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Adherence of *Candida albicans* to Human Buccal Epithelial Cells: Host-Induced Protein Synthesis and Signaling Events

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The synthesis of proteins by *Candida albicans* was studied following adherence of blastoconidia to human buccal epithelial cells (HBEC). Initially, labeling of HBEC, *C. albicans*, and HBEC-*C. albicans* with [^{35}S]methionine was performed. After a 3-h incubation and prior to labeling with [^{35}S]methionine, the cultures were treated with cycloheximide to prevent HBEC protein synthesis. The HBEC-*C. albicans* mixture as well as *C. albicans* and HBEC incubated separately were extracted with β-mercaptoethanol (β-ME). These extracts as well as the cell residue (solubilized by boiling with sodium dodecyl sulfate [SDS]) were examined by SDS-polyacrylamide gel electrophoresis and autoradiography. In comparison to cultures of *C. albicans* incubated without HBEC, proteins with molecular masses of approximately 52 to 56 kDa from β-ME extracts and from SDS-solubilized cells were observed only from adhering cultures. In addition, unlabeled β-ME extracts were electrotransferred to nitrocellulose and immunoblotted with antiphosphotyrosine antibodies to determine whether cell signaling events were occurring during adherence. Proteins with molecular masses of 54 and 60 kDa were recognized only in mixed cultures of *C. albicans* and HBEC. These data indicate that following adherence of *C. albicans* to HBEC, new *Candida* proteins are expressed. Further, these events are accompanied by the expression of signal proteins, presumably of *Candida* origin.

The adherence of *Candida albicans* to a variety of host cells, host cell ligands, and plastics has been described (1, 4–10, 12–20, 22–25, 27–45, 47–49). The interaction of the organism with mucosal cells is believed to be one of the critical initial events in the development of candidiasis. Supporting this conclusion are the observations that nonadhering strains of *C. albicans* cause less disease in animal models (8, 15, 21) and that pretreatment of animals with a ligand recognized by *C. albicans* reduces the tissue load of the organism (32). Also, carriage of *C. albicans* in the oral cavity is lower in individuals who secrete a fucosyl-containing ligand recognized by the organism (7). Presumably, soluble ligand binds to a cell surface adhesin(s) of the organism, thus preventing the adherence of *C. albicans* to host cells. Conversely, *C. albicans* binds readily to cells in those individuals who do not secrete this ligand (7).

The adhesins of *C. albicans* appear to be diverse, reflecting the ability of the organism to colonize and invade a variety of host cells and tissues (8, 10). The host cell ligands recognized by *C. albicans* are also diverse but appear to be broadly classified into at least two types, glycosides (fucosyl or other) (6, 47) and, in part, the peptides (arginine-glycine-aspartic acid [RGD]) of several extracellular matrix proteins (4, 5, 8–10, 12, 19, 20, 22, 23, 25, 29, 30, 34, 43, 48, 49). The infection process continues following adherence of the organism to host cells and plastics (as in the case of indwelling catheters). However, the events subsequent to adherence are not entirely understood. Presumably, the conversion of blastoconidia to hyphae (pseudohyphae) and the secretion of essential enzymes (i.e., secreted aspartyl proteases) promote invasion to subepithelial or subendothelial tissues and the vascular compartment (15).

Hawser and Douglas (24) have observed that the adherence of *C. albicans* blastoconidia to plastic catheters is followed by conversion to hyphae. These events indicate that a contact-induced regulation of gene expression may be occurring. Other investigators have demonstrated a thigmotropism-like effect in *C. albicans* when hyphal forms were grown on Nuclepore membranes (46). Following its horizontal growth pattern, the organism entered pores and grew to the underside of the filters, directional changes which probably require upregulation of genes or the transcription of a new gene(s). The directed growth may be important during the transgression of mucosal epithelial surfaces by *C. albicans.*

In order to examine some of the key events which occur following adherence of the organism to human buccal cells, we have compared adhering with nonadhering *Candida* cultures and determined if (i) new *Candida* proteins are made and (ii) signal events occur during adherence. Our measurement of signaling utilizes Western blotting (immunoblotting) of extracted *Candida* proteins with antiphosphotyrosine antibodies, similar to the approaches used for characterizing the mating signal transduction pathways of *Saccharomyces cerevisiae* (2, 3).

MATERIALS AND METHODS

Cultures and buccal epithelial cells. *C. albicans* 4918 was used throughout these studies. This isolate was originally obtained from a patient with an endocarditis infection and has been described previously (9). Blastoconidia were grown and standardized as described below. Human buccal epithelial cells (HBEC) were collected on cotton swabs from healthy male and female volunteers and transferred to phosphate-buffered saline (PBS) (pH 7.2). HBEC was washed three times with PBS and counted by using a hemocytometer.

Adherence assays. *C. albicans* cells (2×10^7/ml) were incubated with HBEC (1×10^5 to 2×10^5/ml) in PBS (pH 7.2) for the times indicated below. At each time interval, cultures were washed with sterile, 0.02 M PBS (pH 7.2) through filters with a pore size of 12.0 μm (Nuclepore [PC]; Costar, Cambridge, Mass.) and the contents of the filter were transferred to microscope slides and stained with Gram stain to determine the percent adherence. Adherence was expressed as the percentage of HBEC with adhering *Candida* cells.

Methionine labeling of cultures. *C. albicans* was grown for 48 h at room temperature on yeast extract-peptone-glucose agar or, in some cases, on Luria-Bertani agar (Thomas Scientific). Cells were collected in sterile 0.02 M PBS (pH 7.2) and washed three times in PBS containing gentamicin (5 μg/ml), 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.02% sodium azide, 1 mM leu-

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peptin, and 1 mM pepstatin to prevent protein degradation. The cell suspension was uniformly composed of blastoconidia. For all experiments, a concentration of 2×10^7 blastoconidia per ml of PBS was used. The concentration of HBEC was approximately 1×10^5 to 2×10^5 cells per ml of PBS. Cultures of *C. albicans* and HBEC were mixed at a 1:1 ratio in a total volume of 2 ml and incubated at 37°C for a total of 1 or 3 h. At 1 or 3 h, the HBEC-*C. albicans* cultures were pulsed with 10 μCi of [^{35}S]methionine (1,144 Ci/mmol; ICN, Irvine, Calif.) for 30 min and were subsequently pulsed for an additional 30 min with 100 μM cold methionine. Cycloheximide (10 μg/ml; Sigma Chemical Co., St. Louis, Mo.) was added 30 min prior to labeling with methionine to inhibit HBEC protein synthesis. Cultures of HBEC or *C. albicans* incubated alone were treated in the same fashion. After labeling, all cultures (HBEC, *C. albicans*, and HBEC-*C. albicans*) were centrifuged in a microcentrifuge and washed with sterile, cold water.

Extraction of proteins. The same procedure was used for both the radiolabeled and the nonlabeled cell suspensions. β-Mercaptoethanol (β-ME) (1%) in 10 mM phosphate buffer, pH 7.5 (100 to 200 μl), was added to each of the cell pellets (from 2-ml cultures), and the suspension was incubated at 37°C for 30 min with shaking. After centrifugation, the supernatant was transferred to microconcentrators (Microcon; Amicon, Beverly, Mass.) and washed several times with water to remove the β-ME. These steps were performed at 4°C. Finally, the washed, concentrated extracts were frozen at −70°C and lyophilized. The sediment from the radiolabeled cells after β-ME extraction was also washed with water and frozen at −20°C. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, the cell sediments were suspended in 20 μl of SDS sample buffer, boiled for 10 min, and clarified by centrifugation. Fifteen microliters of the cell lysate and 20 μl of the β-ME extracts from the labeled cultures were applied to wells of a 1-mm-thick gel. SDS-PAGE was performed under reducing conditions by using a Mini-gel system with 10% polyacrylamide as described previously (21, 49). Following electrophoresis, the gel was dried for autoradiography on Kodak X-Omat film (Kodak, Rochester, N.Y.) and exposed for 2 h (cell lysate) or 2 days (β-ME extracts) at −70°C before the film was developed. The molecular weights of the radiolabeled bands were calculated by using radiolabeled standard markers (GIBCO-BRL, Grand Island, N.Y.).

Immunoblotting. The nonlabeled culture suspensions were processed in the same way as the radiolabeled ones. Generally, only the β-ME extracts were assayed for immunoreactivity. The lyophilized extracts were dissolved in water, and total protein concentration was determined according to the Lowry procedure, as described previously (49). Equal amounts of 1.5 to 2.0 μg of protein (diluted 1:3 for Aurodye staining) were loaded on 10% polyacrylamide slab gels (thickness, 0.75 mm). The SDS-PAGE-separated proteins were transferred to nitrocellulose membranes (pore size, 0.45 μm) and stained with Aurodye or immunoblotted according to methods previously described (9, 49). An antiphosphotyrosine monoclonal antibody, immunoglobulin G subclass 1 (IgG1), from ascites fluid (PT-66; Sigma Chemical Co.), was employed in most of the experiments. In addition, two other antiphosphotyrosine antibodies, a rabbit polyclonal IgG and a monoclonal IgG2b(κ) (Upstate Biotechnology, Inc., Lake Placid, N.Y.) were also tested. All antibodies were used at a concentration of 1 μg/ml and incubated at 4°C overnight. As a secondary reagent, rabbit IgG and mouse IgG alkaline phosphatase-conjugated antibodies were used in accordance with the manufacturer's protocol (Promega). Protein standards (Bio-Rad) were used for the estimation of the molecular weights of specific bands.

RESULTS

Adherence of *C. albicans* to HBEC. The adherence of *C. albicans* 4918 to HBEC was measured at 1, 2, 5, and 24 h. Adherence, measured as the percentage of HBEC with adhering *Candida* cells, reached its maximum at 2 h (85%) and did not increase significantly thereafter (data not shown). Germination of adhering blastoconidia in cell suspensions was not observed at any time interval. On the basis of this observation, we chose 1- and 3-h incubation times for *C. albicans* with HBEC for metabolic labeling and signal studies.

Host-induced proteins of *C. albicans*. To determine if HBEC induced the synthesis of new proteins in *C. albicans* 4918, we compared protein profiles of blastoconidia incubated in PBS alone with those of HBEC and blastoconidia (3 h). All cultures were metabolically labeled with [^{35}S]methionine. *C. albicans*-specific proteins could be determined for adhering cultures since cycloheximide inhibited the synthesis of new proteins in HBEC (Fig. 1, lanes 1 and 4). The spectrum of the metabolically labeled *Candida* proteins incubated without HBEC (Fig. 1, lanes 2 and 5) was compared with that for the cultures of HBEC-*C. albicans*. Proteins with molecular masses of 56 kDa (from β-ME extracts of cells) and 52 and 54 kDa (from SDS-solubilized cell residues) were observed (Fig. 1, lanes 3 and 6)

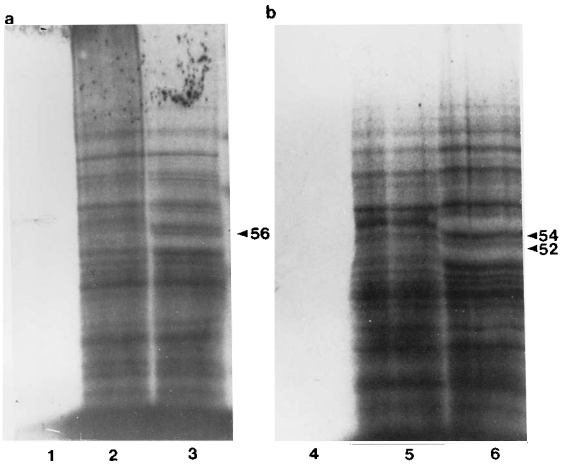

FIG. 1. [^{35}S]methionine-labeled proteins of *Candida albicans*. HBEC (lanes 1 and 4), *C. albicans* (lanes 2 and 5), and HBEC-*C. albicans* (lanes 3 and 6) cultures were incubated for 3 h at 37°C and pulsed for 30 min with labeled methionine subsequent to treatment with cycloheximide. All cultures were extracted with β-ME, and the β-ME extracts (a) or SDS-solubilized cell residues (b) were subjected to SDS-PAGE and autoradiography. The numbers to the right of each panel are molecular sizes in kilodaltons.

only from cultures of HBEC-*C. albicans*. The autoradiograms otherwise appeared to be similar. The data indicate that the 56-kDa protein (Fig. 1, lane 3) is probably associated with the cell wall (and/or plasma membrane), since it was solubilized by β-ME; the 52- and 56-kDa proteins may also be associated with similar cell locations or may be cytoplasmic. Results at 1 h were similar to those at 3 h.

Signaling events during adherence. We next compared HBEC, *C. albicans*, and HBEC-*C. albicans* cultures for signaling events after an either 1- or 3-h incubation at 37°C. Proteins from β-ME extracts of each of the three cultures were separated by SDS-PAGE and transferred to nitrocellulose. Immunoblotting was performed with three antiphosphotyrosine antibodies, as described in Materials and Methods. In most instances, the monoclonal antibody PT-66 (Sigma) was employed. The data from a representative Western blot of β-ME extracts (3 h) are shown in Fig. 2. In panel A, an Aurodye-stained nitrocellulose membrane of HBEC (lane 1), *C. albicans* (lane 2), and HBEC-*C. albicans* (lane 3) reveals a spectrum of proteins in regard to their molecular masses. We also compared the effects of two media on signaling responses in *C. albicans*. Thus, in Fig. 2A, lanes 2 and 3 show extracts from blastoconidia grown on yeast extract-peptone-glucose agar for adherence assays and lanes 4 and 5 show extracts from blastoconidia grown on Luria-Bertani agar. In Fig. 2B, Western blots of HBEC, *C. albicans*, and HBEC-*C. albicans* β-ME extracts with the monoclonal antibody PT-66 are shown. For the control cultures, a reactive protein with a molecular mass of about 50 kDa from HBEC is observed (Fig. 2B, lane 1), while a few faint protein bands can be detected in *C. albicans* blastoconidia incubated in PBS (Fig. 2B, lanes 2 and 4). In comparison, from β-ME extracts of cultures of HBEC-*C. albicans*, proteins with molecular masses of approximately 54 and 60 kDa reacted with the antiphosphotyrosine monoclonal antibody PT-66 (Fig. 2B, lanes 3 and 5); however, it appeared that signal responses were greater for cells prepared on yeast extract-peptone-glucose agar than for cells prepared on Luria-Bertani agar (Fig. 2B, compare lanes 3 and 5). The results obtained from an additional five Western blot analyses were similar. The 54- and 60-kDa proteins were also precipitated by

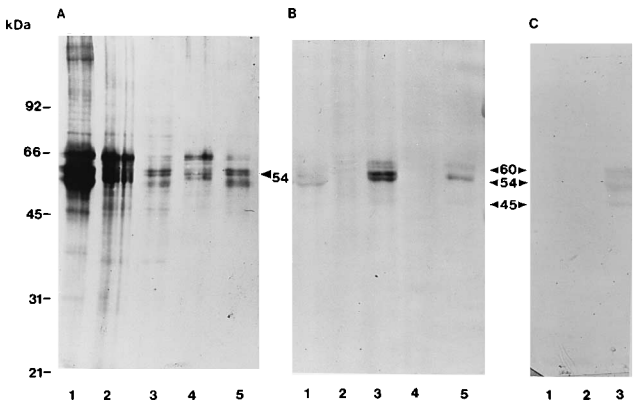

FIG. 2. Aurodye staining (A) and Western blots (B and C) of proteins extracted from HBEC (lanes 1), *C. albicans* (lanes 2 and 4 [A and B]; lane 2 [C]), and HBEC-*C. albicans* (lanes 3 and 5 [A and B]; lane 3 [C]). Lanes 2 and 3, *C. albicans* grown on yeast extract-peptone-glucose agar; lanes 4 and 5, *C. albicans* grown on Luria-Bertani agar for use as inoculum in the adherence studies. Two dominant bands at 54 and 60 kDa are observed with antiphosphotyrosine antibody (PT-66) and the HBEC-*C. albicans* culture. (C) The antiphosphotyrosine monoclonal antibody IgG2b(κ) (Upstate Biotechnology, Inc.) immunoprecipitated proteins of 45, 54, and 60 kDa only from HBEC-*C. albicans* cultures.

the purified monoclonal antibody IgG2b(κ) (four experiments [Fig. 2C, lane 3]) and the polyclonal antibody (two experiments) described in Materials and Methods (Upstate Biotechnology, Inc.). The monoclonal antibody [IgG2b(κ)] also precipitated a 45-kDa protein to a greater extent than did PT-66 (Fig. 2C, lane 3). While the 54-kDa protein was consistently observed in all Western blots, the 60-kDa protein was not always detected. Additionally, the polyclonal antibody (Upstate Biotechnology, Inc.) also strongly precipitated a 68-kDa protein (data not included). For HBEC cells, the presence of a precipitated protein was observed only in some experiments and may have been a function of the source of HBEC. There was no difference in the Western blot profiles of the 1- and 3-h cultures.

DISCUSSION

The adherence of *C. albicans* to host cells is thought to be an essential event in the pathogenesis of candidiasis. Clearly, the organism must possess virulence determinants even though host abrogations, such as neutropenia, T-cell defects, prolonged use of antibacterial antibiotics, etc., contribute greatly to the invasiveness of the organism.

In this study, we focused upon events occurring during adherence and chose HBEC as the host cell with which to study these events. Adherence to HBEC has been extensively studied; recognition by *C. albicans* occurs through a cell surface mannoprotein and a fucosyl glycoside of HBEC (6, 47).

We used cycloheximide to inhibit HBEC protein synthesis and, in this way, could directly determine if new proteins were synthesized by *C. albicans* during adherence. By autoradiography, proteins with molecular masses of 52 to 56 kDa were present only in HBEC-*C. albicans* cultures. These proteins have not been characterized; however, their presence in β-ME extracts indicates their location in the cell wall or plasma membrane, although proteins with similar molecular masses were also found in SDS lysates of the β-ME-insoluble cell residue. β-ME extracts of the HBEC-*C. albicans* culture also reacted with antiphosphotyrosine antibodies. While we cannot totally determine the source(s) of these proteins (HBEC, *C. albicans*, or both), there are several indirect observations which indicate that the phosphotyrosine proteins are of fungal origin. First, Ballard et al. have demonstrated that cycloheximide-treated *S. cerevisiae* was significantly inhibited in generating a 40-kDa protein which reacted with an antiphosphotyrosine antibody when cells of mating type **a** were incubated with α-factor (2). A similar observation has been made with the epidermal growth factor receptor signal transduction of mammalian cells (11). Thus, ongoing protein synthesis seems to be a requirement for signal transduction events, and as such, HBEC signal transduction should be at least partially inhibited in the presence of cycloheximide, since new protein synthesis was not observed. Second, the molecular masses of the *C. albicans*-specific proteins detected by autoradiography (Fig. 1, lanes 3) approximate those seen with Western blot analyses (Fig. 2). Aside from these results, obtained by using antiphosphotyrosine antibody, we are now in the process of purifying phosphotyrosine proteins from metabolically labeled *C. albicans* cultured with HBEC in the presence of cycloheximide.

The induction of new proteins following infection by fungi has been observed with the plant pathogen *Cladosporium fulvum* (26). Joosten and De Wit have shown that infection of susceptible plants with *C. fulvum* (compatible reaction) resulted in the synthesis of a 14-kDa protein not observed in vitro or in reactions with resistant plants (incompatible reaction). This protein appears to be of fungal origin; Northern (RNA) analyses have demonstrated its presence during compatible reactions only. The function of the protein is unknown but seems to be related to the establishment of a successful parasitic relationship.

Clearly, *C. albicans* can respond to its host in a number of ways. First, the conversion of blastoconidia to hyphae occurs during colonization of surfaces, phagocytosis, and adherence to plastic (15, 24, 46). The conversion may be associated with the virulence of the organism (15). Secondly, growth patterns of the organism on mucosal surfaces resemble a contact-sensing mechanism recently described in vitro by Sherwood et al. as a thigmotropism (46). Hyphal growth of the organism occurred along the surfaces of Nuclepore filters until the organism reached the membrane pores. Upon contacting a pore, the organism turned inside the pore in a manner much like that observed during the penetration of mucosal surfaces by hyphae. The change in the direction of growth would seem likely to require upregulation and/or new gene transcription, i.e., cytoskeletal and wall synthesis, etc., events often dictated by signal transduction events. Mating, cell wall synthesis, and growth of yeast cells in hypo-osmotic media have been shown to be controlled by the mitogen activated protein kinase pathway (3).

We have focused upon one of the early events during the infection process, i.e., adherence. Our data indicate that following adherence, specific proteins are made by *C. albicans*; further, phosphorylation of proteins occurs, indicating possible signal events. These observations are new to studies involving *C. albicans*-host cell interactions.

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