. After the electrophoresis, the IEF gel was fixed (30 min) in methanol-acetic acid-water (5:1:4) and soaked (30 min) in En^3 Hance solution (New England Nuclear Corp., Boston, Mass). Finally, the gel was dried and exposed to a Kodak X-Omat film at −80°C.

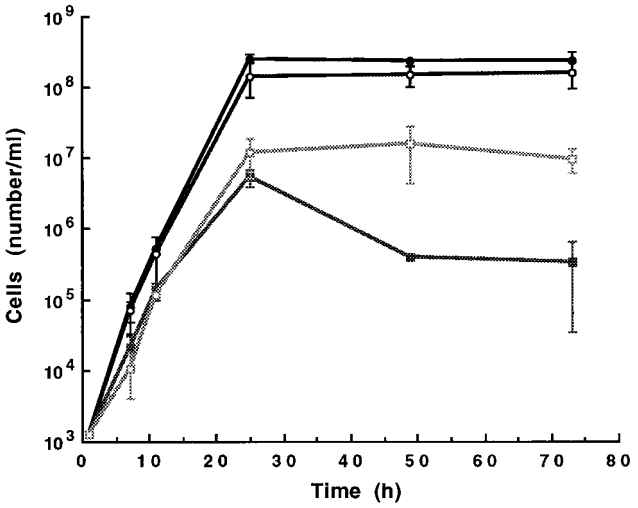

FIG. 1. Growth of blastoconidia of *C. albicans* LAM-1 in YNB cultures all containing mucin and supplemented with glucose and ammonium sulfate (●), only glucose (○), only ammonium sulfate (■), or neither glucose nor ammonium sulfate (□). The cultures were incubated at 37°C, and the cells were counted with a hemacytometer. Values represent the means ± standard deviations of samples from three independent experiments.

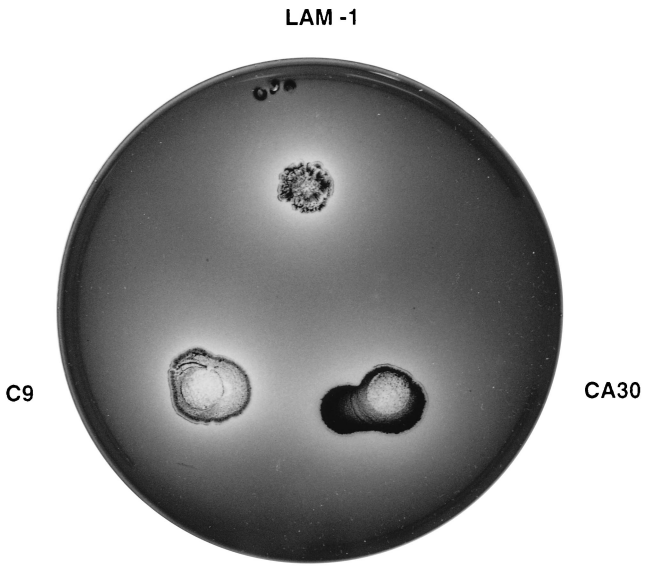

FIG. 2. Zones of mucin lysis around colonies of *C. albicans* LAM-1, C9, and CA30 grown on YNB-glucose-mucin. The plates were incubated at 37°C for 3 days and stained with amido black.

N-terminal sequencing. After IEF, the regions of the gel corresponding to pIs 4.1 and 5.3 were excised and immersed in two-dimensional electrophoresis equilibrium buffer for 15 min. The gel slices were then inserted on top of an SDS–5% (wt/vol) polyacrylamide stacking gel, and migration was performed as mentioned above for SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes (pore size, 0.2 μm; Bio-Rad) in 10 mM CAPS buffer (pH 11)–10% (vol/vol) methanol, by using a Trans-Blot cell (Bio-Rad) at 90 V and 250 mA for 60 min. After rinsing with water, the membrane was stained with 0.1% (wt/vol) Coomassie blue R-250 in 50% (vol/vol) methanol and destained in 50% (vol/vol) methanol–10% (vol/vol) acetic acid. The membrane was allowed to dry, and the bands were excised and sequenced by automated Edman degradation with an Applied Biosystems sequencer model 473A (Service de Séquence de Peptides de l'Est du Québec, CHUL, Ste-Foy, Québec, Canada).

RESULTS

Mucin as a nitrogen or carbon source. The ability of *C. albicans* yeast cells to use mucin as the sole nitrogen or carbon source was evaluated by growing the blastoconidia in media containing mucin instead of glucose and/or ammonium sulfate. Figure 1 shows that mucin and ammonium sulfate were equivalent as nitrogen sources in supporting cell growth in the presence of glucose, and the culture reached the stationary phase after 24 h of incubation. Thus, the cells were able to use mucin as the sole nitrogen source. The initial pH of the culture containing glucose and mucin was 4.5 and fell to 3.5 after 24 h of incubation. Preculture of blastoconidia in conditions of ammonium starvation (1.7% [wt/vol] YNB, 1% [wt/vol] glucose, 0.02% [wt/vol] ammonium sulfate), rather than Sabouraud liquid broth, had no effect on subsequent cell growth in media containing glucose and mucin or mucin alone. Limited growth occurred when the yeast cells were inoculated in media in which no carbohydrate source other than mucin was included, suggesting that mucin is less efficiently utilized as a carbon source by *C. albicans*. Finally, growth in YNB without amino acids, ammonium sulfate, glucose, or mucin was further limited and only reached 5.8×10^4 and 8.4×10^4 cells per ml after 24 and 72 h of incubation, respectively.

Clear zones of mucin lysis were apparent around colonies of strains C9, LAM-1, and CA30 grown on solid medium containing mucin and glucose (Fig. 2). The diffusion of mucinolytic activity into the medium surrounding the colonies suggested the release of a soluble mucinolytic enzyme(s). However, no zones of mucin lysis were observed on medium containing mucin, glucose, and ammonium sulfate (data not shown). On the basis of these observations, we selected YNB containing mucin and glucose as the inducer medium and the virulent strain LAM-1 used in the model of oral candidiasis (29) for the subsequent experiments.

Characterization of mucinolytic activity. Culture supernatants from LAM-1 cells were purified and concentrated by ultrafiltration, and the mucinolytic activity was quantified in a microplate assay, with biotin-labelled mucin as the substrate. Raising the pH to 6.0 and concentrating the supernatant before freezing were all essential to maintaining mucinolytic activity. Maximal protease activity was detected at pH 3.5 in 24-h culture supernatants (20% ± 7.6% [mean ± standard deviation of three independent experiments]). After incubation with pepstatin A (2 μg/ml), the activity decreased to 7.5% ± 1.9% and proteolysis was thus inhibited by 64%. Finally, when the culture supernatant was boiled, the residual activity was 9.5% (9). The inhibition of mucin degradation by pepstatin A suggested the involvement of an aspartyl proteinase (EC 3.4.23.6) in mucinolytic activity. An incubation period of 24 h was selected for the subsequent experiments.

Upon SDS-PAGE, two protein bands with apparent molecular weights of 42 and 45 kDa appeared in the mid-log phase (15 h) (Fig. 3, lane 6). Both bands persisted throughout a 7-day extended incubation period. Failure to initially raise the pH of the culture supernatant from 3.5 to 6.0 resulted in a marked weakening of the 42-kDa band. The 42-kDa band was more prominent relative to the 45-kDa band and was the only one to react with the anti-Sap2p monoclonal antibody along with a lower-molecular-weight product most likely resulting from autodegradation (44) (Fig. 3, lane 8). Induction of Sap2p expression under standard conditions was done by growing the known aspartyl proteinase-producing strain C9 (28) in YNB medium containing glucose and BSA. Culture supernatant from strain C9 also showed a 42-kDa band which was recognized by the anti-Sap2p monoclonal antibody (Fig. 3, lanes 2 and 4). These

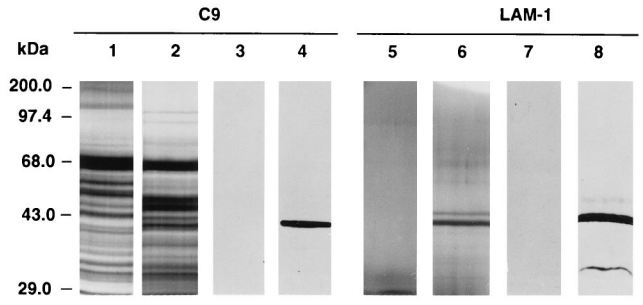

FIG. 3. SDS-PAGE and Western blot analysis of culture supernatants from *C. albicans* C9 and LAM-1 grown in YNB-glucose-BSA and YNB-glucose-mucin, respectively. Lanes: 2 and 6, SDS-PAGE of supernatants from C9 and LAM-1, respectively; 4 and 8, Western blot of supernatants from C9 and LAM-1, respectively, probed with anti-Sap2p monoclonal antibody; 1, 3, 5, and 7, samples from uninoculated media included as controls (1 and 5, SDS-PAGE; 3 and 7, Western blot). The gels were silver stained.

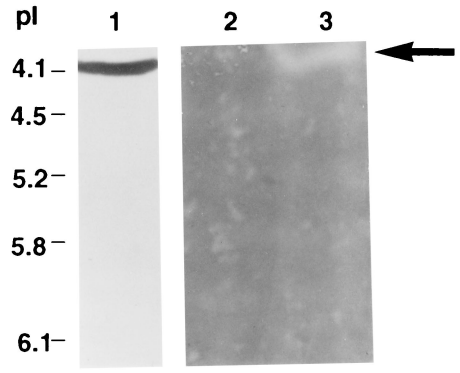

FIG. 5. Western blot and zymogram from a native IEF gel of culture supernatant from *C. albicans* LAM-1. Lanes: 1, Western blot, probed with anti-Sap2p monoclonal antibody; 2, zymogram of heat-inactivated culture supernatant; 3, zymogram of culture supernatant, showing degradation of mucin (arrow).

results provide evidence that the 42-kDa band was antigenically related to *C. albicans* Sap2p.

IEF and two-dimensional electrophoresis. To further characterize the 42- and 45-kDa bands, they were subjected to native IEF and two-dimensional electrophoresis. Figure 4 shows that the pIs of the 42- and 45-kDa components were 4.1 and 5.3, respectively. No other bands were detected in the second-dimension gel, indicating the absence of Sap isoforms in the culture supernatant. The IEF procedure resulted in complete separation of the 42- and 45-kDa bands, allowing us to perform the N-terminal protein sequencing without risk of contamination.

Western blot and zymogram from IEF gel. The identity of the pI 4.1 protein was confirmed by Western blot from the IEF gel, since it was the only one recognized by the anti-Sap2p monoclonal antibody (Fig. 5, lane 1).

To determine whether the mucinolytic activity was a consequence of cooperative action of both 42- and 45-kDa bands, we performed a zymogram from the native IEF gel, with biotin-labelled mucin as the substrate. The proteolytic activity was uniquely identified at pI 4.1 as a clear band against a dark blue-violet background (Fig. 5, lane 3), demonstrating that the 42-kDa protein was the only component responsible for mucin degradation.

Metabolic labelling. Autoradiography revealed that during incubation in mucin-containing medium, the 42-kDa band was the only component which incorporated [^{35}S]methionine or -cysteine. The 45-kDa component failed to incorporate the radiolabelled precursor, suggesting that it may be a breakdown product of mucin.

N-terminal sequencing. The N-terminal sequence of the first 20 amino acids from the 42-kDa protein was QAVPVTLH NEQVTYAADITV, matching that reported by others (22) for *C. albicans* Sap2p. The most-probable glycine-rich sequence of the 45-kDa protein was GEGGSGGEGGQGGQGGPXGA, and a BLAST search (1 May 1996) revealed homology with type II keratin, a mesenchyme-specific cell surface glycoprotein precursor from the sea urchin (*Strongylocentrotus purpuratus*), and a glycine-rich cell wall structural protein from the tomato (*Lycopersicon esculentum*).

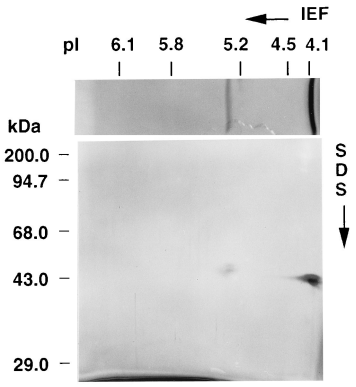

FIG. 4. Native IEF gel (top) and two-dimensional electrophoresis gel (bottom) of culture supernatant from *C. albicans* LAM-1. The pIs and positions of molecular weight markers are indicated. The gels were silver stained.

DISCUSSION

Invasion of the bowel wall by *Candida* species and systemic spread to deep organs are triggered by complex interactions resulting from modifications of normal bacterial flora and host defenses. However, virtually nothing is known of the impact of *Candida* virulence factors on the ability of the fungus to engage in this process. Several clues suggest that *Candida* virulence factors may play a role. (i) *C. tropicalis* disseminates more easily than *C. albicans* from the gastrointestinal tract of humans (59) and mice (15, 58), suggesting the presence of specific virulence determinants. (ii) An advancing border of necrosis is seen at the leading edge of gastrointestinal invasion by *C. tropicalis* hyphae (54). (iii) Progressive extracellular digestion of the mucin layer is observed in 5-day-old mice inoculated intragastrically with *C. albicans* CA30 (8). The latter observation prompted the hypothesis that a mucinolytic enzyme(s) contributes to the virulence of *Candida* species by facilitating penetration of the mucus barrier and subsequent adherence to and invasion of epithelial cells.

In this study, we provide evidence that Sap2p contained in concentrated culture filtrate of *C. albicans* blastoconidia has mucinolytic activity. Mucins are the major constituents of mucus and play a role in protection against invasion by potential pathogens because of their rich and heterogeneous oligosaccharide composition and ability to form a gel (19). For a microorganism such as *C. albicans*, which colonizes mucosal surfaces, mucin degradation by Sap2p may allow closer approximation to epithelial cells and/or modification of cellular surfaces to create receptors, promoting invasion and spread of the fungus within the host. Because porcine stomach mucin is

structurally similar to the major gastric glycoproteins of humans and resembles human intestinal mucin in general composition (25), these findings suggest a potential role for Sap2p in the pathogenesis of gastrointestinal candidiasis. We have previously shown that concentrated culture filtrate from *C. albicans* LAM-1 degrades porcine stomach mucin (9). The glycoprotein structure of mucins renders them potentially susceptible to attack by both glycosidases and proteinases. The proteolytic nature of the mucinase activity of Sap2p is suggested by loss of portions of mucin molecules which were labelled with biotin specifically and exclusively on their protein moieties. The activity was probably directed against the minor or naked regions of the mucin molecule which are poorly glycosylated as well as the link peptide of 118 kDa (18), which are known to be susceptible to proteolytic digestion.

The present in vitro experimental evidence suggests that Sap2p may be involved in the previously described in vivo progressive extracellular digestion of the intestinal mucus barrier observed after oral-intragastric inoculation of *C. albicans* in the infant mouse model (8). Most indirect evidence which associates proteinase production and virulence during mucosal candidiasis is derived from the detection of the expression of SAP1 and SAP2 in experimental vaginitis (13).

The production of mucin-degrading enzymes has been implicated as a virulence determinant for a number of enteropathogens, including *Vibrio cholerae* (10), *Bacteroides fragilis* (42), *Shigella* spp. (20), *Helicobacter pylori* (49), and *Yersinia enterocolitica* (32). Extensive mucin degradation would require the secretion of neuraminidase, endo-β-*N*-acetylhexosaminidase, and proteases (50). While we cannot exclude the possibility of expression of secreted or wall-associated glycosidase activity by *C. albicans* grown in the presence of mucin, it would appear more likely that the major mucin-degrading enzyme may be Sap2p.

Our results are in agreement with reports indicating that Sap2p is the major isoenzyme of the Sap family produced by the yeast form of a majority of clinical isolates of *C. albicans* (22, 55). The apparent molecular mass of 42 kDa and pI of 4.1 were in close agreement with those reported for Sap2p (57). It is well known that Sap2p expression is induced in media containing protein as a single nitrogen source, stimulated by peptides of eight or more amino acid residues and repressed by low-molecular-weight nitrogen components (31). Induction of Sap2p by mucin is consistent with the observation that several protein substrates, including casein, bovine serum, and hemoglobin, all have the ability to induce Sap (55). In addition, inhibition of mucinolytic activity around the colonies of *C. albicans* C9, LAM-1, and CA30 grown on solid medium containing mucin, glucose, and ammonium sulfate concurred with the known repression of Sap2p levels by ammonium salts (55). Despite its glycosylated structure, mucin seems to be a highly utilizable nitrogen source, inducing Sap2p expression and supporting cell growth in the presence of glucose. We have also examined the role of mucin as a sole carbon source. Cultures supplemented with mucin but lacking an added carbon source grew poorly. The simplest explanation for these results is that glucose acts as a preferred growth substrate for *C. albicans*, while mucin is a poor carbon source, probably because no glycosidases capable of mucin degradation were secreted under these conditions. In addition, these results are consistent with the observation that *C. albicans* requires a carbon and energy source for growth and expression of Sap2p (22). Detection of Sap2p in the mid-log phase and persistence in the medium for 7 days are in agreement with the results of White et al. (57) and occurred concurrently with a decrease in the pH of the culture medium to 3.5, which is optimal for expression of Sap2p mRNA (22, 55).

A less-prominent band of 45 kDa was also present in the concentrated culture supernatants from cells grown in mucinase-inducing medium. Its N-terminal amino acid sequence did not show any homology with the N-terminal segments of mature Sap proteins reported to date, making it thus unlikely that it is a Sap isoenzyme. The absence of metabolic labelling suggested that it may be a breakdown product of mucin resulting from Sap2p digestion. However, the possibility that it may be an extracellular product of *C. albicans* could not be formally excluded because of its low methionine-cysteine content and/or possible weak de novo synthesis. The N-terminal amino acid sequence did not show conclusive homology to sequences of known proteins.

Candida-mucin interactions may involve not only mucinolysis but also adhesion to host surfaces. In the gastrointestinal tract, mucin glycoproteins were associated with adhesion of *C. albicans* to intestinal epithelium (27), and two different studies have demonstrated adhesion of *C. albicans* to salivary mucin, which may either act to inhibit adhesion with some surfaces in the oral cavity or promote adhesion to other surfaces (17, 21). *C. albicans* contains a mannoprotein adhesin with a lectin-like affinity for fucose, a component of mucins (4). It is thus conceivable that *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.

The results of this study demonstrate that Sap2p is responsible for proteolysis of mucin by *C. albicans* in vitro and may be involved as a virulence factor in the breakdown of mucus and penetration of the mucin barrier by *C. albicans*.

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