Involvement of a Cell Wall Receptor in the Mode of Action of an Anti-Candida Toxin of Pichia anomala

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Hanes-Woolf, Dixon, and Hill plots of growth rates of Candida albicans RC1 grown in various concentrations of glucose and a Pichia anomala WC65 toxin suggested the presence of toxin-binding sites. Indirect immunofluorescence microscopy with antitoxin antibodies demonstrated binding of the toxin to the cell wall. Resistance to the toxin of a mutant Saccharomyces cerevisiae deficient in cell wall β-1,6-D-glucan suggests that the glucan either served as the receptor or influenced the number or composition of the receptor. Immunofluorescence that appeared to be associated with the cell membrane of toxin-treated spheroplasts of C. albicans was also observed. Spheroplasts of the resistant mutant of S. cerevisiae were sensitive to the toxin.

Of the various yeast killer toxins, the double-stranded RNA plasmid-encoded toxins of strains of Saccharomyces cerevisiae have been studied the most extensively (2, 20, 22). The mechanism of action of these S. cerevisiae toxins involves an initial energy-independent binding of the toxin to a cell wall receptor. The toxin is then transported from the cell wall by an energy-dependent process to the cell membrane, which becomes permeable to protons, thereby causing lethality (20). The cell wall receptor on susceptible S. cerevisiae strains for the S. cerevisiae K1 toxin is β-1,6-D-glucan (3, 7, 11), and that for S. cerevisiae KT28 is a mannanoprotein (17). The possible existence of a second receptor for the S. cerevisiae toxin on the cell membrane of susceptible cells has been suggested since suicidal mutants of killer S. cerevisiae strains and immune mutants of susceptible S. cerevisiae strains that show normal cell wall binding to the toxin have been isolated (3, 20). Recently, Zhu and Bussey (23) have also shown that the S. cerevisiae K1 toxin kills spheroplasts of insusceptible strains of Candida albicans, Kluyveromyces lactis, and Schwannomyces aluvius. Inhibition of DNA synthesis in S. cerevisiae by yeast killer toxin KT28 has also been suggested (16).

The toxin of Pichia kluyveri causes leakage of potassium ions and ATP, decrease of cellular pH, and inhibition of amino acid uptake (8, 9). The mode of action of the killer toxin of Pichia anomala WC65, which is active against yeasts of various genera, including the animal pathogen C. albicans, has not been defined (14). The toxin is a glycoprotein with a molecular weight of about 83,300 and consists of about 86% protein and 14% carbohydrate. The toxin shows saturation kinetics at increased toxin concentrations, which suggests a receptor-mediated action (14). In this study we examined the possible involvement of receptors in toxin activity.

**Production and purification of toxin.** Crude concentrates of toxin were produced and purified as described earlier (15). Briefly, P. anomala WC65 was grown in Sabouraud dextrose broth, culture filtrates were concentrated by ultrafiltration, and the crude concentrate was purified by steps involving ethanol precipitation and fast-performance liquid chromatography on Mono Q and Superose 12 columns (Pharmacia, Uppsala, Sweden).

**Growth model.** The model for growth of microbes proposed by Monod (10), $\frac{k}{k_{\text{max}}}$ $= [s]/(K_s + [s])$, where $k$ is the actual specific growth rate, $k_{\text{max}}$ is the maximum specific growth rate, $[s]$ is the substrate concentration, and $K_s$ is the growth constant (1; also see references 10 and 13), was applied to logarithmic-phase cells of C. albicans RC1 grown in yeast nitrogen base (YNB) broth (Difco) buffered at pH 4.6 with 10 mM citrate phosphate buffer containing various concentrations of glucose (2 to 100 mM). Growth rates were determined as described previously (14). A nonlinear regression data analysis computer program (Enzfitter; Elsevier Science Publishers, Amsterdam, The Netherlands) was used to analyze growth rate data.

**Kinetic analyses.** C. albicans was grown to an optical density of 1.0 at 600 nm in YNB broth with limiting concentrations of glucose (0 to 30 mM). The cells were diluted to an optical density of about 0.058 (about $2 \times 10^6$ cells per ml) in fresh broth with the appropriate glucose concentration. Purified toxin (500 μl) was added to 3.0 ml of culture in cuvettes and incubated at 28°C on a roller drum (30 rpm). The optical densities at 600 nm were detected at time zero and at 30-min intervals for up to 6 h. The control cells for each glucose concentration were treated with heat-denatured toxin (100°C for 5 min). Growth rates were also determined in the presence of various concentrations of toxin at a nonlimiting (100 mM) concentration of glucose in YNB broth. Data were analyzed with reciprocal plots, a Dixon plot, and a Hill plot and interpreted as described by Segel (18) and Dixon and Webb (4).

**Antisera.** New Zealand White rabbits were immunized with purified toxin (65 μg/ml) mixed with adjuvant (Ribi Immunochim Inc., Hamilton, Mont.) by the protocol suggested by the manufacturer. Anti-Candida antibodies in the antisera were eliminated as described earlier (19). Briefly, we adsorbed fluorescein isothiocyanate (FITC)-conjugated goat immunoglobulin G (IgG) to rabbit IgG (Sigma Chemical

**MATERIALS AND METHODS**

**Cultures and media.** P. anomala WC65 and the susceptible yeast C. albicans RC1 were from the culture collection at Georgia State University. S. cerevisiae ATCC 28684 (S14) and S. cerevisiae ATCC 28685 (krel-I) (S14;96) were obtained from the American Type Culture Collection, Rockville, Md. All yeasts were maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.).

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Co., St. Louis, Mo.) with heat-killed *C. albicans* RC1 for 30 min.

**Immunofluorescence.** *C. albicans* RC1 was grown to the logarithmic phase in Sabouraud dextrose broth, washed three times with 0.1 M citrate phosphate buffer (pH 4.4) containing 0.5% Formalin, and stored for 48 h at 4°C. The killed cells were incubated overnight in a 1:10 dilution of normal goat serum in citrate phosphate buffer (pH 4.4) at 4°C. The cell density was adjusted to about 10^7 cells per ml, and 10 μl of the cell suspension was smeared on a microscope slide. The smear was heat fixed, treated with 10 μl of purified toxin in citrate phosphate buffer (pH 4.4), and incubated at 28°C for 20 min. The slides were washed in three changes of citrate phosphate buffer (pH 4.4 over 5 min), air dried, and treated with 10 μl diluted rabbit antiserum diluted 1:100 in phosphate-buffered saline (PBS); NaCl, 8.0 g; KCl, 0.20 g; KH₂PO₄, 0.12 g; Na₂HPO₄, 0.91 g; deionized H₂O, 1 liter (pH 7.2)). The slides were incubated at 28°C for 1 h and washed three times in PBS. The slides were then treated with 10 μl of FITC-conjugated goat anti-rabbit IgG (1:2,400 dilution in PBS) mixed with normal goat serum (1:100 dilution in PBS) and incubated for 15 min at 28°C in a moist chamber. The slides were washed in three changes of PBS over 15 min. Controls included cells that were processed but not exposed to toxin, cells treated with toxin but not treated with antitoxin rabbit antisera, and cells treated with only the goat anti-rabbit FITC-labeled antibody.

The slides were mounted in 90% glycerol containing 0.1% p-phenylenediamine and viewed on a microscope (BH2-RFL; Olympus) equipped with an automatic photomicrographic system. Photographs were taken on a Tri-X Pan film (Eastman Kodak Co., Rochester, N.Y.) and developed in Acufine (Acufine, Inc., Chicago, Ill.).

**Spheroplast and protoplast generation and fixation.** The procedures described by Harlow and Lane (5) were used for protoplast production. *C. albicans* RC1 cells were grown to the mid-logarithmic phase in Sabouraud dextrose broth, washed three times in 0.1 M citrate phosphate buffer, and held in buffer containing 4% paraformaldehyde for 90 min. The cells were then washed three times in 0.1 M potassium phosphate (pH 6.5) and harvested by centrifugation. The pellet was suspended in 33 mM citrate phosphate buffer (pH 5.9) containing 1.2 M sorbitol, 1 mg of zymolase-20T (Seikagaku Kogyo Co. Ltd., Tokyo, Japan) per ml, and a 1:10 volume of β-glucuronidase (Sigma) and incubated for 90 min at 30°C. The cells were washed three times in the citrate phosphate buffer containing sorbitol. Ten microliters of cell suspension was applied to slides coated with poly-L-lysine (Polysciences, Inc., Warrington, Pa.), and the slides were incubated for 15 min at room temperature. The spheroplasts and protoplasts were fixed in methanol at −20°C for 6 min followed by immersion in acetone at −20°C for 30 s. The slides were rinsed in four changes (5 min each) of PBS containing 1% bovine serum albumin. The slides were treated with 10 μl of toxin and stained, along with the controls, for immunofluorescence as described above.

**Role of the cell wall component β-1,6-glucan in toxina**

for S. cerevisiae ATCC 28684 (S14) and *S. cerevisiae* ATCC 28685 (krel-1) (S14.96) were tested for their susceptibilities to the *P. anomala* killer toxin by the well assay procedure of Woods and Bevan (21) and by a growth rate reduction assay (14). Spheroplasts were made as described above, except that cells were not treated with 4%...
paraformaldehyde. Spheroplasts were regenerated as described earlier (6).

RESULTS

Growth model. Nonlinear regression data analysis indicated that the equation of Monod (10) appropriately described the growth under experimental conditions because the plot of residual (experimental growth rate minus the theoretical growth rate; Δy) versus glucose concentration (x) showed a good fit (Fig. 1). The plot of the relative residual versus the calculated growth rate (Δy/Δx) showed a characteristic pattern (inverse hyperbolic or saddle point) (Fig. 1, inset B). This pattern is an indicator of the appropriateness of simple weighting for regression analysis.

Kinetic analysis. Growth rates of C. albicans RC1 determined at various limiting substrate (glucose) concentrations in the presence and absence of the toxin were analyzed by a Hanes-Woolf plot ([S]/k versus [S]) (Fig. 2). The plot intersected below the x axis and to the left of the y axis, which indicated a mixed type of inhibition. The growth constant (K_s) of C. albicans on glucose obtained from the Hanes-Woolf plot was 0.86 mM, and the k_max was 1.18 h^{-1}. The data, which were obtained from inhibition of growth at various concentrations of toxin at nonlimiting glucose concentrations and plotted as a Dixon plot (1/k versus [I], where [I] is the toxin concentration) (Fig. 3), were linear (r = 0.99), which suggests a mixed inhibition system (4, 18). The secondary plot of the slopes against the reciprocals of the glucose concentrations was nonlinear (Fig. 3, inset). Kinetic data obtained at various toxin concentrations gave a Hill coefficient of 1.48 (Fig. 4).

Immunofluorescence. Rabbit antiserum to purified toxin which was adsorbed with formalinized cells of C. albicans RC1 reacted with the toxin. Cell surfaces of C. albicans fluoresced after treatment with toxin followed by treatment with antitoxin rabbit antibodies and anti-rabbit FITC-labeled

FIG. 4. Hill plot of log k/(k - k_i) versus log [toxin]. Growth rates were determined at various toxin concentrations in the presence of nonlimiting concentrations of glucose. The slope for a linear fit was 1.476 (r = 0.995). A second-order regression (r = 0.998) had limiting slopes of −3.013 and 0.425.

FIG. 5. Phase-contrast (A) and immunofluorescence (B) photomicrographs of C. albicans RC1 treated with toxin and stained with antitoxin antisera and phase-contrast (C) and immunofluorescence (D) photomicrographs of cells not treated with the toxin. Phase-contrast (E) and immunofluorescence (F) photomicrographs of spheroplasts treated with toxin and phase-contrast (G) and immunofluorescence (H) photomicrographs of spheroplasts not treated with toxin are also shown. Note the diffused fluorescence around the lysed spheroplasts treated with toxin that show binding to membranes compared with binding to intact cells. Magnification, × 891.
goat antibody (Fig. 5B), whereas cells that were not treated with toxin but that were treated with antitoxin antibodies and anti-rabbit FITC-labeled goat antibody did not show fluorescence (Fig. 5D). Spheroplasts of C. albicans RC1 treated with toxin also demonstrated fluorescence that appeared to be associated with the cell membrane (Fig. 5F). Spheroplasts that were not treated with the toxin showed no immunofluorescence (Fig. 5H).

Role of β-1-6-d-glucan. The binding of the toxin to cells suggested by kinetic analyses and observed by indirect immunofluorescence microscopy prompted us to determine whether cell wall β-1-6-d-glucan was involved in toxin activity. We tested the activity of the P. anomala toxin against a wild-type S. cerevisiae strain that was susceptible to the toxin and against a mutant (krel-1) that had about 50% less β-1-6-d-glucan compared with that of the wild type. S. cerevisiae wild-type strain S14 was susceptible to the P. anomala toxin, whereas S. cerevisiae krel-1 strain S14.96 showed reduced susceptibility (Fig. 6). The spheroplasts of both the wild-type and the mutant strains were also sensitive to the toxin.

**DISCUSSION**

Analysis of the kinetic data of growth rates of C. albicans RC1 on various glucose concentrations at fixed toxin concentrations and various toxin concentrations at a nonlimiting fixed concentration of glucose (Hanes-Woolf plot and the Dixon plot) suggested the presence of two mutually nonexclusive binding sites for the toxin (4, 18). The Hill plot indicated the presence of cooperativity between at least two binding sites (4, 18). Since kinetic analysis cannot distinguish between n molecules with one binding site per molecule and one molecule with n independent, identical binding sites, the data obtained from the Hill plot could not be used for estimating the quantity of sites present on the cell (18). The mixed type of inhibition indicated by kinetic studies may be interpreted as follows: one of the binding sites (logically, the surface β-1-6-d-glucan-related binding site) binds noncompetitively and the secondary site binds competitively. Alternatively, the kinetic data could also be interpreted as an indication of a nonlinear post-primary-binding cascade reaction that is initiated by the toxin. However, in view of our observations and those of others (20, 23), because spheroplasts of susceptible and some resistant strains of yeasts are sensitive to killer toxins, such a cascade effect seems unlikely.

The fluorescence of the walls of antibody-toxin-treated whole cells of C. albicans, as well as the decreased susceptibility to the toxin of a glucan-deficient mutant of S. cerevisiae, supports the role of a cell wall receptor. Killing of spheroplasts of the resistant S. cerevisiae mutant and the fluorescence of C. albicans spheroplasts treated with toxin and antitoxin antibodies may be because of a second receptor on the cell membrane. However, further investigations with isolated membranes and with mutant C. albicans strains that show cell wall binding but that are resistant to the toxin are necessary to establish definitively the role of the cell membrane in toxin sensitivity. Results of this study and our previous investigations (15) indicate general similarities in the role of cell wall-binding sites on susceptible yeasts for *Pichia* and *Saccharomyces* toxins. Whether different strains of *C. albicans* with different susceptibilities (12) have different types of binding sites or simply vary in the numbers of a specific binding site has not been established.

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**LITERATURE CITED**


