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Photoaffinity Analog of the Semisynthetic Echinocandin LY303366: Identification of Echinocandin Targets in *Candida albicans*

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The echinocandins are a family of cyclic lipopeptides with potent antifungal activity. These compounds inhibit the synthesis of β-1,3-glucan in fungi. The new semisynthetic echinocandin LY303366 was derivatized to produce a photoactivatable cross-linking echinocandin analog with antifungal activity. This analog was radioiodinated and used as a probe in microsomal membrane preparations of *Candida albicans* which contain glucan synthase activity. The photoaffinity probe identified two major proteins of 40 and 18 kDa in both membrane preparations. Labeling of these proteins was specific in that it required irradiation with UV light and was effectively competed against with unlabeled echinocandin analogs. In addition, the abilities of echinocandin analogs to compete with the photoaffinity probe correlated to their relative antifungal potencies and glucan synthase inhibition. The 40-kDa protein was isolated, and partial sequences were obtained from internal peptide fragments of the protein. Analysis of the sequences of these internal peptides of the 40-kDa protein revealed that it was a new protein not previously described as being involved in glucan synthesis or the mode of action of echinocandins.

In the past 15 years, there has been a dramatic increase in the number of immunocompromised individuals, which, in part, accounts for the dramatic increase in the incidence of systemic mycoses (36). *Candida albicans* is the fungal pathogen in a majority of fungal infections, accounting for 72% of nosocomial fungal infections (35). Recently, *Candida* spp. have surpassed *Escherichia coli* as the third most frequently isolated organism from nosocomial bloodstream infections (38). Antifungal therapy to treat fungal infections is limited at present. The treatment of choice, with amphotericin B, suffers from the drawbacks of being an intravenous treatment and being limited in total dosage due to its toxicity (19). The oral azole fluconazole is well tolerated, but its antifungal spectrum is limited in that it is not active against some of the other *Candida* spp. (6). More importantly, the frequency of reports describing resistance to fluconazole in *C. albicans* is increasing (41). A recent study demonstrated the presence of fluconazole-resistant *C. albicans* in a patient population which had not been previously treated with the drug (21). In addition, the emergence of new fungal pathogens, such as *Candida krusei* and *Fusarium* spp., which are intrinsically less susceptible to fluconazole is of recent clinical concern (36).

The echinocandins are a family of cyclic lipopeptides with intrinsic antifungal activity (3). Recent advances in semisynthetic echinocandins have led to the development of highly potent, orally bioavailable agents, such as LY303366 (Fig. 1) (8). The MICs of this compound for most common fungal pathogens are very low, and this compound has shown oral efficacy in animal models of systemic candidiasis and pneumocystis pneumonia (7). It has also shown efficacy in animal models with *Aspergillus fumigatus*, one of the more difficult pathogens to treat, when administered by the intravenous route (39). LY303366 is presently in initial clinical trials in humans.

The echinocandins inhibit the synthesis of the major structural glucan in the cell walls of fungi, (1,3)-β-D-glucan. This polymer of glucose is synthesized by the enzyme glucan synthase (EC 2.4.1.34). Crude membrane preparations of *C. albicans* contain glucan synthase activity, which is inhibited by echinocandin analogs in vitro (42, 46). Substantial effort has gone into the purification of glucan synthase from many fungal organisms, including the model yeast *Saccharomyces cerevisiae*, *Neurospora crassa*, and *C. albicans* (2, 23, 47). In addition to classical biochemical purification, genetic approaches using *S. cerevisiae* have recently identified genes involved in resistance to the echinocandins. One gene, *ETG1*, is believed to encode the catalytic subunit of glucan synthase and has been isolated by a number of groups using several different approaches (4, 11, 12, 20, 40). A second gene encoding resistance to echinocandins, *GNS1*, has also been characterized (15). Direct binding of echinocandin analogs to the products of these genes has not yet been demonstrated.

In this study, a photoactivated cross-linking echinocandin analog was used as a probe for direct binding of an echinocandin analog to proteins in crude microsomal membrane preparations prepared from a synchronously budding yeast culture of *C. albicans*. The echinocandin analog was prepared by modification of the aminal hydroxyl of LY303366 with ethanolamine and subsequent coupling of an aryl azide photoactivatable cross-linker to the resulting amine. This photoactivated cross-linking analog both has antifungal activity and is competed for in the photocross-linking studies by LY303366. The covalent cross-linking of echinocandin to proteins in membrane preparations of *C. albicans*, which contain glucan synthase activity, demonstrated interaction of echinocandin with two proteins with apparent molecular masses of 40 and 18 kDa.

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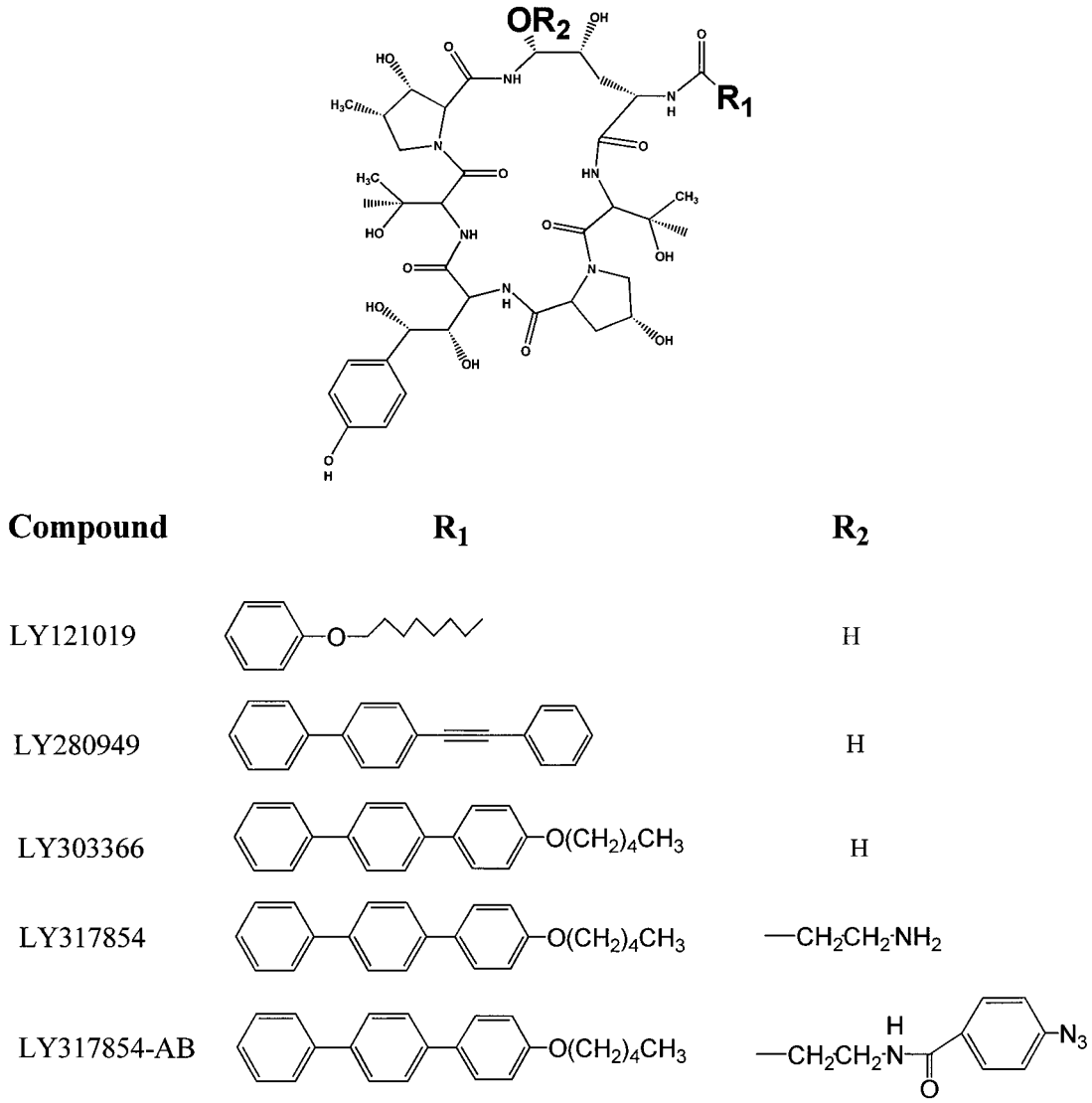

FIG. 1. Structures of compounds used in this study.

MATERIALS AND METHODS

Preparation of LY317854. Aminoethylation of the aminal hydroxyl of LY303366 was accomplished as previously described for tetrahydroechinocandin B (24).

Preparation of 4-azidobenzamide derivative of LY317854 (LY317854-AB). One milligram of LY317854 was dissolved in 100 μl of neat dimethylformamide. *N*-Hydroxysuccinimide ester of 4-azidobenzoic acid (HSAB; Pierce) was dissolved in neat dimethylformamide at a concentration of 10 mg/ml. The HSAB solution (100 μl; 5 molar excess over LY317854) was added to LY317854 in an amber vial. One microliter of triethylamine was added, and the reaction mixture was allowed to stir overnight at room temperature in the dark. The reaction mixture was transferred to a microcentrifuge tube, and the product was precipitated by addition of a 10× volume of cold 10% NaCl. The suspension was kept on ice for 10 min and then centrifuged at 14,000 × *g* for 10 min at 4°C. The resulting pellet was washed once more with cold 10% NaCl, followed by a wash with cold water. The pellet was then dried in vacuo, yielding 300 μg of product, which was then redissolved in 100% dimethyl sulfoxide (DMSO) at 2 mg/ml. The product was analyzed by high-performance liquid chromatography, and the free amine (LY317854) was not detected.

Radioiodination of LY317854-AB. In a screw-cap microcentrifuge tube, 5 μl of 317854-AB (2 mg/ml in DMSO) was diluted with 100 μl of acetonitrile, and 20 μl of 0.5 M sodium phosphate, pH 6.8, was added. The solution was mixed vigorously by vortexing. Carrier-free $Na^{125}I$ (5 μl; 500 μCi) was added, and the reaction was initiated by addition of a single IodoBead (Pierce). The reaction was allowed to proceed for 5 min and then the product was purified on a Sep-Pak C_{18} cartridge equilibrated with 20% acetonitrile-water. The product was eluted with 3 ml of 90% acetonitrile. The solution was transferred to an amber vial, and the solvent was removed in vacuo.

Purification of ^{125}I-labeled LY317854-AB. The radioiodinated product was purified by absorption chromatography on a Sephadex LH-20 column. The radioiodinated compound in DMSO was diluted into 1 ml of water. This was loaded on a Sephadex LH-20 column (total column volume, 6 ml) equilibrated with water. The column was then washed with 3 column volumes of water. The bound radioactive material was eluted with 80% acetonitrile-water. Fractions were collected and aliquots were counted in an LKB gamma counter. Peak fractions, examined by high-performance liquid chromatography, gave a single radioactive peak. The specific activity of the compound was 11 mCi/μmol.

Preparation of membranes from *C. albicans*. *C. albicans* 3153 (obtained from H. Buckley) was routinely cultured on Sabouraud's solid medium at 30°C. Stocks were maintained at 4°C. A single colony from a stock plate was inoculated into 5 ml of Lee's medium and allowed to shake overnight at room temperature (28). This culture was then used to inoculate 250 ml of Lee's medium, grown to saturation, and then diluted to obtain synchronously budding cells (32). After synchronous evagination had occurred, cultures were chilled in ice water for 10 min.

Cells were suspended in 50 mM HEPES-Na (pH 8.0)–1 M sucrose–10 mM NaF–5 mM EDTA–1 mM dithiothreitol (DTT) (GS buffer) containing 100 μM 5′-guanylylimidodiphosphate (GMP-PNP), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μM pepstatin A, 2 mM benzamidine, and 0.6 μM leupeptin and were disrupted with glass beads. Crude microsomal membranes were prepared as

described previously (18). The membranes were resuspended in ice-cold GS buffer without DTT and were transferred to a small Dounce homogenizer on ice and gently homogenized. The membrane suspensions were flash frozen in dry ice-ethanol at a protein concentration of 2 mg/ml and were stored at −90°C.

Preparation of detergent-washed membranes. Crude microsomal membranes were thawed and diluted with an equal volume of ice-cold GS buffer containing 50 mM NaCl, 100 μM GTP, and 0.02% W-1 detergent. The mixture was gently vortexed and kept on ice. The membrane preparation was then diluted with an equal volume of 50 mM HEPES–5 mM EDTA–10 mM NaF (pH 8.0) containing 50 mM NaCl, 1 mM DTT, 100 μM GTP, and 0.01% W-1 detergent to drop the sucrose concentration to 0.5 M. The membranes were harvested by ultracentrifugation at 200,000 × *g* at 4°C for 1 h. The pellet was resuspended in cold GS buffer containing 50 mM NaCl, 100 μM GTP, and 0.03% W-1. The resuspended membrane preparation was diluted by addition of an equal volume of the buffer described above, but without sucrose. The membranes were harvested as described above by ultracentrifugation. The pellet was resuspended in GS buffer containing 50 mM NaCl, 100 μM GTP, and 0.03% W-1 detergent and was gently stirred overnight at 4°C. The homogenate was diluted fivefold with buffer without sucrose, and the membranes were harvested by ultracentrifugation as described above. The detergent-washed membranes were homogenized in a Dounce homogenizer in GS buffer without DTT and were stored in aliquots at −90°C.

Determination of protein concentration. Protein concentration was determined by the bicinchoninic acid method according to the manufacturer's instructions (Pierce). Samples were read at 590 nm in a Beckman DU-70 spectrophotometer. Standard curves were prepared by using bovine serum albumin (2 mg/ml; Pierce).

SDS-PAGE. Proteins labeled from the photoaffinity experiments were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-protean II system (Bio-Rad) with 12% acrylamide gels containing 4 M urea (27). The stacking gel was 4% acrylamide and contained 4 M urea. Samples for electrophoresis were dissolved in SDS-urea loading buffer, consisting of 0.05 M Tris-HCl (pH 6.8), 10% SDS, 4 M urea, and 0.16 M DTT, and were warmed at 50°C for 5 min, followed by centrifugation at 14,000 × *g*. Samples were electrophoresed and stained with Coomassie brilliant blue G-250. After destaining and drying, the gels were subjected to autoradiography, and the autoradiograms were quantitated by laser densitometry. Inhibition of photoaffinity labeling by competitor echinocandins was calculated by comparison to a control lacking competitor compounds on a given gel and subsequent autoradiogram. The degree of inhibition is therefore a relative measure compared to the control in any given experiment.

Susceptibility testing. Susceptibility testing of echinocandin analogs was performed in a microdilution broth assay. *C. albicans* 3153 was grown overnight in Sabouraud's dextrose medium (10). Cells were diluted to 10^5/ml in test medium, Lee's medium, or Bacto Antibiotic Assay Medium 3 (10). Compounds were prepared as stock solutions in 100% DMSO. Twofold serial dilutions in test medium at twice the final desired concentration in 2% DMSO-test medium were prepared in the microtiter plate (100 μl/well). Cell suspensions were added to each well (100 μl per well). The final compound concentrations ranged by twofold serial dilution from 20 to 0.02 μg/ml in a final concentration of 1% DMSO. Plates were incubated at their respective temperatures for up to 72 h depending on the medium and were read on a Molecular Devices ThermoMax plate reader at 650 nm. The MIC was the lowest concentration of compound which inhibited growth of the organism by more than 90%, as determined by turbidity.

Glucan synthase assays. Membrane preparations were assayed for glucan synthase activity as described previously (47). The product was identified as 1,3-β-D-glucan by high-performance anion-exchange chromatography and by enzymatic degradation as described previously (48).

Photolabeling of *C. albicans* microsomal membranes. Membrane preparations were thawed on ice and diluted 10-fold in GS buffer without DTT. Diluted membrane preparations (200 μg/ml; 25 μl) were transferred to screw-cap microcentrifuge tubes on ice. To these membrane preparations, 20 μl of 62.5 mM sodium [2-(*N*-morpholino)ethanesulfonic acid] (MES-Na) (pH 5.0)–1 M sucrose–10 mM NaF–5 mM EDTA was added, giving a final pH of 6.7 to 6.8. Echinocandin analogs were dissolved in DMSO at 20 times the stated concentrations. Compound in DMSO or 100% DMSO (2.5 μl) was then added to the membrane preparation on ice. In reduced light, 2.5 μl of the probe echinocandin analog (0.126 nmol; 10^5 cpm) in DMSO was added. The membranes were gently mixed and kept on ice. The final concentration of the probe echinocandin was 3.4 μg/ml.

Samples prepared as described above were transferred to a water bath at 30°C for 5 min. The membranes were returned to the ice. Samples were transferred to a separate ice tray and individually photolyzed for 45 s at a distance of 3 cm by using a hand-held short-wave UV lamp with the filter removed. Immediately after photolysis, the sample was precipitated by addition of ice-cold 80% methanol (800 μl) and was transferred to −20°C for a minimum of 1 h.

Samples were harvested by centrifugation at 14,000 × *g* at 4°C for 30 min. The solvent was removed, and the pellet was resuspended in 500 μl of cold acetone. The samples were placed at −20°C for an additional hour and were centrifuged as described above. The pellets were then resuspended in 250 μl of chloroform-methanol (2:1) at room temperature. Pellets were collected by centrifugation and then dried in vacuo. The samples were then dissolved in SDS-urea PAGE loading buffer and subjected to electrophoresis as described above.

Purification of the 40-kDa EBP. The 40-kDa echinocandin binding protein (EBP) was trace labeled with the ^{125}I-echinocandin photoaffinity probe in crude microsomal membranes of *C. albicans* 3153. This trace-labeled preparation was diluted with cold 20 mM Tris-HCl (pH 7.9)–150 mM NaCl–300 mM sucrose–1 mM EDTA–1.0% *n*-octyl-β-D-glucoside (NOG)–0.5% cholamidopropyl-dimethyl-ammonio-1-propane sulfonate (CHAPS) containing 1 mM PMSF, 2 μg of pepstatin A/ml, and 2 μg of aprotinin/ml. The membrane preparation was kept on ice for 20 min and was subsequently centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was collected and stored at −90°C.

C. albicans 3153 cells were grown in 6 liters of Lee's medium at room temperature overnight. Cells were harvested as previously described, and a crude microsomal membrane preparation was prepared as described above. The membranes were resuspended in 10 ml of cold 20 mM Tris-HCl (pH 7.9)–150 mM NaCl–300 mM sucrose–1 mM EDTA–1.0% NOG–0.5% CHAPS containing 1 mM PMSF, 2 μg of pepstatin A/ml, and 2 μg of aprotinin/ml. The membranes were transferred to a glass Dounce homogenizer on ice and were gently homogenized with 10 strokes of the pestle. Additional buffer was added to adjust the protein concentration to 1 mg/ml. The solubilized membrane preparation was collected after centrifugation at 100,000 × *g* for 1 h at 4°C. The resulting pellet was resuspended in buffer and homogenized as described above in half the original volume. The supernatant was pooled with the first solubilization fraction after ultracentrifugation as described above.

Trace-labeled membranes (1 ml; 2.4×10^6 cpm) were thawed and added to the solubilized microsomal membranes (25 ml; 7.5 mg of protein). The solubilized membrane preparation was applied to a Sephacryl S-400HR column (2.8 by 45 cm) equilibrated with 20 mM Tris-HCl (pH 7.9)–150 mM NaCl–1 mM EDTA–0.1% NOG–0.05% CHAPS. The column was eluted at 30 ml/h at 4°C. Six fractions per hour were collected, and aliquots were counted in an LKB model 1282 Compugamma counter. The protein concentration in each fraction was determined by the bicinchoninic acid method. Fractions containing a peak of radioactivity and protein were pooled and dialyzed against 20 mM Tris-HCl (pH 7.9)–1 mM EDTA–0.1% NOG–0.05% CHAPS.

The pooled and dialyzed fractions were then loaded onto a DEAE-Sepharose column (0.8 by 7 cm) equilibrated with 20 mM Tris-HCl (pH 7.9)–1 mM EDTA–0.1% NOG–0.05% CHAPS. Bound radioactivity was eluted with a 150-ml linear gradient from 0 to 500 mM NaCl. Fractions were analyzed for radioactivity and protein as described above. Fractions containing a peak of radioactivity were pooled and analyzed by SDS-PAGE as described above.

Sequence analysis of the 40-kDa EBP. The 40-kDa protein was further purified by SDS-PAGE as described above and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore) in 25 mM Tris–192 mM glycine–15% methanol at room temperature (7 V/cm for 1.5 h). Membranes were rinsed with distilled water after transfer to reduce the background and then were stained with 0.025% Coomassie brilliant blue R-250 in 40% methanol for 5 min. The membrane was then destained with 40% methanol and allowed to air dry. Appropriate bands were excised with a razor blade and sent for automated Edman sequencing (30).

Preparation of peptide fragments of the 40-kDa EBP. Partial proteolysis of the 40-kDa protein was accomplished by the method of Cleveland using endoproteinase-Glu C (endo-Glu C; Boehringer Mannheim) (5). Cleavage with cyanogen bromide was performed within the gel slice according to the method of Sokolov et al. (44). Cleaved fragments of the 40-kDa protein were resolved by standard SDS-PAGE (no urea) and transferred to PVDF membranes as described above.

Sequence analysis. Peptides were sequenced by gas-phase Edman degradation. Peptide and nucleotide sequence comparisons and analysis were performed with the University of Wisconsin Genetics Computer Group program and by BLAST (1, 9).

RESULTS

Biological activity of LY317854-AB. The potency of the echinocandin compounds is dependent on the test medium. Generally, the potency of the echinocandins is greater in Bacto Antibiotic Assay Medium 3 than in the standard antifungal susceptibility medium recommended by the National Committee for Clinical Laboratory Standards (37). In the testing of echinocandin derivatives, it has been found that the use of Bacto Antibiotic Assay Medium 3 as a test medium gives a broader range of potency for these compounds, thereby allowing for discrimination of more-potent analogs from less-potent ones. Results of in vitro testing in Bacto Antibiotic Assay Medium 3 also correlated well with the observed potency of these compounds in animal models of experimental *Candida* infections (data not shown).

The antifungal activity of the aminoethylated compound (LY317854) was lower than that of the parent compound,

TABLE 1. MICs of echinocandin derivatives against *C. albicans* 3153 in two media

Medium (°C) and compound	MIC (μg/ml)
Lee's medium (25)	
LY303366 (parent)	0.02
LY317854	0.31
LY317854-AB	0.31
LY280949[a]	0.16
Bacto Antibiotic Assay Medium 3 (30)	
LY303366 (parent)	0.0025
LY280949	0.04
LY121019[b]	0.31
LY317854	0.02
LY317854-AB	0.04

[a] Compound 13b (8).
[b] Cilofungin.

LY303366, when tested against *C. albicans* in Lee's medium and in Bacto Antibiotic Assay Medium 3 (Table 1) (10, 28). Acylation of the amine in LY317854, forming the 4-azidobenzamide derivative, resulted in a decrease in the aqueous solubility of the compound, but the antifungal activity was comparable to that of LY317854 under the given conditions. The introduction of the photoactivatable cross-linking moiety thus had no significant effect on the antifungal activity of LY317854.

Photoaffinity labeling of microsomal membranes from *C. albicans*. The labeling of membranes from the budding form of *C. albicans* is shown in Fig. 2A. The first lane represents the photoreaction of the probe molecule in the absence of competitor echinocandin. Two proteins are significantly labeled by the photoaffinity probe: one intensely labeled protein of approximately 40 kDa and a second, less intensely labeled protein with a molecular mass of approximately 18 kDa. The band at the bottom of the gel is at the dye front and is due to the interaction of the probe with lipid components in the crude membrane preparations. The second lane represents the dark control (not photolyzed, but otherwise treated the same as the membranes in lane 1). This control demonstrates that insertion of the photoaffinity probe is due to UV irradiation and subsequent cross-linking and is not the result of insertion through a nonphotolytic event. Lanes 3 to 5 are the patterns resulting from competition by increasing concentrations of LY303366, demonstrating that the photoaffinity analog and the parent compound, LY303366, interact with the same proteins in the membrane preparation. Other proteins, of 32 and 50 kDa, are slightly labeled in this particular experiment, but these bands are not consistently noted when other membrane preparations are used in other experiments. In addition, as mentioned above, significant amounts of the probe echinocandin react with lipid components in the membrane. Thus, some nonspecific background is usually noted in the photoaffinity labeling of membrane preparations. Only the 40- and 18-kDa proteins were reproducibly labeled and inhibited by competitor echinocandins in multiple experiments.

The antifungal activity and inhibition of glucan synthase by echinocandins require the cyclic-peptide portion to be covalently linked to the lipophilic side chain. Separation of these two parts of the molecule results in a loss of antifungal activity and glucan synthase inhibition (45). Both the echinocandin cyclic-peptide nucleus and the terphenyl side chain in combination, but not covalently linked, and at comparable concentrations to that of the intact echinocandin, do not significantly inhibit labeling with the photoaffinity reagent (Fig. 2B). Similar lack of competition was found with either the cyclic peptide nucleus or the side chain alone at comparable concentrations (data not shown).

To support the biological significance of the photoaffinity labeling experiments, the competition of the compounds for the 40-kDa protein with the photoaffinity probe was compared to both the antifungal potency of the competitor compounds and their ability to inhibit glucan synthase activity.

The inhibition of glucan synthase activity in crude microsomal membranes by echinocandins of differing potency was also examined in an effort to correlate the photoaffinity labeling results with glucan synthase inhibition. Surprisingly, in crude microsomal membranes, inhibition of glucan synthase by echinocandins did not seem to correlate with antifungal potency. However, when the crude microsomal membranes were washed with detergent, the washed membranes had significantly higher overall glucan synthase activity, similar to previous observations of glucan synthase activity in *S. cerevisiae*, and

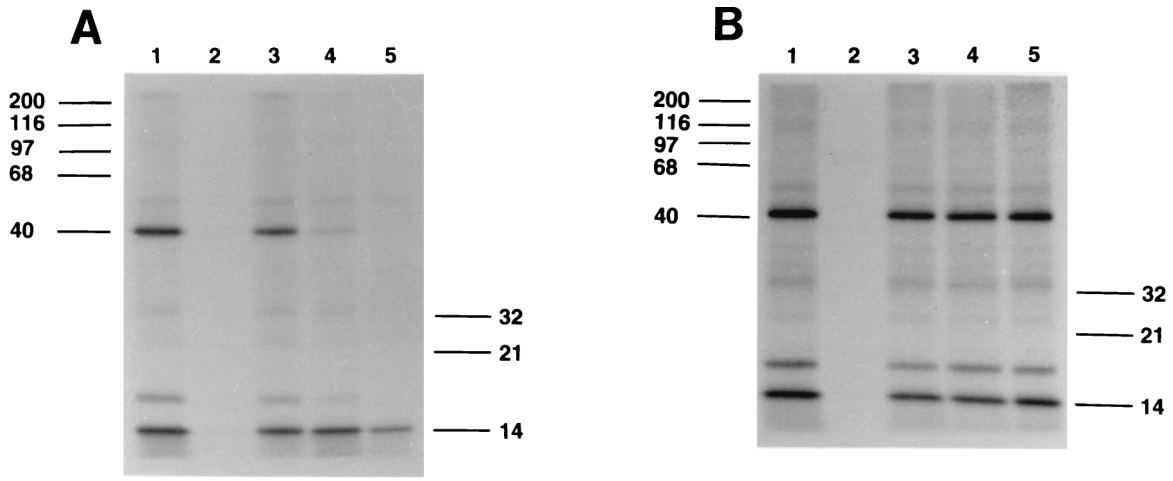

FIG. 2. Autoradiograms of crude microsomal membranes from *C. albicans* 3153 reacted by photolysis with ^{125}I-labeled echinocandin photoaffinity probe. Lanes 1, probe alone; lanes 2, nonphotolyzed control. Molecular size markers are in kilodaltons. (A) Lanes 3 to 5, probe in the presence of 1, 10, and 100 μg of LY303366/ml, respectively. (B) Competitors in lanes 3 to 5 are equimolar mixtures of the cyclic-peptide nucleus and the lipophilic side chain of LY303366 at concentrations comparable to echinocandin concentrations in panel A.

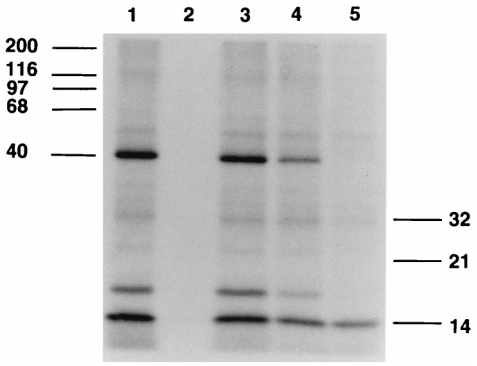

FIG. 3. Autoradiogram of crude microsomal membranes from *C. albicans* 3153. Experimental conditions and lanes 1 and 2 were the same as for Fig. 2. Lanes 3 to 5, probe in the presence of 10 μg of LY121019 (cilofungin), LY280949, and LY303366/ml, respectively.

the potencies of the echinocandin analogs now correlated with their abilities to inhibit glucan synthase activity (17). It is not clear why this difference in glucan synthase activity between crude and detergent-washed membranes was observed. Dose-response curves of glucan synthase activity in the detergent-washed membranes were obtained with LY303366, LY280949, and LY121019 (cilofungin), and the 50% inhibitory concentration (IC_{50}) was determined for each compound. Detergent-washed membranes were photolabeled with the probe echinocandin as described for the crude microsomal membranes.

The competition of echinocandins of differing potency with the photoaffinity probe in crude microsomal preparations is shown in Fig. 3. Identical results were obtained with detergent-washed membranes (data not shown). With either type of membrane preparation, the relationship between competition in the photolabeling experiments and antifungal potency was logarithmic (Table 2). The absolute values for inhibition varied slightly from experiment to experiment, but the relative inhibitions of the three competitor compounds in the photolabeling experiments correlated with the antifungal potencies of these compounds in several independent experiments.

In addition, competition in the photoaffinity labeling experiments correlated with glucan synthase inhibition in detergent-washed membranes (Table 2). Although the IC_{50}s for glucan synthase inhibition with the echinocandin analogs fell within a relatively narrow range, there was a linear relationship between glucan synthase inhibition and ability to compete in the photoaffinity labeling experiments.

Purification of the 40-kDa EBP. Two-dimensional gel analysis of the labeled 40-kDa EBP indicated that the pI of the protein was approximately 5.6 (data not shown). Initial attempts at sequencing the protein from two-dimensional gels failed, suggesting that the amino-terminal end may be blocked.

TABLE 2. Correlation of inhibition of photoaffinity labeling with biological activities of echinocandins

Compound[a]	% Inhibition[b]	MIC (μg/ml)[c]	IC_{50} (μg/ml)[d]
LY121019	39.0	0.31	1.30
LY280949	54.2	0.04	1.00
LY303366	84.7	0.0025	0.70

[a] Semisynthetic echinocandins (see Fig. 1).
[b] Inhibition of photoaffinity labeling of the 40-kDa protein (see Fig. 3).
[c] In Bacto Antibiotic Assay Medium 3.
[d] Glucan synthase IC_{50}.

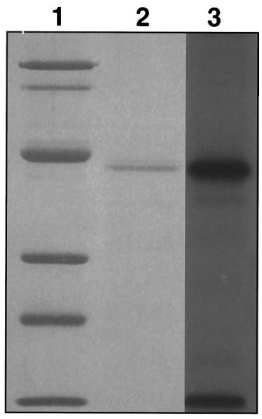

FIG. 4. Photoaffinity labeling of isolated 40-kDa protein. Lane 1, molecular weight markers stained with Coomassie brilliant blue G-250: phosphorylase B (92 kDa), bovine serum albumin (68 kDa), ovalbumin (42 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). Lane 2, partially purified 40-kDa protein from *C. albicans* stained with Coomassie brilliant blue G-250; lane 3, corresponding autoradiogram of photoaffinity-labeled partially purified 40-kDa protein from *C. albicans*.

To obtain sequence from internal fragments, the protein was purified by conventional methods by using the ^{125}I-labeled EBP from photolabeling experiments as a tracer.

Photoaffinity labeling of the purified 40-kDa EBP. To confirm that the isolated protein was the desired 40-kDa EBP, the partially purified protein (10 μg) was photoaffinity labeled. The isolated protein reacts strongly with the echinocandin probe. Competition with increasing concentrations of LY303366 demonstrated that the isolated protein is the 40-kDa EBP (Fig. 4).

Partial proteolysis of the 40-kDa EBP by endo-Glu C. The partially purified 40-kDa protein was further purified by SDS-PAGE, and gel slices were subjected to proteolytic digestion with endo-Glu C as described previously (5). By using increasing amounts of protease, progressive digestion of the 40-kDa EBP was demonstrated (Fig. 5, lane 2). The resulting doublet bands at 18 and ~10 kDa were subjected to amino acid sequence analysis. The doublet bands at 18 kDa resulted in no sequence information. The doublet bands at ~10 kDa gave a single sequence: IGELEDQYIDK YDQYRIXLK. The sequence was compared to the database

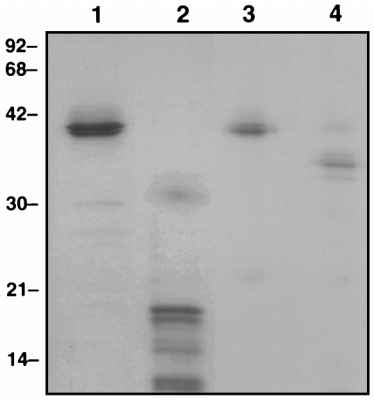

FIG. 5. SDS-PAGE of fragmented 40 kDa protein. Lanes 1 and 3, isolated 40-kDa protein stained with Coomassie brilliant blue G-250; lane 2, endo-Glu C partial digest of the 40-kDa protein; lane 4, cyanogen bromide digest of the 40-kDa protein. Molecular size markers (in kilodaltons) are shown on the left.

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          1                                                   50
YPL004c   MHRTYSLRNQ RAPTAAELQA PPPPPSSTKS KFFGKASIAS SFRKNAAGNF
YGR086c   MHRTYSLRNS RAPTASQLQN PPPPPSTTKG RFFGKGGLAY SFRRSAAGAF

         51                                                  100
          GPELARKLSQ LVKTEKGVLR AMEVVASERR EAAKQLSLWG ADNDDDVSDV
          GPELSRKLSQ LVKIEKNVLR SMELTANERR DAAKQLSIWG LENDDDVSDI
Ca CNBr                    MR AVEVTSRERK DVAK
         101                          (T)                   150
          TDKLGVLIYE LGELQDQFID KYDQYRVTLK SIRNIEASVQ PSRDRKEKIT
          TDKLGVLIYE VSELDDQFID RYDQYRLTLK SIRDIEGSVQ PSRDRKDKIT
Ca Endo Glu-C       IGELEDQYID KYDQYRIXLK
         151                          (S)                   200
          DEIAHLKYKD PQSTKIPVLE QELVRAEAES LVAEAQLSNI TREKLKAAYS
          DKIAYLKYKD PQSPKIEVLE QELVRAEAES LVAEAQLSNI TRSKLRAAFN

         201                                                 250
          YMFDSLRELS EKFALIAGYG KALLELLDDS PVTPGEARPA YDGYEASRQI
          YQFDSIIEHS EKIALIAGYG KALLELLDDS PVTPGETRPA YDGYEASKQI

         251                                                 300
          IMDAESALES WTLDMAAVKP TLSFHQTVDD VYEDEDGEEE EEPEIQ....
          IIDAESALNE WTLDSAQVKP TLSFKQDYED .FEPEEGEEE EEEDGQGRWS

         301                                                 350
          NGDIPGQVVE EEEVEWTTEV PVDDEAHEAD HHVSQNGHTS GSENI*..
          EDEQEDGQIE EPEQE..... ..EEGAVEEH EQVGHQQSES LPQQTTA*
```

FIG. 6. Alignment of homologous *S. cerevisiae* translation products of YPL004c and YGR086c and comparison to the peptides sequenced from the *C. albicans* 40-kDa EBP.

sequences by using the BLAST algorithm. A single high-scoring segment was found to match pir:S52527, a hypothetical protein from the *S. cerevisiae* genome (13 of 17 amino acids; 76% identity). This hypothetical yeast protein is the putative translation product of the yeast open reading frame YPL004c, located on chromosome XVI.

Cyanogen bromide digestion of the 40-kDa EBP. Digestion of the 40-kDa EBP with CNBr was accomplished in the gel slice. Separation of the resulting polypeptides by SDS-PAGE demonstrated a single major band with an approximate molecular mass of 33 kDa (Fig. 5, lane 4). The digestions were repeated, and the resulting polypeptide was transferred to PVDF membranes and sequenced. The 33-kDa band gave a single amino acid sequence: RAVEVTSRER(K/T)DV. The sequence was compared to database sequences by using the BLAST algorithm. The highest-scoring match for this peptide (when residue 11 was T) was pir:B552872, human neurofibromatosis type 1 protein (NF-1) (9 of 12 amino acids; 75% identity). The second-highest-scoring segment was the hypothetical yeast protein pir:S52527, described above (8 of 16 amino acids; 50% identity).

The high level of homology between the *C. albicans* polypeptides and the translation product of YPL004c prompted a search of the databases by BLAST using the entire putative translation product of YPL004c. A second highly homologous protein was detected in the *S. cerevisiae* genome, corresponding to a second hypothetical yeast protein of unknown function translated by the open reading frame YGR086c, located on chromosome VII. Both proteins are highly homologous (69% identical, 82% similar), but there are no recognizable functional motifs and their function is unknown. There were also several lower-scoring segments indicative of low-level homology to tropomyosins from several sources. The significance of these homologies from a functional perspective is not clear. Figure 6 shows the amino acid sequences of the translation products of YPL004c and YGR086c, with a comparison of the homologous *C. albicans* peptides sequenced from the 40-kDa EBP.

DISCUSSION

The echinocandins are noncompetitive inhibitors of glucan synthase (46). Although a number of genes involved in glucan synthase activity have been identified, there has been no evidence of a direct interaction of the echinocandins or pneumocandins with any of these molecules. In the photoaffinity labeling studies presented herein, it has been demonstrated that the echinocandins interact directly with at least two polypeptides in crude and detergent-washed microsomal membrane preparations of *C. albicans*, the 40- and 18-kDa proteins.

In competition studies using LY303366, there is a clear dose-response relationship in the inhibition of labeling of the 40-kDa protein with the photoaffinity probe at a fixed concentration. In addition, compounds of differing antifungal potency demonstrated a correlation between competition for labeling of the 40-kDa protein and both potency and glucan synthase inhibition. Both experimental results indirectly support the role of the 40-kDa protein in the mechanism of action of the echinocandins and as part of the glucan synthase complex. Specific enrichment of a 39-kDa protein along with other higher-molecular-mass proteins has been reported in the partial purification of the glucan synthase complex from *C. albicans* by product entrapment (16). This enriched 39-kDa protein could be the 40-kDa protein identified in this work.

GNS1 is a gene identified through resistance to pneumocandin in *S. cerevisiae* (15). Gns1p is believed to be involved in glucan synthesis, as disruption of the gene resulted in a 90% loss of glucan synthase specific activity, although the β-glucan content of whole cells appeared normal. The predicted product of the *GNS1* gene is a 40-kDa protein with five to six putative transmembrane domains. The authors suggested that Gns1p is a subunit of the glucan synthase complex, but *GNS1* has recently been shown to be allelic with *ELO2*, a gene involved in very-long-chain fatty acid elongation (34). Isolation of the 40-kDa EBP from *Candida* and subsequent sequencing of internal peptide fragments demonstrated that the 40-kDa EBP is not the gene product of a *GNS1* homolog in *C. albicans*. By using peptide sequences, two homologous hypothetical proteins were identified in the *S. cerevisiae* genome, but no functional role has been assigned to these genes. We have cloned the two homologous genes from *S. cerevisiae*, and their characterization will be reported elsewhere. We are currently isolating the gene coding for the 40-kDa EBP from *C. albicans* in order to construct a *C. albicans* strain with a deletion of this gene.

The nucleotide GTP is known to activate glucan synthase in vitro in *S. cerevisiae* and other fungi (43). In *S. cerevisiae*, the GTP-activating component of the glucan synthase complex may be separated from the catalytic component of the enzyme by extraction with salt and detergent (33). The GTP-binding protein associated with glucan synthase activity in *S. cerevisiae* has been characterized as a 20-kDa protein and has been identified as the translation product of the *RHO1* gene (14). The role of Rho1p in glucan synthase activity has been supported by inactivation of glucan synthase activity by ADP ribosylation with exoenzyme C3 from *Clostridium botulinum*, which specifically targets Rho1p (31).

One clue to the possible function of the 40-kDa EBP is the homology of the CNBr fragment to the human protein NF-1. Protein NF-1 is a GTPase-activating protein (GAP) involved in small GTPase cycling (49). One of the targets of protein NF-1 is the small GTPase ras p21 (29). This suggests a potential role of the 40-kDa EBP as a regulator of glucan synthase activity, possibly through a GAP-like function, although the region of homology of the CNBr fragment of the 40-kDa EBP to NF-1 is outside the ras-GAP-related domain of NF-1 (22). Given the

importance of GTP as a regulator of glucan synthesis as described above, it is intriguing to speculate that the 40-kDa EBP is involved in GTPase cycling.

A similar relationship between labeling and antifungal activity is also qualitatively observable for the 18-kDa protein, but due to the significantly less intense labeling of the protein, the relationship is not readily quantified. In addition, the physical amount of the 18-kDa protein is very small compared to that of the 40-kDa protein. We were therefore unable to directly sequence this protein from gels. Due to its low labeling efficiency and low physical abundance, we have been unable to identify this protein by classical biochemical methods.

The role of GTP in glucan synthase activity, the homology of the 40-kDa EBP to a ras-GAP protein, and the molecular size of the 18-kDa protein suggested a possible role for the 18-kDa protein as a small GTPase, possibly the *Candida* homolog of Rho1p. The identity of the 18-kDa protein and its possible functional role remain to be determined, but preliminary evidence obtained from ADP ribosylation with exoenzyme C3 in *Candida* suggests that the 18-kDa protein is not a Rho1p homolog in *C. albicans* (unpublished data). This does not exclude the possibility that the 18-kDa protein is another type of small GTPase.

Genetic studies with *S. cerevisiae*, using a phenotype of resistance to pneumocandins or echinocandins, suggest that the target for these cyclic lipopeptides is encoded by the *ETG1* gene (11, 13). This gene encodes a large 215-kDa membrane protein with 16 putative transmembrane segments. This gene has also been isolated through other phenotypes, such as resistance to papulacandin B, another glucan synthase inhibitor (*PBR1*), hypersensitivity to calcofluor white (*CWH53*), hypersensitivity to the immune suppressant FK506 in a calcineurin-deficient background (*FKS1*), and synthetic lethality with calcineurin mutants (*CND1*) (4, 12, 20, 40). In addition, the protein encoded by the *ETG1* locus was identified biochemically through selective enrichment of glucan synthase by glucan product entrapment (23). Homologs of the *ETG1* locus have been identified in *C. albicans* and *Aspergillus nidulans* (25, 26). Surprisingly, there was no evidence of direct interaction of the probe with a large polypeptide corresponding to the putative gene product of the *C. albicans ETG1* homolog. This result could be an artifact of the positioning of the cross-linker group on the cyclic-peptide nucleus or could possibly be due to a low abundance of the *C. albicans ETG1* gene product in crude membranes. Alternatively, it may be that noncompetitive inhibition of glucan synthase is mediated by proteins other than the *ETG1* gene product.

In summary, using an analog of LY303366, we have constructed a photoaffinity probe for examining the direct interaction of echinocandins with polypeptides in membrane preparations of *C. albicans*. The photoaffinity experiments described in this work detected two polypeptides but did not detect the gene product of the *C. albicans* homolog of *ETG1*, previously identified through genetic and biochemical studies to be the putative catalytic domain of glucan synthase. The major labeled polypeptide of 40 kDa possesses limited homology to the human NF-1 protein, a protein involved in regulation of small GTPase cycling, and therefore may be involved in GTP regulation of glucan synthase activity.

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