Specific Adherence of *Candida tropicalis* to Lysophospholipids†

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ABSTRACT: *Candida* species are usually commensal organisms, but they become invasive when the host is immunocompromised. Mechanisms by which these organisms adhere to, colonize, and then invade host tissues are poorly understood. To detect potential host receptors, members of a lipid library were chromatographically separated and then overlaid with *Candida tropicalis*; components to which the organisms bound were visualized by autoradiography. In initial experiments no interactions with either glycolipids or intact phospholipids were detected. However, lysophospholipids supported adherence of *C. tropicalis* but not *Saccharomyces cerevisiae*. These results were confirmed by a second assay; *C. tropicalis* adhered to certain lysophospholipids, but not intact phospholipids, that were immobilized on microtiter plates. Using [^{14}C]-1-palmitoyl-*sn*-glycero-3-phosphocholine, we showed that *C. tropicalis* adherence is accompanied by rapid conversion of the labeled lipid to a number of compounds. Thus, the interaction of *C. tropicalis* with lysophospholipids results in significant changes in both the organism and the lysophospholipid to which it binds. We hypothesize that this interaction could be an important component of the infection process.

The genus *Candida* is a collection of 150 asporogenous yeast species that are classified as fungi. They exist in several morphologically distinct forms (Odds, 1985). The yeast or blastoconidial form is a commensal organism that colonizes epithelial surfaces of most healthy people without causing harm. Blastoconidia can grow by budding, form elongations termed germ tubes, or convert to filamentous (hyphal) forms. Conversion from the yeast to the filamentous form is associated with invasion, which usually occurs when the host is immunocompromised, as is the case with elderly, pregnant, diabetic, and human immunodeficiency virus-infected patients (Odds, 1988). Under these circumstances opportunistic *Candida* species can cause infections of the mouth, other parts of the gastrointestinal tract, and vagina. *Candida albicans* is the species most commonly isolated from infected sites, followed by *Candida glabrata*, *Candida tropicalis* and *Candida parapsilosis*. The other pathogenic *Candida* species are *Candida stellatoidea*, *Candida guilliermondii*, *Candida krusei* and *Candida pseudotropicalis*.

Although *C. albicans* is isolated more frequently from sites of infection, there is evidence that *C. tropicalis* may be more virulent (de Repentigny et al., 1992). Virulence of the various *Candida* spp. is a complex phenomenon that is associated with morphological changes (Brawner & Mori, 1992), production of proteinases (Cutler, 1991), surface hydrophobicity (Hazen et al., 1991), phenotypic switching (Soll et al., 1987), and molecular mimicry (Mayer et al., 1990). Adherence [reviewed by Calderone and Braun (1991)] is also an important virulence factor and is the necessary first step in the colonization and invasion of host tissues. As with bacterial infection, it is important to note that these stages often overlap temporally, suggesting that the underlying molecular mechanisms may not be entirely separable. For example, the *Candida* aspartyl proteinases could have an important role in both initial adhesion and subsequent invasion (Borg & Ruchel, 1988; White et al., 1993).

For bacteria, initial adhesion is often the result of specific interactions between protein complexes termed adhesins on the cell surfaces of the microorganisms and the carbohydrate portions of host cell surface receptors (Ofek & Perry, 1985; Yeung et al., 1987; Ofek & Sharon, 1990). Examples include the interactions of a *Streptococcus sanguis* protein, SSP-5, with the sialic acid residues of human salivary agglutinin (Demuth et al., 1990), the binding of uropathogenic *Escherichia coli* to Galα1→4Gal-containing glycolipids (Leffler & Svanborg-Eden, 1980; Bock et al., 1985), and the adherence of *Fusobacterium nucleatum* via the unsubstituted lactosamine residues carried by a highly glycosylated human salivary proline-rich glycoprotein (Gillece-Castro et al., 1991).

Adhesin–carbohydrate interactions have similarly been implicated in candidal adherence. Specific adhesins have not been identified, but binding characteristics have suggested their existence. Chitin inhibited adhesion of *C. albicans* to vaginal epithelial cells in vitro, suggesting a role for *N*-acetylglucosamine residues in adherence (Segel et al., 1982). Critchley and Douglas (1987) reported that at least two different glycosides containing L-fucose and *N*-acetylglucosamine can function as epithelial receptors for *C. albicans*. Of the five *Candida* strains examined in this study, four interacted primarily with fucose-containing receptors and one bound via *N*-acetylglucosamine. Finally, adhesion of *C. albicans* to human buccal epithelial cells can be blocked by Fucα1→2Galβ-bearing complex carbohydrates (Brassart et al., 1991), whereas adhesion to cultured human keratinocytes can be blocked by the addition of amino sugars and fucose (Ollert et al., 1993). Together these findings suggest that there are several different candidal adhesins, that more than one carbohydrate receptor sequence is present on host cells, and that certain adhesive characteristics may be strain- or switch phenotype-specific.

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The infection process is likely to involve interactions between other classes of molecules as well. With regard to protein–protein interactions, *C. albicans* can adhere to extracellular matrix components such as fibronectin (Klotz & Maca, 1988; Jakab et al., 1993). Furthermore, peptides containing the sequence arginine-glycine-aspartic acid reduced the number of *C. albicans* abscesses in a rabbit model of systemic infection (Klotz et al., 1992). Since this tripeptide sequence is recognized by some members of the integrin family of cell–extracellular matrix adhesion receptors, there is a great deal of interest in the possibility of a *Candida* integrin homologue (Klotz et al., 1993). A synthetic adhesive peptide from laminin (CDPGYIGSR-NH_2) blocked *C. albicans* adherence to cultured human keratinocytes (Ollert et al., 1993), and glycosaminoglycans inhibited adherence to extracellular matrix proteins (Klotz & Smith, 1992) suggesting that other matrix-binding proteins could also play an important role in adherence.

Evidence also exists of interaction between candidal surface carbohydrates and an unknown host receptor. Adherence of *Candida* to epithelial cells is blocked by selective inhibition of mannoprotein synthesis (Kennedy, 1988) and by anti-mannan antibodies (Miyakawa et al., 1992). This mechanism may be particularly important with regard to adherence of the yeast forms of the organism to spleen and lymph node tissues (Kambe et al., 1993). Recently, the structure of a *C. albicans* cell wall surface and plasma membrane epitope recognized by an antibody that blocks adhesion was shown to be β 1,2-mannotetraose (Li & Cutler, 1993).

Although certain general features of molecules that can serve as *Candida* receptors have been described, the identity of these molecules is still largely unknown. In identifying potential receptors for various organisms, overlay techniques have been extremely useful. Lipids, separated by thin-layer chromatography (Hansson et al., 1985), or glycoproteins, separated by SDS–polyacrylamide gel electrophoresis (Prakobphol et al., 1987), are incubated with radiolabeled microorganisms and interactions are detected by autoradiography. Jimenez-Lucho et al. (1990) used a thin-layer chromatogram overlay to show that *Candida*, like many bacteria and *Saccharomyces cerevisiae*, can adhere to lactosylceramide. Here we used two different adhesion assays, thin-layer chromatogram overlay and adherence to lipids immobilized on microtiter plates, to show that certain lysophospholipids, but not their corresponding phospholipids, can mediate *C. tropicalis* adherence. Binding resulted in rapid metabolism of the substrate, suggesting that the organism's lipid-metabolizing enzymes are linked to the adherence process.

EXPERIMENTAL PROCEDURES

Materials. Purified phospholipids (summarized in Table 1), molybdenum blue reagent, and dextrose were obtained from Sigma Chemical Co., St. Louis, MO. Yeast extract, peptone, and Sabouraud's dextrose agar were purchased from Difco Laboratories, Detroit, MI. [^{35}S]Methionine (1159 Ci/mmol) and [^{14}C]-1-palmitoyl-*sn*-glycero-3-phosphocholine (57 mCi/mmol) were obtained from New England Nuclear, Boston, MA. Hyperfilm was a product of Amersham, Arlington Heights, IL. Butylmethacrylate/isobutylmethacrylate was from Aldrich Chemical Co., Milwaukee, WI. Silica high-performance thin-layer plates (HPTLC S-60, aluminum-backed) were purchased from EM Science, Cherry Hill, NJ. Gelatin was from Allied Chemical, Morristown, NJ. Chloroform, methanol, and hexane were from Fisher Scientific, Santa Clara, CA. Falcon flexible flat-bottomed poly(vinyl chloride) 96-well microtiter plates (Microtest III flexible assay plates) were obtained from Becton Dickinson and Co., Oxnard, CA. Pressure-sensitive, double-sided tape was purchased from Scotch 3M, St. Paul, MN. Construction of the lipid library was described previously (Gillece-Castro et al., 1991).

Preparation of Labeled Candida. C. tropicalis (ATCC 750) and *S. cerevisiae* (ATCC 60782) were obtained from the American Type Culture Collection. *S. cerevisiae* (EG 123), isolated from an HIV-negative subject, was kindly provided by Dr. Shelley Miyasaki, University of California San Francisco. All organisms were grown on Sabouraud's dextrose agar at 25 °C overnight. Stock cultures were maintained at 4 °C until use. The cultures were transferred to new plates at least once a month.

For metabolic labeling, a single colony was transferred from the plate to yeast peptone broth (1% yeast extract, 2% peptone, and 2% dextrose) and grown at 25 °C for 16 h. The organisms were then subcultured in fresh broth containing 50 μCi/mL [^{35}S]methionine and grown at 25 °C for 16 h. In a few experiments the organisms were grown and labeled at 37 °C. Radiolabel incorporation was 0.2–0.4 cpm/cell. The organisms were harvested by centrifugation, washed three times in phosphate-buffered saline (PBS)[1] and resuspended in PBS containing 0.2% gelatin (PBS-gel). The suspension was then adjusted to a final concentration of either (1–2) $\times$ 10^7 cells/mL (for the overlay assays) or 5 $\times$ 10^6 cells/mL (for the centrifugal force assays) by A_{620} determinations related to cell number by direct counting in a hemocytometer. For heat killing, labeled *C. tropicalis* was autoclaved for 30 min. This was the minimum heating time that consistently prevented subsequent growth of this organism.

Determining the Effect of Lipids on C. tropicalis Growth. The cells (1 $\times$ 10^4 cells/mL) were incubated for 2 h at room temperature in 250 μL of yeast–peptone broth containing either 0 (control), 25, 50, or 100 μg/mL 1-palmitoyl-*sn*-glycero-3-phosphocholine. An aliquot (25 μL) from each suspension was then plated on Sabouraud's dextrose agar and cultured at room temperature for 48 h, after which growth was estimated by counting the number of colonies.

Overlay Method for Assessing Candida Binding to Lipids. Adherence of *Candida* to lipids chromatographed on high-performance silica gel plates was detected essentially as described by Bock et al. (1985). Briefly, glycolipid and phospholipid members of a lipid library, as well as purified phospholipids and lysophospholipids (see Table 1), were spotted (2 μg total, unless otherwise indicated) onto silica gel high-performance plates that were developed with chloroform/methanol/water (60:35:8 v/v/v). For every experiment, two plates on which identical components were spotted were run in parallel. After chromatography, the plates were dried. One plate was used for chemical detection of lipids; the phospholipid bands were detected by spraying with molybdenum blue reagent (Dittmer & Lester, 1964) and the glycolipid bands were detected by spraying with anisaldehyde (Bock et al., 1985). The other plate was coated with butylmethacrylate/isobutylmethacrylate, incubated for 60 min in PBS-gel to prevent nonspecific binding, and then overlaid with the ^{35}S-labeled yeast cell suspension [(1–2) $\times$ 10^7 cells/mL]. After 2 h, this plate was washed three times with PBS to remove the unbound cells. Components to which the *Candida* bound were detected by autoradiography. Each experiment was performed a minimum of three times.

[1] Abbreviations: HIV, human immunodeficiency virus; PBS, phosphate-buffered saline; PAF, platelet-activating factor.

Centrifugal Force Assay of Candida Adhesion. We have adapted an assay which was devised to quantify the strength of eukaryotic cell–cell and cell–extracellular matrix adhesive interactions (McClay et al., 1981; Lotz et al., 1989; Burdsal et al., 1991) to the study of bacteria–saliva interactions (A. Prakobphol, C. Burdsal, and S. J. Fisher, submitted for publication). Here we used this method to study *Candida*–phospholipid interactions. The organisms are centrifuged onto lipid-coated microtiter wells. After incubation, the plates are inverted and centrifuged again. In this assay, the centrifugal force required to remove the organisms is directly related to the adhesive strength of this interaction.

Flat-bottomed poly(vinyl chloride) microtiter plates were lipid-coated by adding 50 μL of a 50 μg/mL solution of lipid dissolved in methanol. Methanol (50 μL) was added to control wells. Then the plates were incubated for 1 h at room temperature (RT), during which time the methanol completely evaporated. To quantify the adsorbed lipid, lipid-coated wells were washed 1× with PBS and then the bound material was redissolved in methanol and subjected to thin-layer chromatography. Routinely, every other row of wells was used so that during the subsequent centrifugation steps, overflow would run into empty wells. The coated wells were washed 1× with PBS, and nonspecific binding was blocked by incubation with PBS-gel for 1 h at RT. The blocking solution was removed from the wells and 140 μL of fresh PBS-gel, 25 μL of radiolabeled *Candida* (5×10^6/mL), and another 135 μL of PBS-gel were added. This volume (300 μL total) produced a slight positive meniscus in the well which helped to eliminate trapping of air. The plate was then carefully sealed with double-sided, pressure-sensitive tape. To bring the organisms into contact with the wells, the plates were centrifuged in a microtiter plate carrier at RT for 10 min at 2*g* (spin-on). After incubation at RT for 1 h, the plates were inverted and centrifuged again for 10 min at RT (spin-off); replica plates were subjected to different centrifugal forces ranging from 10*g* to 450*g*. The plates were then removed from the centrifuge in the inverted position and submerged in ethanol/dry ice to freeze the contents of each well. The bottom 3 mm of each well was clipped off and transferred to a scintillation vial, and the number of candidal cells that remained bound was determined by quantifying the radioactivity in each well.

The data were plotted as percent of cells bound as a function of relative centrifugal force. The percentage of cells bound = number of cells bound after the spin-off step/number of cells brought into contact by the spin-on step × 100. Dyne force per cell was calculated by F = (specific density of the cell − specific density of the medium) × volume of the cell × relative centrifugal force (g, where g = 980 dyn/g). The specific density of the *C. tropicalis* cells (1.138 g/cm^3) was calculated by Percoll density gradient centrifugation. The specific density of the PBS was 1.0 g/cm^3. Calculation of the cell volume was based on published measurements (Odds, 1988). The cell volume, calculated as an oval for *C. tropicalis*, was 8.38 μm^3.

Initial experiments in which we varied the spin-on centrifugal force showed that 2*g* was the minimum force at which the maximum number of cells was brought into contact with the wells. In addition, we determined if the spin-on step changed the strength of the *Candida*–lipid interactions. The cells were allowed to attach to lipid-coated microtiter wells for 1 h at 1*g*. Some of the wells were then frozen immediately to determine the total number of adherent cells, whereas others were subjected to the spin-off step to measure the strength of adhesion. No difference in the strength of adhesion was observed.

Analysis of Lysophospholipid Metabolism by Adherent Candida. [^{14}C]-1-Palmitoyl-*sn*-glycero-3-phosphocholine was spotted on thin-layer chromatography plates. The blocking step was omitted and the plates were incubated with one of the following: fresh culture medium in which no organisms had grown, PBS, *C. tropicalis* conditioned medium, or unlabeled *C. tropicalis* suspended in PBS. After incubation (2 h) the plates were washed sequentially with PBS (1×) and distilled H_2O (1×) and then dried and developed as described above, and bands were detected by autoradiography. To analyze the ^{14}C-labeled products formed as a result of *C. tropicalis* adherence to the labeled lipid, the band that remained at the origin was scraped from the plate, extracted with chloroform/methanol/H_2O (4:8:3), and rechromatographed.

RESULTS

We first performed experiments (data not shown) to determine, by overlay assay, whether *C. tropicalis* would adhere either to human salivary glycoproteins or to members of a lipid library that contains glycolipids and phospholipids. When nitrocellulose blots of electrophoretically separated proteins were overlaid with ^{35}S-labeled *Candida*, no interactions with salivary components were detected. However, adherence of *C. tropicalis* to a component of a complex lipid mixture isolated from dog intestine and separated by thin-layer chromatography was consistently observed. The relative chromatographic mobility and staining reaction with molybdenum blue reagent suggested that the potential *Candida* receptor was a phospholipid; no interactions with any of the glycolipid members of the library (Gillece-Castro et al., 1991) were detected.

We then used the overlay assay to screen a number of purified phospholipids and their corresponding lyso forms for their ability to support adhesion of *C. tropicalis*. In these experiments, examples of which are shown in Figures 1 and 2, duplicate thin-layer chromatography plates were developed. One was sprayed with molybdenum blue reagent, for chemical detection of phospholipids, and the other was overlaid with radiolabeled *C. tropicalis* for autoradiographic identification of potential receptors. *C. tropicalis* did not adhere to 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (Figure 1, lane 1), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (Figure 1, lane 7), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-DL-glycerol (Figure 2, lane 1), or 1,2-diacyl-*sn*-glycero-3-phosphoserine (Figure 2, lane 3). In these initial experiments the only exception was that in about one-third of the experiments *C. tropicalis* adhered, at barely detectable levels, to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (Figure 1, lane 3).

In contrast, *C. tropicalis* adhered consistently to the lyso forms of these compounds. Figure 1 shows binding of *C. tropicalis* to 1-palmitoyl-*sn*-glycero-3-phosphocholine (lane 4), 1-oleoyl-*sn*-glycero-3-phosphocholine (lane 5), 1-stearoyl-*sn*-glycero-3-phosphocholine (lane 6), and 1-palmitoyl-*sn*-glycero-3-phosphoethanolamine (lane 8). Figure 2 shows binding to 1-acyl-*sn*-glycero-3-phospho-DL-glycerol (lane 2) and 1-palmitoyl-*sn*-glycero-3-phosphoserine (lane 4). Compounds that had an acetyl group at C-2 (1-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine and 3-hexadecyl-2-acetyl-*sn*-glycero-1-phosphocholine; Figure 2, lanes 5 and 6) also supported *C. tropicalis* adherence, as did 1-acyl-*sn*-glycero-3-phosphoinositol (data not shown). The only exception to this pattern was that weak binding to 1-oleoyl-*sn*-glycero-3-phosphate (Figure 1, lane 2) was inconsistently observed.

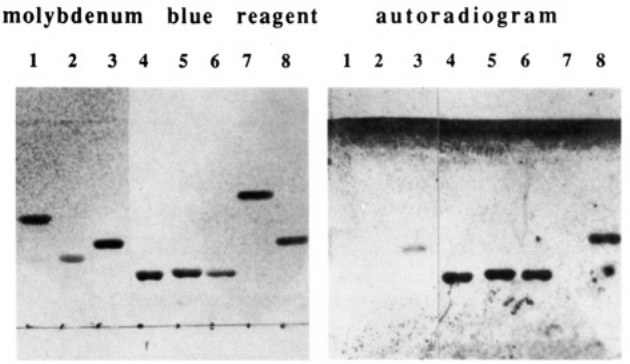

FIGURE 1: Adherence of ^{35}S-labeled *C. tropicalis* to phospholipids. Identical plates were spotted with 2 μg of 1,2-dipalmitoyl-*sn*-glycero-3-phosphate (lane 1), 1-oleoyl-*sn*-glycero-3-phosphate (lane 2), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (lane 3), 1-palmitoyl-*sn*-glycero-3-phosphocholine (lane 4), 1-oleoyl-*sn*-glycero-3-phosphocholine (lane 5), 1-stearoyl-*sn*-glycero-3-phosphocholine (lane 6), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (lane 7), and 1-palmitoyl-*sn*-glycero-3-phosphoethanolamine (lane 8). The plate on the left was sprayed with molybdenum blue reagent, and the plate on the right was overlaid with radiolabeled *C. tropicalis* and then exposed to X-ray film, producing the autoradiogram shown.

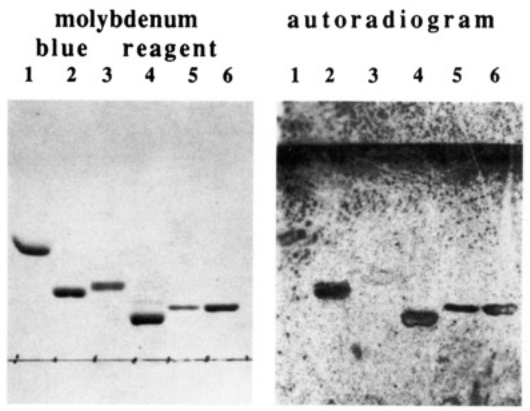

FIGURE 2: Adherence of ^{35}S-labeled *C. tropicalis* to phospholipids. Identical plates were spotted with 2 μg of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-DL-glycerol (lane 1), 1-acyl-*sn*-glycero-3-phospho-DL-glycerol (lane 2), 1,2-diacyl-*sn*-glycero-3-phosphoserine (lane 3), 1-palmitoyl-*sn*-glycero-3-phosphoserine (lane 4), 1-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (lane 5), and 3-hexadecyl-2-acetyl-*sn*-glycero-1-phosphocholine (lane 6). The plate on the left was sprayed with molybdenum blue reagent, and the plate on the right was overlaid with labeled *C. tropicalis* and then exposed to X-ray film, producing the autoradiogram shown.

We also investigated the amount of lysophospholipid that was required to detect adherence. Figure 3 shows adherence to 0.1 μg of 1-palmitoyl-*sn*-glycero-3-phosphocholine that was detected by overlay with radiolabeled *Candida* (lane 3, autoradiogram) but not by spraying with the molybdenum reagent (lane 3, molybdenum reagent). This was also true for 0.1 μg of 1-palmitoyl-*sn*-glycero-3-phosphoethanolamine (data not shown). Thus, binding of radiolabeled *C. tropicalis* was observed using an amount of lysophospholipid that could not be detected chemically.

After we made these initial observations, we became aware of a report that the thin-layer chromatogram assay of bacterial receptors could give false-positive and false-negative results (Yiu & Lingwood, 1992). Therefore, we used a second assay to quantify the relative strength of *Candida*–phospholipid interactions. Radiolabeled cells were brought into contact with lipid-coated microtiter well bottoms by low-speed centrifugation (spin-on). The wells were sealed with pressure-sensitive tape and the cells were allowed to bind. Then the plates were inverted and centrifuged again at different speeds

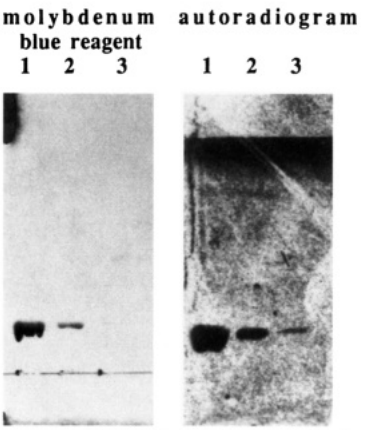

FIGURE 3: Adherence of ^{35}S-labeled *C. tropicalis* to 1-palmitoyl-*sn*-glycero-3-phosphocholine. Identical plates were spotted with 10 μg (lane 1), 1 μg (lane 2), and 0.1 μg (lane 3) of 1-palmitoyl-*sn*-glycero-3-phosphocholine. The plate on the left was sprayed with molybdenum blue reagent, and the plate on the right was overlaid with labeled *C. tropicalis* and then exposed to X-ray film, producing the autoradiogram shown. Adherence was detected at phospholipid levels that were below the level of chemical detection (lane 3).

(spin-off); the centrifugal force required to remove the organisms from the coated well allows calculation of the adhesive force of the interaction (McClay et al., 1981; Lotz et al., 1989; Burdsal et al., 1991; A. Prakobphol, C. Burdsal, and S. J. Fisher, submitted for publication).

To determine the quantity of adsorbed lipid which mediates optimal adherence, microtiter wells were coated with methanol solutions containing 10–100 μg/mL 1-palmitoyl-*sn*-glycero-3-phosphocholine, a lysophospholipid that preliminary experiments showed mediates adherence of *C. tropicalis* in this assay. Maximum binding occurred with a 50 μg/mL coating solution, the concentration that was routinely used in all subsequent experiments. We also determined, by thin-layer chromatography of the adsorbed lipid after resolubilization, that 0.5 μg/well 1-palmitoyl-*sn*-glycero-3-phosphocholine adsorbed to the plate from a 50 μg/mL coating solution. Using this same method we compared the relative adsorption of all the lipids tested. In comparison to adsorption of lysophospholipids, adsorption of phospholipids and fatty acids was somewhat higher.

Next, we assayed the number of cells bound to either 1-palmitoyl-*sn*-glycero-3-phosphocholine or 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine as a function of time (Figure 4A; 0–240 min). In this experiment the spin-off centrifugal force was held constant at 40*g*. Maximum adherence to 1-palmitoyl-*sn*-glycero-3-phosphocholine occurred within 30 min. In contrast, a very low level of adherence to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and to wells coated with methanol alone (data not shown) was observed at 30 min and increased only slightly during the course of the experiment.

In additional experiments (Figure 4B) we compared the strength of adhesive interactions between *C. tropicalis* and either intact phospholipids or lysophospholipids. Throughout the range of centrifugal forces tested (10–450*g*), significantly more organisms bound to the lysophospholipid than to the corresponding intact phospholipid. For example, at 200*g* (2.27×10^{-7} dyn) 70% of the cells remained bound to 1-palmitoyl-*sn*-glycero-3-phosphocholine. In contrast, about 20% of *C. tropicalis* adhered to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, a value that was not significantly different from the percentage of organisms that adhered to control methanol-coated wells. In addition, the force of *C. tropicalis* adherence to the lysophospholipid was significantly greater than to the

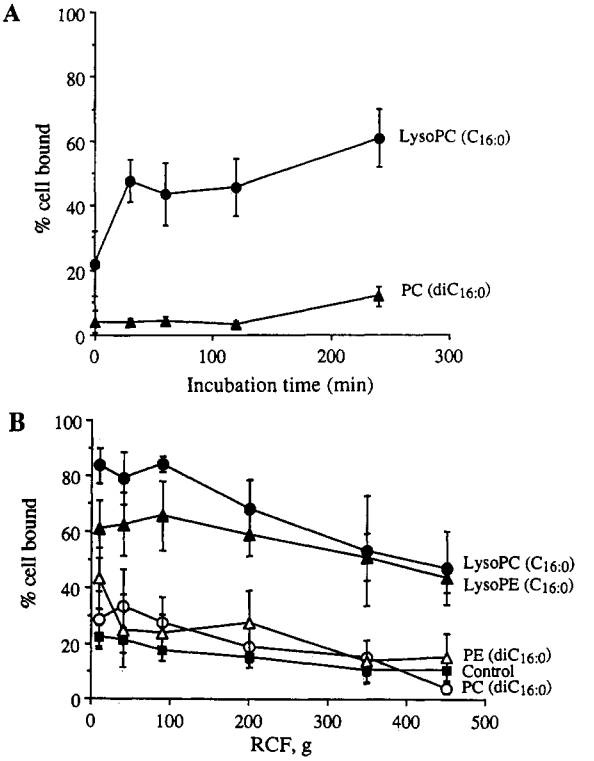

FIGURE 4: Centrifugal force assay of ^{35}S-labeled *C. tropicalis*–phospholipid interactions. (A) Effect of incubation time on the number of adherent organisms. Microtiter wells were coated with either 1-palmitoyl-*sn*-glycero-3-phosphocholine [lysoPC($C_{16:0}$)] or 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine [PC($diC_{16:0}$)] as described in Experimental Procedures. The centrifugal force with which the organisms were removed from the plates was held constant at 40*g*. (B) Effect of centrifugal force on the number of adherent organisms. Wells were coated with the lipids used in (A), as well as with 1-palmitoyl-*sn*-glycero-3-phosphoethanolamine [lysoPE($C_{16:0}$)], 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine [PE($diC_{16:0}$)], or methanol (control). At all centrifugal forces tested, the adhesive strength of *C. tropicalis* interaction with the lysophospholipid was greater than the interaction with the corresponding phospholipid. Each point is the mean and each bar is the standard deviation calculated from six replicate wells.

corresponding phospholipid. Finally, comparison of Figure 4 panels A and B shows the variation in the actual number of adherent organisms we observed among experiments (compare percent of cells that adhered to 1-palmitoyl-*sn*-glycero-3-phosphocholine in both experiments). Nevertheless, we always observed the pattern of preferential adherence of *C. tropicalis* to lysophospholipid; in every experiment at least 3-fold more cells bound to lysophospholipid-coated wells than to those coated with phospholipids.

The data obtained concerning the ability of phospholipids to mediate adhesion of *C. tropicalis* as assayed by both thin-layer chromatogram overlay and the centrifugal force adhesion assay are summarized in Table 1. Screening additional compounds by both the overlay and the centrifugal force assays showed that the former technique occasionally resulted in false-positives. Structures 5–9 (intact phospholipids) mediated adherence only in the overlay assay; no activity was detected in the centrifugal force assay. Certain lysophospholipids mediated adherence in both assays (structures 14–18, 21, and 22). Substantially weaker binding to some of the fatty acid chains was also demonstrated (structures 25–27).

Next, we used two methods to determine whether the organism was killed by the lysogenic properties of the phospholipids with which it interacts. First, incubation of *C. tropicalis* for 2 h at room temperature with 1-palmitoyl-*sn*-glycero-3-phosphocholine (25, 50, or 100 μg/mL) did not affect the subsequent growth of the organism. Second, thin-layer plates of chromatographically separated lipids were incubated for 2 h with unlabeled *C. tropicalis* and then inverted onto Sabouraud's dextrose agar plates. Within 24–48 h, live organisms were recovered in colonies that were the same shape and in the same corresponding location as the lysophospholipid band to which they were adherent (data not shown). The latter result shows that lysophospholipids mediate adhesion of intact, live candidal cells. Heat killing, however, abolished the ability of *C. tropicalis* to adhere to lysophospholipids, even though in two separate experiments heat treatment did not alter the appearance of the organisms at the light microscopic level and they retained 70% and 75% of their incorporated radioactivity, respectively. These results suggest either that the adherence patterns we observed require live *C. tropicalis* or that heat treatment itself alters the ability of the *Candida* ligand(s) to interact with lysophospholipids.

Table 1: Binding of *C. tropicalis* to Phospholipids and Other Lipids

no.	name	TLC[a]	centrifugal force assay[a]
	Phospholipids		
1	1,2-dipalmitoyl-*sn*-glycero-3-phosphate	–	ND
2	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine	–	ND
3	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine	(+)	(+)
4	1,2-distearoyl-*sn*-glycero-3-phosphocholine	–	–
5	1,2-dioleoyl-*sn*-glycero-3-phosphocholine	++	–
6	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine	++	–
7	1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine	++	–
8	1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine	++	–
9	1-oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine	++	–
10	1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine	–	–
11	1,2-diacyl-*sn*-glycero-3-phosphoserine	–	ND
12	1,2-diacyl-*sn*-glycero-3-phosphoinositol	–	–
13	1,2-dipalmitoyl-*sn*-glycero-3-phospho-DL-glycerol	–	–
	Lysophospholipids		
14	1-oleoyl-*sn*-glycero-3-phosphate	(+)	(+)
15	1-palmitoyl-*sn*-glycero-3-phosphocholine	++	++
16	1-stearoyl-*sn*-glycero-3-phosphocholine	++	++
17	1-oleoyl-*sn*-glycero-3-phosphocholine	++	++
18	1-hexadecyl-*sn*-glycero-3-phosphocholine	++	+
19	1-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine	++	–
20	3-hexadecyl-2-acetyl-*sn*-glycero-1-phosphocholine	++	ND
21	1-palmitoyl-*sn*-glycero-3-phosphoethanolamine	++	++
22	1-palmitoyl-*sn*-glycero-3-phosphoserine	++	+
23	1-acyl-*sn*-glycero-3-phosphoinositol	++	–
24	1-acyl-*sn*-glycero-3-phospho-DL-glycerol	++	–
	Other Lipids		
25	palmitic acid	–	+
26	stearic acid	+	+
27	monopalmitoylglycerol	+	+
28	dipalmitoylglycerol	–	(+)
29	tripalmitin	ND	–
30	tristearin	ND	–

[a] ++ = maximum binding detected, + = 40–60% maximum binding, (+) = 10–20% maximum binding, ND = not determined.

In additional experiments we investigated possible mechanisms by which *C. tropicalis* interacts with lysophospholipids. First, we studied the effect of temperature, using the centrifugal force assay. The impetus for these experiments was the observation that *Candida* spp. are usually more hydrophobic when they are grown at room temperature (the temperature that was routinely used in our experiments) than when they are grown at 37 °C (Hazen et al., 1986). When adherence at these two temperatures was compared, we found that fewer (50%) cells bound at the higher temperature, but the patterns of phospholipid preference were identical.

Next, we investigated whether the phospholipid was altered during the adhesion process. We spotted [^{14}C]-1-palmitoyl-*sn*-glycero-3-phosphocholine at the origin of thin-layer plates

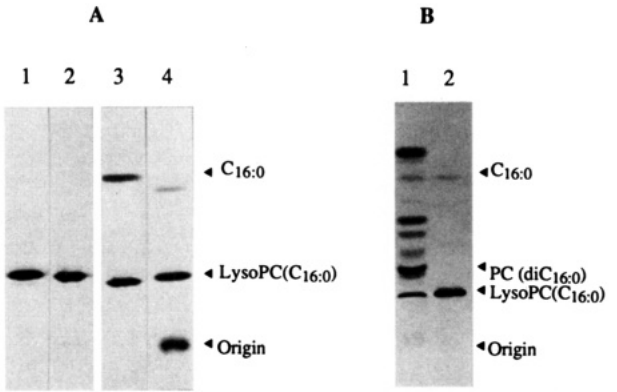

FIGURE 5: *C. tropicalis* adherence to [^{14}C]-1-palmitoyl-*sn*-glycero-3-phosphocholine results in modification of the labeled lipid's structure. (A) [^{14}C]-1-palmitoyl-*sn*-glycero-3-phosphocholine was spotted on thin-layer chromatography plates and incubated with either fresh culture medium in which no organisms had grown (lane 1), PBS (lane 2), *C. tropicalis* conditioned medium (lane 3), or *C. tropicalis* suspended in PBS (lane 4). The plates were developed as described in Experimental Procedures. (B) The prominent band that remained at the origin of the sample incubated with a *C. tropicalis* suspension (panel A, lane 4) was scraped from the plate, extracted in solvent, and rechromatographed (panel B, lane 1). [^{14}C]-1-palmitoyl-*sn*-glycero-3-phosphocholine, which contained a small amount of palmitic acid ($C_{16:0}$), was chromatographed in lane 2. A prominent band which moved slightly behind the solvent front is a product of free fatty acid esterified to methanol, usually observed after storage of [^{14}C]-1-palmitoyl-*sn*-glycero-3-phosphocholine in this solvent. Abbreviations are the same as those in Figure 4.

and then incubated the plates with either fresh *C. tropicalis* culture medium, PBS, *C. tropicalis* conditioned medium, or *C. tropicalis* suspended in PBS. After incubation, the labeled lipid was subjected to thin-layer chromatography to determine if its structure had changed (Figure 5A). No additional bands were visible when samples of [^{14}C]-1-palmitoyl-*sn*-glycero-3-phosphocholine were incubated with either fresh culture medium in which no organisms had grown (lane 1) or PBS (lane 2). In contrast, samples of [^{14}C]phospholipid that were incubated in *C. tropicalis* conditioned medium (lane 3) contained an additional band that had the same chromatographic mobility as palmitic acid, suggesting that this organism secretes a lysophospholipase. The most striking chromatographic pattern was found in the sample that had been incubated with *C. tropicalis* cells (lane 4). In addition to a relatively faint band with a chromatographic mobility slightly less than that of palmitic acid, a prominent band remained at the origin of the plate.

This band was scraped from the plate and extracted by incubating for 1 h at 80 °C in a mixture of chloroform/methanol/H_2O. As an additional control [^{14}C]-1-palmitoyl-*sn*-glycero-3-phosphocholine was spotted onto a plate and extracted in parallel. The solvent was evaporated and the products were redissolved in methanol and again subjected to thin-layer chromatography (Figure 5B). The band that had remained at the origin after the first thin-layer chromatographic step (Figure 5A, lane 4) was now separated into a number of components (Figure 5B, lane 1). Bands that had the same R_f values as 1-palmitoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and palmitic acid were detected, as well as at least three additional bands. In contrast, no additional bands were detected in the control sample which was extracted in parallel (data not shown). These results show that *C. tropicalis* adherence is accompanied by its metabolism of the lysophospholipid.

DISCUSSION

The centrifugal force adhesion assay showed that *C. tropicalis* did not interact with phospholipids with two fatty acid substituents. In contrast, the lyso forms of certain phospholipids supported adherence of the organisms in this assay. Free fatty acids also supported adherence, although attachment of the fatty acid to the glycerol backbone significantly enhanced binding activity. From the lysophospholipids we studied it was not clear whether this fatty acid chain had to be located on C1 or C2 of the glycerol backbone. However, our results did suggest other structural requirements. First, a synthetic lyso analogue of a choline phospholipid (1-hexadecyl-*sn*-glycero-3-phosphocholine) supported binding, but 1-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine did not. This suggests that a free hydroxyl group on C2 of the glycerol backbone is necessary to support *Candida* adherence. Second, lysophosphatidylcholine (Table 1, structures 15–17) and 1-palmitoyl-*sn*-glycero-3-phosphoethanolamine strongly supported adhesion, but 1-acyl-*sn*-glycero-3-phosphoinositol and 1-acyl-*sn*-glycero-3-phospho-DL-glycerol were inactive in the centrifugal force assay; 1-dipalmitoyl-*sn*-glycero-3-phosphoserine had intermediate activity. This suggests that the nature of the polar head group also influences binding.

Interactions of *Candida* with the lyso form of phospholipids as a possible mechanism mediating interactions with host cells have not, to our knowledge, been previously described. The functions of the organism's endogenous lipids, including their roles in transport, growth, and morphogenesis, have been studied because these compounds are important targets for antifungal therapy and drug design [reviewed by Mishra et al. (1992)]. Ghannoum et al. (1986) investigated the role of lipids extracted from three *Candida* spp. in blocking adherence of these organisms to human buccal epithelial cells. Of the compounds tested, only phospholipids (diacylglycerophosphoethanolamines, diacylglycerophosphoglycerols, diacylglycerophosphocholines, diacylglycerophosphoinositols, and diacylglycerophosphoserines), sterols, and steryl esters blocked adhesion; triacylglycerols and free fatty acids were inactive in this regard. Although these results are not totally consistent with our own regarding the specific structural features of potential phospholipid receptors, they do implicate this class of molecules in the process.

From our studies it is not yet possible to identify the *Candida* ligand that interacts with lysophospholipids. One possibility is that the adherence of these organisms to lysophospholipids is the result of an interaction between a *Candida* enzyme and its substrate. For example, some species produce lysophospholipase (Price & Cawson, 1977; Barrett-Bee et al., 1985) and lysophospholipase–transacylase (Banno et al., 1985; Takahashi et al., 1991), either of which could potentially function as enzymatic ligands for potential lysophospholipid receptors. Here we present evidence suggesting that enzymes which use lysophospholipids as substrates are involved in the adhesive interactions we observed; adhesion of *C. tropicalis* to [^{14}C]-1-palmitoyl-*sn*-glycero-3-phosphocholine resulted in the formation of at least five compounds. A major product had the same R_f as 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, suggesting the involvement of a lysophospholipase–transacylase. A less prominent band cochromatographed with palmitic acid, evidence for lysophospholipase activity. Interestingly, the lane containing labeled lipid that was incubated with conditioned medium shows only a single product that had the same R_f as palmitic acid, suggesting the medium primarily contains a lysophospholipase activity. Currently, we are determining the time course during which the cell-

associated compounds are formed as a means of gaining insight into the enzymatic activities involved. Furthermore, it is clear that additional experiments will be necessary to determine if these enzymatic activities mediate, or ensue from, *C. tropicalis* adhesion.

It is possible that other lipid-metabolizing enzymes produced by *Candida* spp. could be involved in similar types of adhesive interactions. For example, some of these organisms also produce phospholipases, including phospholipase A (Price & Cawson, 1977; Barrett-Bee et al., 1985), raising the possibility that *Candida* phospholipase activity is part of a cascade that results in phospholipid hydrolysis and production of lysophospholipids that can mediate adhesion. It is interesting to note that this family of enzymes is associated with infection by other pathogenic organisms. Lipases have been correlated with the entry of rickettsiae into the cytoplasm (Winkler, 1990) and with the virulence of *Listeria monocytogenes* (Geoffroy et al., 1991; Mengaud et al., 1991).

Although we have focused on *C. tropicalis*, we are very interested in the possibility that other *Candida* spp. might utilize this type of adherence mechanism. In preliminary experiments we have begun to address this question. By thin-layer chromatogram overlay, many other *Candida* spp. (several ATCC and clinical strains of *C. albicans*, *C. krusei*, *C. guilliermondii*, and *C. parapsilosis*) showed the same adherence pattern, preferring adherence to lysophospholipids rather than the corresponding intact molecules. However, the results of these experiments often showed that fewer organisms adhered during the 1-h course of the experiments. Additional experiments using the centrifugal force adhesion assay to analyze *C. albicans*–phospholipid interactions showed that the time course of this interaction is different; substantial binding is not detected until 3 h of incubation. These results suggest that other *Candida* spp. might use a variation of the *C. tropicalis* adhesion strategy.

The downstream consequences of interactions between *C. tropicalis* and lysophospholipids could potentially have several interesting effects on both the organism and the host. With regard to the organism, it is clear that adherence triggers a cascade of events during which the lysophospholipid is converted into a number of different compounds. We are very interested in determining if these products have effects on the organism itself, particularly with regard to growth and differentiation. With regard to the host, interactions with phospholipids that are components of signal transduction pathways could deplete the intracellular pools of these compounds, thus impairing the functions they mediate. For example, we have shown that *C. tropicalis* adhered to lysophosphatidylcholine compounds. Therefore, it is possible that the cellular responses attributed to these lipids, including proliferation and differentiation [reviewed in Asaoka et al. (1992)], are altered by even a subclinical infection.

The biological relevance of the highly specific interactions we detected is not yet clear. The candidal organisms used for this study were in the blastoconidial form, which is not usually associated with invasion. Thus, it is likely that the binding we observed may play a role in adherence and/or colonization, functions usually associated with yeast forms, rather than invasion. However, it is possible that the same lysophospholipid binding characteristics are shared by both the blastoconidial and the hyphal forms, a possibility we have not yet investigated.

With regard to the biological relevance of lipids as potential *Candida* receptors, we found that, of the major classes of plasma membrane lipids, only sphingomyelin, as evidenced by its relatively weak interactions with *C. tropicalis*, was a potential receptor (data not shown). Likewise, we did not detect adherence to lactosylceramide, previously identified by thin-layer chromatogram overlay as mediating *C. albicans* adherence (Jimenez-Lucho et al., 1990). Although glycosphingolipids and intact glycerolphospholipids were unable to interact with *C. tropicalis*, the lyso forms of the latter compounds did support adherence. Normally these lipids, which are sequestered inside the cell, are found only in trace amounts in the plasma membrane, usually as the result of dynamic remodeling and recycling. Nevertheless, it is tempting to speculate that the lysophospholipids which we have shown can mediate adherence of *C. tropicalis in vitro* are a link in the chain of molecular events that results in infection by these organisms *in vivo*. For example, *Candida* might interact with these lysophospholipids after the plasma membrane of the host cell has been damaged by other underlying disease processes. In this case the lysophospholipids may be more readily available to mediate initial adhesive interactions between *C. tropicalis* and host cells.

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