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Characterization of a Fucoside-Binding Adhesin of *Candida albicans*

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***Candida albicans* GDH 2346 produces extracellular polymeric material (EP) which contains a mannoprotein adhesin with a lectin-like affinity for fucose-containing glycosides. EP isolated from culture supernatants of this strain was used as starting material for purification of the adhesin. The purification protocol involved a stepwise treatment of EP with *N*-glycanase, papain, and dilute alkali to cleave the protein and carbohydrate portions of the mannoprotein molecule. Fucoside-binding protein fragments were then recovered by affinity adsorption with the trisaccharide determinant of the H (type 2) blood group antigen which terminates in a residue of L-fucose. The purified adhesin was devoid of carbohydrate and inhibited yeast adhesion to buccal epithelial cells 221 times more efficiently, on a protein weight basis, than did EP. Adhesion inhibition reached a maximum of 78 to 80% at an adhesin concentration of 10 μg ml^{-1}. Our results indicate that this protein is the major adhesin of yeast-phase cells of *C. albicans* GDH 2346 but that one or more secondary adhesion mechanisms may operate.**

Although wall components such as chitin (19) and lipids (11) have been proposed as possible adhesins for the opportunistic fungal pathogen *Candida albicans*, most experimental evidence indicates a role for surface mannoprotein in mediating yeast attachment to buccal and vaginal epithelial cells (4, 7, 8). Adhesion can be inhibited by pretreating the epithelial cells with a crude mannoprotein preparation obtained from culture supernatants of yeasts grown in medium containing a high concentration of galactose (17). This extracellular polymeric material (EP) is thought to originate, at least in part, from a surface fibrillar layer whose synthesis is promoted by growth under such conditions (16). The ability of EP to inhibit adhesion indicates that it contains an adhesin capable of binding to, and thus blocking, epithelial cell receptors. Binding is specific (17), since EP from one *C. albicans* strain (GDH 2023) fails to inhibit adhesion of a second strain (GDH 2346).

The predominant interaction between yeasts and epithelial cells appears to involve the protein portion of the mannoprotein adhesin. Pretreatment of crude adhesin (EP) with heat, dithiothreitol, or proteolytic enzymes (except papain) either partially or completely abolishes its ability to inhibit adhesion to buccal cells (5), whereas pretreatment with sodium periodate or α-mannosidase has little or no effect. Moreover, the protein-rich fraction obtained by incubating crude adhesin with endoglycosidase H inhibits attachment more than does the carbohydrate-rich fraction (5). These findings are consistent with earlier studies (13, 20) in which adhesion to vaginal cells was shown to be severely inhibited by treating yeasts directly with various proteolytic enzymes or reducing agents but not by similar treatment with glycosidases.

By analogy with many bacterial adhesion mechanisms (12), a proteinaceous yeast adhesin might be expected to interact in a lectin-like manner with a glycoside receptor (either glycoprotein or glycolipid) on the host cell surface. Adhesion inhibition tests with sugars and lectins have been widely used to characterize epithelial cell receptors. Early tests of this type with *C. albicans* produced apparently contradictory data (reviewed in reference 7), but more recent work indicates that a receptor containing L-fucose is required for most strains of the yeast (6). However, addition of L-fucose to assay mixtures caused only partial inhibition of adhesion with the sensitive strains (6), suggesting that the natural mucosal receptor is larger than an L-fucose residue or that a particular stereochemical configuration is necessary.

In this study, we have exploited this knowledge of receptor specificity in developing a scheme for purification of the yeast adhesin. Our protocol involves sequential treatment of crude adhesin (EP) with *N*-glycanase, papain, and dilute alkali to cleave the protein and carbohydrate portions of the mannoprotein molecule. Fucoside-binding protein fragments are then recovered by affinity adsorption with the trisaccharide determinant of the H (type 2) blood group antigen which terminates in a residue of L-fucose.

MATERIALS AND METHODS

Organism. *C. albicans* GDH 2346 (NCYC 1467) was used throughout the study. This strain was originally isolated at Glasgow Dental Hospital from a patient with denture stomatitis. It was maintained on slopes of Sabouraud dextrose agar (Difco) and subcultured monthly. Every 2 months, cultures were replaced by new ones freshly grown from freeze-dried stocks.

Growth conditions. The organism was grown at 37°C, with shaking, in yeast nitrogen base medium (Difco) containing 500 mM galactose as described previously (16). It grows exclusively in the budding yeast phase under these conditions. Cells were harvested after 24 h (stationary phase of growth) and washed twice in 0.15 M phosphate-buffered saline, pH 7.2 (PBS).

Isolation of EP. EP was prepared by freeze-drying dialyzed culture supernatant, using a minor modification of a method previously described (5). Batches of medium (500 ml in 2-liter Erlenmeyer flasks) were inoculated with overnight yeast cultures (50 ml) and incubated at 37°C for 5 days in an orbital shaker operating at 150 rpm. Yeasts were removed by

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centrifugation, and the culture supernatant fluid was subjected to ultrafiltration in an Amicon DC5 hollow-fiber concentration system, using a 5K filter. It was then dialyzed at 4°C for 3 days against five changes (5 liters each) of distilled water. The retentate (crude EP) was freeze-dried and weighed.

Analysis of EP and EP fractions. Protein was determined by the method of Lowry et al. (14), with bovine serum albumin as a standard. Total carbohydrate was estimated according to the procedure of Dubois et al. (10), with mannose as a standard.

Adhesion assays. Yeast adhesion to exfoliated buccal epithelial cells was determined by light microscopy as described previously (9) except that a lower yeast cell concentration (10^7 organisms ml^{-1}) was used. The ability of crude EP or EP fractions to inhibit adhesion was tested as follows. Epithelial cell suspensions (10^5 cells ml^{-1} in PBS; 1 ml) were centrifuged in a bench centrifuge at 5,000 × *g* for 5 min, and the pellets were resuspended in EP solution (1 ml). Crude EP was used at a concentration of 10 mg ml^{-1} in PBS, while EP fractions were used at lower concentrations which varied according to the experiment; these variations were accounted for in calculating the adhesion inhibition index (AII). After incubation at 37°C for 30 min with gentle shaking, the buccal cells were recovered by centrifugation and resuspended in PBS (1 ml) for use in adhesion assays. All adhesion values quoted represent mean figures derived from three independent assays, each carried out in triplicate. Epithelial cells were obtained from the buccal mucosa of a single, healthy donor (blood group A; nonsecretor) and were always collected at the same time of day to minimize variability.

Treatment of EP with papain and dilute alkali. Papain (Sigma) was used at a concentration of 0.1 mg ml^{-1} in 0.01 M potassium phosphate buffer, pH 6.2. EP (10 mg ml^{-1}; 1 ml) was incubated in this enzyme solution at 25°C for 30 min, and then α_2-macroglobulin (2 mg ml^{-1}; 0.05 ml) was added to prevent further proteolytic activity.

Treatment with dilute alkali involved dissolving EP (20 mg) in 0.1 M NaOH (1 ml) in a screw-cap bottle. The solution was incubated at 30°C for 24 h and then neutralized with HCl (0.1 M; 1 ml) to give a final EP concentration of 10 mg ml^{-1}.

In experiments involving a combined treatment with papain and dilute alkali, the initial EP concentration, as well as the volume and concentration of alkali and acid added, was adjusted such that the final solution (for incubation with buccal cells) had an EP concentration of 10 mg ml^{-1}.

Purification of adhesin by affinity adsorption. Synsorb affinity adsorbents (Chembiomed Ltd., Edmonton, Alberta, Canada) containing blood group determinants (Lewis[a], Lewis[b], and H [types 1 and 2]) covalently linked to a silica matrix were used in the batch adsorption procedure recommended by the manufacturer. Synsorb adsorbent (0.05 g) was washed in a plastic screw-cap tube with PBS (2 ml) and allowed to settle. The buffer was removed, and EP solution (10 mg ml^{-1}; 1 ml) or partially purified EP digest (1 ml) was added. The mixture was gently tumbled for 3 h at 4°C. After this time, unbound material was eluted with PBS until the A_{280} value of the eluate returned to zero. Bound material was then eluted by incubating the adsorbent with aliquots (1 ml) of 1.5% ammonium hydroxide in saline for 15 min at 4°C, again until the A_{280} value of the eluate returned to zero. The bound material was freeze-dried, redissolved in water, and desalted on a Sephadex G-25 (5 ml) desalting column.

Complete purification protocol for the adhesin. Batches of purified adhesin were typically prepared as follows. EP (100 mg ml^{-1} in 0.55 M Na_2HPO_4, pH 8.6; 1 ml) was incubated with *N*-glycanase (Genzyme; 250 U ml^{-1}; 9.6 μl) at 37°C for 24 h, and then 0.1 M sodium citrate buffer, pH 4.4 (0.04 ml), was added. The concentration of EP was adjusted to 50 mg ml^{-1} with 0.01 M potassium phosphate buffer, pH 6.2, and the solution was incubated with papain (0.1 mg ml^{-1}) at 25°C. After 30 min, proteolysis was terminated by the addition of α_2-macroglobulin. Dilute alkali was then added to give a final concentration of 0.1 M NaOH, and the mixture was incubated at 30°C for 24 h. After neutralization with HCl, the EP digest was treated with Synsorb H-2 affinity adsorbent, using the batch adsorption procedure described above.

AII. AII is a measure of the relative efficiency with which each EP fraction inhibits adhesion compared with crude, unfractionated EP, which is assigned a value of 1. Preparations of crude EP, at a concentration of 10 mg ml^{-1}, routinely inhibit adhesion by 45 to 50%. In determining the AII, the weight of protein in each component (EP or EP fraction) required to inhibit adhesion by 50% is calculated. Then, AII = W_{EP}/W_F, where W_{EP} is the weight of EP protein required to produce 50% inhibition of adhesion and W_F is the weight of protein in EP fraction required to produce 50% inhibition of adhesion.

RESULTS

Pretreatment of EP with dilute alkali. Dilute alkali releases short oligosaccharides from O-glycosidic linkage with serine or threonine residues in yeast mannoprotein (1). Previous work (5) showed that treatment of EP with 0.1 M NaOH enhances its ability to inhibit adhesion of *C. albicans* GDH 2346 to buccal epithelial cells. In the present study, the effects of four different concentrations of NaOH, ranging from 0.05 to 0.20 M, on adhesion inhibition by EP were investigated. The results (not shown) indicated that pretreatment of EP with 0.1 M NaOH at 30°C for 24 h produces maximal inhibition of adhesion. This concentration was used in all subsequent experiments. Higher concentrations of alkali produced less inhibition, and it is possible that there was some hydrolysis of the protein portion of the mannoprotein under these conditions.

Pretreatment of EP with papain and dilute alkali. Earlier studies (5) showed that partial degradation of EP by limited papain digestion, followed by dilute alkali treatment, substantially increased the effectiveness of EP as an inhibitor of adhesion. These findings were confirmed here by using assays in which buccal cells were preincubated with EP (treated or untreated) at a final concentration of 10 mg ml^{-1}. The combined papain-dilute alkali pretreatment of EP resulted in an adhesion inhibition of 74%. By contrast, alkali-treated and untreated EP preparations produced adhesion inhibition of 56 and 51%, respectively. Control mixtures showed that treatment of EP with either α_2-macroglobulin–inactivated papain or α_2-macroglobulin alone did not affect adhesion inhibition. In these experiments and in the previous study (5), papain was used under nonreducing conditions. However, further work has revealed that inclusion of cysteine and EDTA in the incubation mixture with papain produces an EP preparation which, presumably because of more extensive proteolytic degradation, is completely devoid of any ability to inhibit adhesion (4a).

Purification of adhesin by affinity adsorption. Following our earlier demonstration (6) that a glycoside containing L-fucose was likely to be the major epithelial cell receptor for many *C. albicans* strains, including strain GDH 2346, at-

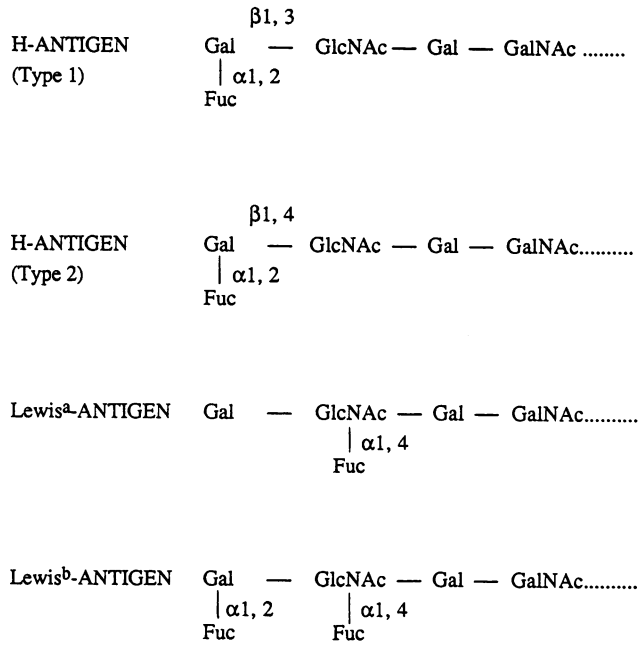

FIG. 1. Structures of H and Lewis blood group antigens. Gal, D-galactose; Fuc, L-fucose; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine.

tempts were made to purify yeast adhesin from crude EP by affinity adsorption with blood group oligosaccharides immobilized on a silica matrix (Synsorb immunoadsorbents). The adsorbents selected for this purpose carried the oligosaccharide determinants of the Lewis[a], Lewis[b], and H (types 1 and 2) blood group antigens (Fig. 1). Preparations of EP from *C. albicans* GDH 2346 were treated with these products by a batch adsorption procedure; after elution, bound material was analyzed for protein and carbohydrate. EP protein bound in greatest amounts to the Lewis[a], Lewis[b], and H-2 oligosaccharides (Table 1). However, H-2-adsorbed material contained considerably less carbohydrate than did that bound to either the Lewis[a] or Lewis[b] oligosaccharides. EP protein bound in lowest amounts to the H-1 oligosaccharide (Table 1).

To determine which of the bound fractions produced the greatest inhibition of yeast adhesion relative to crude EP, a series of adhesion inhibition assays was carried out with *C. albicans* GDH 2346. The results of experiments with EP fractions obtained from the H-2 affinity adsorbent are shown

TABLE 1. Biochemical analysis of EP from *C. albicans* GDH 2346 bound to different affinity adsorbents

Immobilized oligosaccharide	% of crude EP bound as[a]:	
	Protein	Carbohydrate
Lewis[a] determinant	14.4 ± 0.4	20.1 ± 1.0
Lewis[b] determinant	12.2 ± 0.2	16.0 ± 0.5
H determinant		
Type 1	5.1 ± 0.1	7.9 ± 0.1
Type 2	13.4 ± 0.2	8.3 ± 0.1

[a] Bound protein and carbohydrate were calculated as percentages of the protein and carbohydrate contents, respectively, of crude EP. Figures represent means ± standard errors of the means of two independent determinations done in triplicate.

TABLE 2. Inhibition of adhesion of *C. albicans* GDH 2346 to buccal epithelial cells by different EP fractions, using adhesin purification with an H-2 affinity adsorbent

EP fraction used to pretreat buccal cells	Mean no. ± SEM of adherent yeasts/100 epithelial cells	Inhibition of adhesion (%)[a]	AII[b]
H-2 bound	435 ± 26	41	62.2
H-2 unbound	547 ± 27	25	1.7
Crude EP	400 ± 16	45	1.0
None (PBS control)	733 ± 17	0	

[a] Inhibition obtained with EP-treated epithelial cells compared with adhesion to PBS-treated epithelial cells.

[b] AII is a measure of the relative efficiency with which each fraction inhibited adhesion compared with crude EP and was calculated as explained in Materials and Methods.

in Table 2. Preincubation of buccal cells with the H-2-bound fraction inhibited subsequent yeast adhesion by 41%, whereas unbound material and crude EP produced inhibition of 25 and 45%, respectively. However, all of these EP preparations contained different weights of protein. To take account of these differences, an AII was calculated for each fraction as described previously (5). AII relates the inhibition observed to the weight of inhibitor protein used; crude, unfractionated EP is assigned a value of 1. On this basis, the H-2-bound fraction inhibited adhesion with a 62-fold-greater efficiency than did crude EP (Table 2).

Similar experiments were carried out with the EP fractions obtained by using the other affinity adsorbents. Material bound to the Lewis[a], Lewis[b], and H-1 oligosaccharides gave rather lower AII values of 46.7, 17.7, and 51.3, respectively.

Complete purification scheme for the adhesin. On the basis of these findings and our earlier studies on adhesion mechanisms (5, 6), a scheme for purification of the yeast adhesin was devised. The protocol (Fig. 2) involves a stepwise treatment of crude adhesin (EP) with *N*-glycanase, papain, and dilute alkali to cleave the protein and carbohydrate

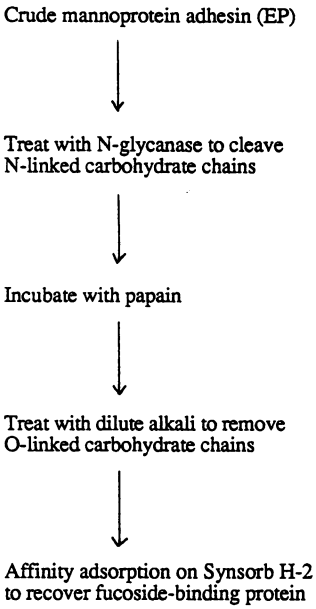

FIG. 2. Complete protocol for the purification of yeast adhesin.

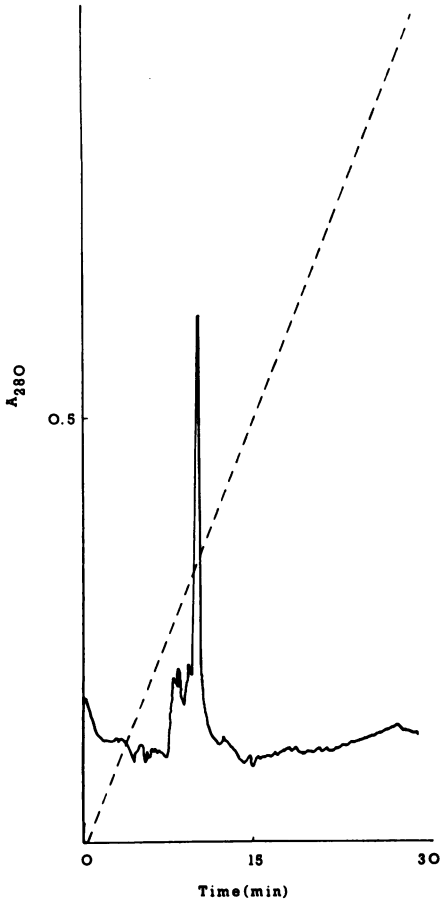

FIG. 3. Reverse-phase FPLC of purified adhesin. Purified material (400 μg of protein ml^{-1}; 100 μl) was applied to a PepRPC HR 5/5 column (Pharmacia LKB) and eluted over 30 min with a gradient of 0 to 100% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 40 ml h^{-1}.

portions of the mannoprotein molecule. *N*-Glycanase breaks the N-glycosidic linkage between *N*-acetylglucosamine and asparagine and so releases polymannose chains attached via diacetylchitobiose bridges; this occurs without disrupting the integrity of the protein (21). Fucoside-binding protein fragments are then recovered by affinity adsorption with the H-2 blood group oligosaccharide.

Purified adhesin isolated by this procedure consisted of one major component as determined by reverse-phase fast protein liquid chromatography (FPLC) (Fig. 3). It inhibited yeast adhesion to buccal cells by 80% and 221 times more efficiently, on a protein weight basis, than did crude EP (Table 3). During the course of the purification procedure, carbohydrate was gradually removed; this correlated with the increasing ability of successive fractions, when tested separately, to inhibit adhesion (Table 4). The final fraction of purified adhesin was completely devoid of carbohydrate. Thus, on an overall weight basis, inhibition of adhesion by the purified adhesin was some 2,000-fold greater than that observed with the starting material because crude EP contains considerable amounts of carbohydrate (Table 4).

In a separate series of experiments, the effects of increasing concentrations of purified adhesin on yeast adhesion to buccal epithelial cells were investigated. Inhibition of adhesion began to level off at an adhesin concentration of 7.5 μg ml^{-1} and reached a maximum of 78% at a concentration of 10 μg ml^{-1} (Table 5). *C. albicans* GDH 2346 is significantly less adherent when grown in medium containing 50 mM glucose as a carbon source (9). However, adhesion of 50 mM glucose-grown yeasts was similarly inhibited to a maximum of 75% by purified adhesin used at a concentration of 15 μg ml^{-1} (results not shown). Our failure to demonstrate adhesion inhibition of 100% with the purified adhesin suggests that one or more additional mechanisms may operate.

TABLE 3. Effect of purified adhesin on adhesion of *C. albicans* GDH 2346 to buccal epithelial cells

EP fraction used to pretreat buccal cells	Mean no. ± SEM of adherent yeasts/100 epithelial cells	Inhibition of adhesion (%)[a]	AII[b]
Purified adhesin	189 ± 29	80	221
Crude EP	508 ± 15	47	1.0
None (PBS control)	965 ± 23	0	

[a] Inhibition obtained with EP-treated epithelial cells compared with adhesion to PBS-treated epithelial cells.
[b] AII is a measure of the relative efficiency with which each fraction inhibited adhesion compared with crude EP and was calculated as explained in Materials and Methods.

DISCUSSION

In an earlier attempt to purify a mannoprotein adhesin from *C. albicans* GDH 2346 (5), crude EP was subjected to chromatography on concanavalin A-Sepharose and DEAE-cellulose. This two-step procedure resulted in the isolation of an active fraction capable of inhibiting yeast adhesion to buccal cells 30 times more efficiently (on a protein weight basis) than did unfractionated EP. The same study also showed that incubation of EP with papain followed by dilute alkali enhanced its ability to block adhesion (5). Our present results have confirmed this finding, and we have incorporated treatment with both reagents into a new scheme for purification of the adhesin. The protocol, which also includes *N*-glycanase and affinity adsorption steps (Fig. 2), produces a fraction capable of significantly greater adhesion inhibition than that isolated previously (AII values of 221 and 31, respectively). Moreover, the purified adhesin is completely devoid of carbohydrate, thus confirming our

TABLE 4. Analysis and inhibitory activities of adhesin fractions isolated during the purification protocol

Fraction[a]	Protein/carbohydrate ratio	AII[b]
1	1:11	1
2	1:1.6	56
3	1:1.2	115
4	1:0.9	136
5	1:0.4	144
6	1:0.0	221

[a] The fractions listed had been processed as follows (in descending order): 1, crude untreated EP; 2, crude EP bound to Synsorb H-2; 3, crude EP treated with papain and dilute alkali and bound to Synsorb H-2; 4, crude EP treated with *N*-glycanase and bound to Synsorb H-2; 5, crude EP treated with *N*-glycanase and dilute alkali and bound to Synsorb H-2; 6, crude EP treated with *N*-glycanase, papain, and dilute alkali and bound to Synsorb H-2 (purified adhesin).
[b] AII is a measure of the relative efficiency with which each fraction inhibited adhesion compared with crude EP and was calculated as explained in Materials and Methods.

TABLE 5. Effects of increasing concentrations of purified adhesin on adhesion of *C. albicans* GDH 2346 to buccal epithelial cells

Component used to pretreat buccal cells	Mean no. ± SEM of adherent yeasts/100 epithelial cells	Inhibition of adhesion (%)[a]
Purified adhesin ($\mu g\ ml^{-1}$)		
1.25	725 ± 19	4
2.50	625 ± 28	17
5.00	280 ± 27	63
7.50	209 ± 19	72
10.00	195 ± 17	78
Crude EP (10 $mg\ ml^{-1}$)	412 ± 15	46
PBS control	752 ± 28	0

[a] Inhibition obtained with adhesin-treated epithelial cells compared with adhesion to PBS-treated epithelial cells.

earlier supposition (5) that it is primarily the protein portion of the mannoprotein molecule which interacts with epithelial cells. We are currently analyzing the purified material by high-pressure liquid chromatography with a view to sequencing studies.

The adhesion inhibition reported here, 80% inhibition by microgram amounts of purified adhesin, is the highest yet recorded for *C. albicans*. However, 100% inhibition was never achieved in any of our experiments, which suggests that one or more secondary adhesion mechanisms operate. Affinity chromatography has shown that crude adhesin preparations from five strains of *C. albicans*, including strain GDH 2346, all contain lectin-like proteins (mannoproteins) capable of binding to *N*-acetyl-D-glucosamine and D-mannose as well as to L-fucose, and there is some evidence that a secondary adhesion system for strain GDH 2346 might involve *N*-acetylglucosamine-containing receptors (6). Alternatively, although our studies indicate that yeasts interact with epithelial cells primarily via the protein portion of a mannoprotein adhesin, the carbohydrate chains of mannoproteins could also participate in attachment to host surfaces. Mutants of serotype A *C. albicans* that lack antigenic factor 6, an epitope located in the outer chain region of cell surface mannoprotein, have been isolated. One such mutant adhered less readily than the parent strain to a cell line of human mouth squamous cell carcinoma, suggesting that the factor 6 determinant may be involved in this particular interaction (18).

Among possible candidates for *Candida* receptors on epithelial cells are the ABO and Lewis blood group antigens, all of which possess residues of L-fucose. Our purification protocol for the adhesin included, as its final step, affinity adsorption with the terminal trisaccharide of the H (type 2) blood group antigen. The purified adhesin was able to block yeast attachment to buccal cells extremely effectively, suggesting that this trisaccharide closely resembles the natural receptor. Support for this idea has been provided recently by Brassart et al. (2), who investigated the ability of various glycopeptides and structurally defined oligosaccharides to inhibit *Candida* adhesion. Mixtures of glycopeptides blocked attachment by up to 55%, but inhibition was completely abolished if fucose residues were removed from the compounds by mild acid hydrolysis. When oligosaccharides were used as inhibitors, the minimal structural requirement for activity was the Fucα1 → 2Galβ determinant of the H antigen (Fig. 1) which is found on all blood group substances of the ABO system.

Unlike ABO antigens, the Lewis antigens are not structural components of epithelial cells but are adsorbed from saliva and other body fluids. Brassart et al. (2) reported that Lewis antigens failed to act as adhesion inhibitors. However, other evidence suggests that the Lewis[a] antigen (which is found in the body fluids of nonsecretors), when adsorbed to epithelial cell surfaces, might function as a receptor for *C. albicans*. In two separate studies, buccal cells from a secretor and a nonsecretor were pretreated with anti-Lewis[a] and anti-Lewis[b] antisera before being used in adhesion assays. In both cases, pretreatment of nonsecretor buccal cells with the anti-Lewis[a] antiserum had a significant inhibitory effect on yeast adhesion, whereas none of the other pretreatments produced statistically significant differences (15, 22). Although these results appear to contradict those of Brassart et al. (2), there is some evidence that the ability of *C. albicans* to bind to the Lewis[a] antigen is strain dependent (15). Overall, the H (blood group O) and Lewis[a] antigens would seem to be prime candidates for fucose-containing epithelial cell receptors. Such a conclusion is consistent with the findings of a clinical survey (3) which indicated that in healthy subjects, blood group O and nonsecretion of blood group antigens are separate and cumulative risk factors for oral carriage of *C. albicans*.

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