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Binding of Yeast Killer Toxin to a Cell Wall Receptor on Sensitive *Saccharomyces cerevisiae*

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^{35}S-labeled killer toxin protein bound to cells of sensitive *Saccharomyces cerevisiae* S14a. Strains that were resistant to toxin through mutation in the nuclear genes *kre1* or *kre2* bound toxin only weakly. Non-radioactive toxin competed effectively with ^{35}S-labeled toxin for binding to S14a, but did not compete significantly in the binding to mutant *kre1*-1. This implied that binding to *kre1*-1 was nonspecific. A Scatchard analysis of the specific binding to S14a gave a linear plot, with an association constant of 2.9×10^6 M^{-1} and a receptor number of 1.1×10^7 per cell. Killer toxin receptors were solubilized from the cell wall by zymolyase digestion. Soluble, non-dialyzable cell wall digest from S14a competed with sensitive yeast cells for ^{35}S-labeled toxin binding and reduced toxin-dependent killing of a sensitive strain. Wall digest from *kre1*-1 competed only weakly for toxin binding with sensitive cells and caused little reduction of toxin-dependent killing. Although the abundant (1.1×10^7 per cell) wall receptor appeared necessary for toxin action, as few as 2.8×10^4 toxin molecules were necessary to kill a sensitive cell of S14a. The kinetics of killing of S14a suggested that some component was saturated with toxin at a concentration 50-fold lower than that needed to saturate the wall receptor.

Killer strains of yeast contain a double-stranded RNA killer plasmid and produce a protein toxin that kills sensitive strains (5, 8). The mechanism of killer toxin action remains unknown, but the process involves binding of toxin to sensitive cells and a second energy-dependent step that results in membrane damage (7). We have purified ^{35}S-labeled toxin and used it to measure the interaction of the toxin with sensitive cells. Evidence for a cell wall receptor comes from previous work which showed, by an indirect assay, that killer-resistant mutations in nuclear genes *kre1* and *kre2* caused reduced binding of killer toxin (1). Here we extend these observations to characterize the binding of ^{35}S-labeled toxin to a cell wall receptor on sensitive cells which is absent or reduced in killer-resistant mutants *kre1*-1 and *kre2*-1.

MATERIALS AND METHODS

Yeast strains and media. The following strains of *Saccharomyces cerevisiae* were used. T158C/S14a, a prototrophic diploid killer obtained by mating super-killer T158C (**a** *his*4C-864) with sensitive S14a (α $ade_{2\cdot5}$) (5), was used for killer toxin production. Killer-resistant mutants derived from S14a were spontaneous mutant *kre1*-1 (S14.96), *kre2-1* (S14.14), and *kre3*-1 (S14.MB6) (1). The liquid growth medium was the minimal medium of Halvorson (3) with 0.5% yeast extract, 0.5% peptone, and 2% dextrose (YEPD).

Preparation of ^{35}S-labeled killer toxin. Killer strain T158C/S14a was grown in Halvorson minimal medium (3), modified by the addition of 5% (wt/vol) glycerol and made sulfate free by replacement of ammonium sulfate with ammonium chloride, 1.62 g/liter, and magnesium sulfate by magnesium chloride, 0.42 g/liter. The trace element solution was also prepared sulfate free by the use of chloride salts as follows: $FeCl_3 \cdot 6H_2O$, 166 mg; $MnCl_2 \cdot 4H_2O$, 65.6 mg; $ZnCl_2$, 42 mg; $CuCl_2 \cdot 2H_2O$, 53 mg; all weights per 100 ml of stock trace element solution. Sodium sulfate was added at 2×10^{-4} M.

To 100 ml of the above growth medium was added 30 mCi of [^{35}S]sulfate, carrier free (Amersham, SJS.1). The medium was inoculated at about 2×10^5 cells per ml, grown at 18°C on a rotary shaker at 150 rpm, and harvested after about 40 h at approximately 6×10^7 cells per ml. The culture was centrifuged at $4000 \times g$ for 5 min, and the supernatant medium was concentrated in an ultrafiltration cell with an Amicon PM-10 membrane. The ^{35}S-labeled material was diluted with approximately 100 ml of unlabeled growth medium concentrated from a 2.5 liter culture of T158C/S14a grown on Halvorson minimal medium. The unlabeled concentrate was added in 15-ml amounts to the ultrafiltration cell and concentrated to 2 to 5 ml to partially dialyze the unincorporated [^{35}S]sulfate. The material was finally concentrated to about 2 ml and then purified by polyethylene glycol precipitation and chromatography on a glyceryl-controlled-pore glass column (51 by 0.9 cm) as described (5). Approximately 40 μg

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of toxin was recovered with a specific activity of 12.5 to 20 μCi/mg of protein. Non-radioactive killer toxin of molecular weight 11,470 was purified as described (5).

Toxin-binding assay. Binding was done under equilibrium conditions. From 20 to 100 μl of toxin was added to cells in 100 μl to 1 ml of YEPD medium without dextrose (YEP), incubated at 20 to 22°C for 10 min in 1.5-ml polypropylene centrifuge tubes, and centrifuged for 30 s at approximately 8,000 × *g* in a microcentrifuge. A known volume of supernatant was removed, leaving the cell pellet plus a remaining known volume of supernatant. The amount of toxin bound to cells was found by counting the cell pellet plus remaining supernatant and subtracting the counts present in the supernatant. To increase the efficiency of detection of radioactive toxin bound to cells, cell pellets were washed into scintillation vials and digested with 200 μl of zymolyase 5000 at 0.5 mg/ml in 0.1 M Tris-hydrochloride (pH 7.5) and 2 mM dithiothreitol for 2 h at 37°C before counting.

Using this method, toxin binding was found to be complete by 3 min and did not decrease for at least 80 min. Measurable dissociation of bound toxin occurred (see Results) if the cell pellets were washed with toxin-free YEP.

Yeast cell wall digests. Spheroplasts were made from S14a or *kre1*-1 by zymolyase digestion as described (2). The spheroplasts were centrifuged at 1,500 × *g* for 5 min, and the supernatant was removed and centrifuged at 12,000 × *g* for 15 min. The supernatant containing the cell wall digest was dialyzed against deionized water, freeze dried, resuspended in 0.1 M sodium acetate buffer (pH 4.6), and stored at −20°C. The amount of polysaccharide in digests from S14a and *kre1*-1 was similar and was from 53 to 89 mg per 2×10^{10} cells, determined as described by Palfree and Bussey (5).

Competition experiments with cell wall digests. To measure reduction of toxin-dependent killing of a sensitive culture, the following procedure was used. Soluble wall digests from S14a or *kre1*-1 at various concentrations in a volume of 750 μl were mixed with killer toxin, plus 750 μl of 2×-concentrated YEPD, and incubated at 20 to 22°C for 30 min. One milliliter of this mixture was added to the pellet from 1 ml of sensitive cells of strain A8207NK in a centrifuge tube. The cell pellet was resuspended, and the tube was incubated on a roller drum for 3 h at 20 to 22°C. Viable cell numbers were estimated after plating and counting on YEPD agar.

To measure competition for ^{35}S-labeled killer toxin binding, a range of concentrations of soluble wall digests (200 μl) were incubated with 20 μl of toxin at 20 to 22°C for 10 min. Sensitive cells of strain S14a at 2×10^7 cells per 200 μl in 2× YEP were added and incubated for a further 10 min. The mixture was centrifuged, 410 μl of the supernatant was removed, and the cell pellet was digested with zymolyase and counted.

RESULTS

Purity of ^{35}S-labeled killer toxin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of radioactive toxin showed a single band after autoradiography (Fig. 1). This band comigrated with pure non-radioactive killer toxin seen by Coomassie staining (5).

Binding of radioactive killer toxin to a sensitive strain and to toxin-resistant mutants. Binding of a constant amount of radioactive toxin to sensitive strain S14a increased with cell number but was essentially complete when some 74% of the input counts were bound (Fig. 2). Binding of killer toxin activity corresponded with binding of radioactivity (data not shown) (1). Resistant mutant *kre1*-1 bound less toxin than its parent S14a (Fig. 2). For example, to bind the same amount of toxin as 4×10^7 cells of S14a per ml required 8.7×10^8 cells of *kre1*-1 per ml. Binding of toxin to *kre1*-1 cells was kinetically distinct from binding to the parent, being more readily dissociable. Dissociation of bound toxin measured after resuspending cells, with bound toxin, in binding medium without free toxin for 15 min was 31% for S14a and 67%

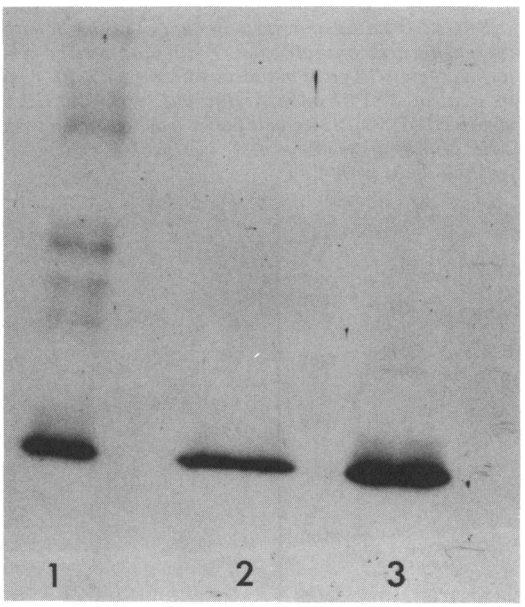

FIG. 1. *Autoradiogram of ^{35}S-labeled killer toxin. Toxin was purified as described, and samples were electrophoresed in a linear 8 to 18% sodium dodecyl sulfate-polyacrylamide gel by the method of Laemmli (4). (Lane 1) Polyethylene glycol-precipitated (partially purified) toxin (10 μl) containing 8.5×10^3 cpm in 1-mg/ml bovine serum albumin. (Lane 2) Pure toxin, 0.2 μg containing 5×10^3 cpm in 1-mg/ml bovine serum albumin. (Lane 3) Pure toxin, 0.68 μg containing 1.7×10^4 cpm. The sample had been precipitated in 50% (vol/vol) ethanol in the presence of cold extracellular material from a killer strain and redissolved in sodium dodecyl sulfate solubilization buffer.*

for *kre1*-1. The altered dissociation can also be seen in Fig. 2, where toxin remaining bound to cells is shown after two washes in toxin-free binding medium; the proportion remaining bound to *kre1*-1 was reduced over that bound to S14a.

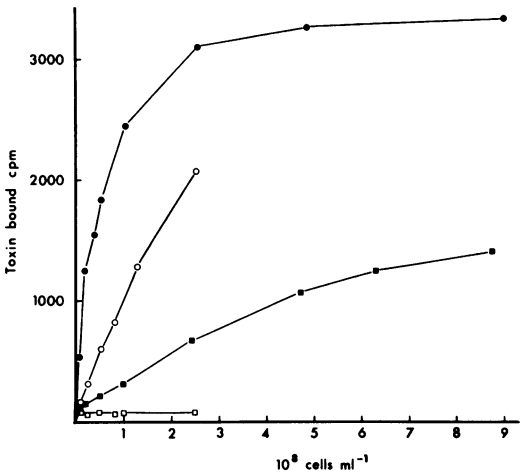

FIG. 2. *Binding of a fixed amount of* ^{35}*S-toxin with increasing cell concentration. Toxin binding was performed using 0.2 μg of toxin containing* 4.7×10^3 *cpm in 1 ml of YEP. Symbols: Binding to (●) sensitive strain S14a; (○) S14a, cell pellet washed twice with YEP. Binding to (■) kre1-1; (□) kre1-1, cell pellet washed twice with YEP.*

Binding of ^{35}S-labeled toxin to resistant mutant *kre2*-1 was also lower than that of S14a, but binding to resistant mutant *kre3*-1 was comparable to S14a (data not shown).

Binding of labeled toxin to a killer strain, A8209K, was similar to that for an isogenic sensitive strain, A8209NK. This suggested that the immunity of killer strains to toxin action was not conferred by absence of the wall receptor.

Competition of ^{35}S-labeled killer toxin with non-radioactive toxin. To show that the radioactive toxin behaved with properties similar to those of non-radioactive toxin, a competition experiment was performed. Increasing concentrations of cold toxin were added to a fixed amount of radioactive toxin, and binding to a constant cell concentration was measured (Fig. 3). The low level of binding of radioactive toxin to *kre1*-1 was not competed significantly with cold toxin, suggesting many low-affinity sites. The radioactive toxin and the cold toxin had biological specific activities that were assayed to be approximately the same by the well test method (9). We have calculated from the competition data the binding of toxin as a function of toxin concentration. A Scatchard analysis of this data is shown in Fig. 4, where the nonspecific binding to the *kre1*-1 mutant has been sub-

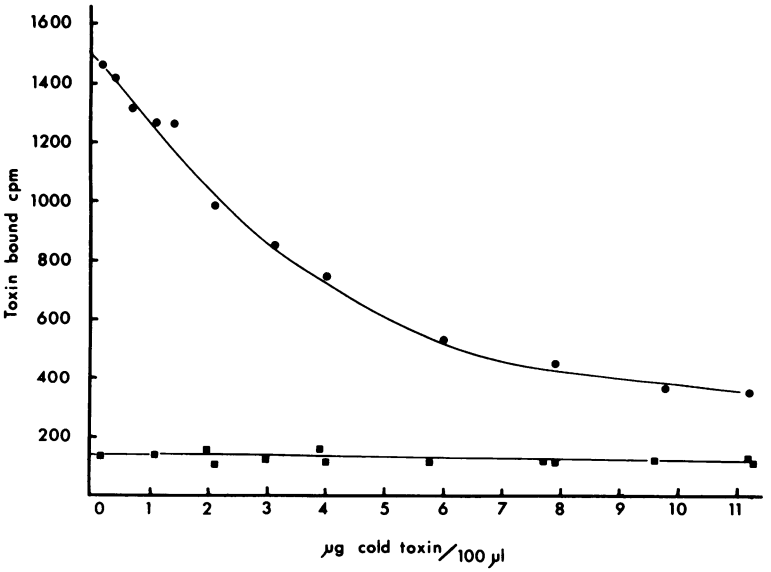

FIG. 3. *Competition of cold killer toxin with* ^{35}*S-labeled toxin for binding to sensitive and resistant yeast strains.* ^{35}*S-labeled toxin (0.16 μg containing* 2.8×10^3 *cpm) and a range of cold toxin concentrations were added to* 5×10^6 *cells in YEP; the total reaction volume was 100 μl. Binding was determined as described in the text. The volume of supernatant remaining was 1.5 μl. Symbols: (●) binding to S14a; (■) binding to kre1-1.*

tracted (6). The data gave an association constant K_a of 2.9×10^6 M^{-1} for toxin binding and a toxin receptor concentration of 1.1×10^7 sites per cell.

Solubilization of a cell wall receptor for killer toxin. Soluble, non-dialyzable cell wall extracts were prepared from sensitive strain S14a and *krel*-1 by digestion of cell walls with zymolyase 5000 (see Materials and Methods). The wall digest from S14a competed with sensitive yeast cells for radioactive killer toxin binding (Fig. 5) and reduced the toxin-dependent killing of a sensitive strain (Fig. 6). Wall digest from *krel*-1 competed only weakly for toxin binding with sensitive cells, and caused little reduction of toxin-dependent killing.

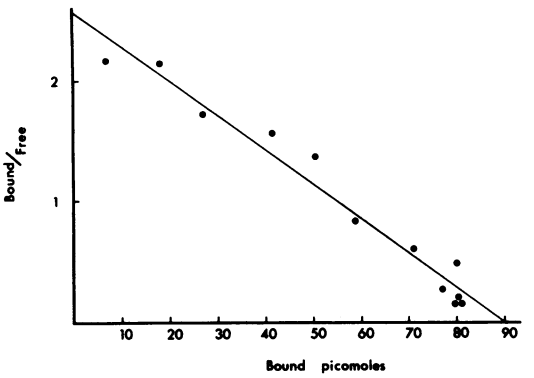

FIG. 4. *Scatchard plot of binding of killer toxin to sensitive strain S14a. The data are derived from Fig. 3. The binding of toxin to krel-1 has been subtracted as described by Rosenthal (6). The line is a least-squares fit to the points.*

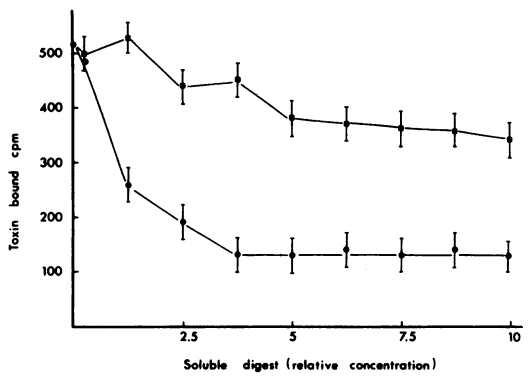

FIG. 5. *Competition of soluble wall digests from S14a and krel-1 with sensitive cells for binding of ^{35}S-labeled killer toxin. Binding of ^{35}S-labeled toxin (0.11 μg containing 2,300 cpm) to sensitive strain S14a was measured in the presence of varying amounts of soluble wall digests from S14a or krel-1 as described in the text. A soluble digest concentration of 10 represents digest products from 3.3×10^8 cells per ml, with a polysaccharide content of 1.47 mg/ml for S14a and 1.25 mg/ml for krel-1. Symbols: (●) soluble digest from S14a; (■) soluble digest from krel-1.*

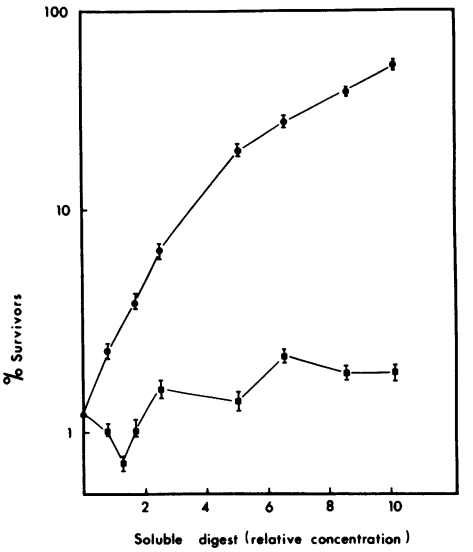

FIG. 6. *Toxin-dependent killing of sensitive strain A8207NK in the presence of increasing amounts of soluble wall digests from S14a and krel-1. A soluble site concentration of 10 represents digestion products from 3.7×10^8 cells per ml, with a polysaccharide content of 0.97 mg/ml for S14a and 1.56 mg/ml for krel-1. Symbols: (●) soluble digest from S14a; (■) soluble digest from krel-1.*

Relationship between toxin binding and biological killing of sensitive cells. Toxin concentrations necessary to saturate the cell wall binding sites in sensitive cells were unexpectedly high (11 μg/100 μl or ~10^{-5} M; Fig. 3) and exceeded the amount of toxin needed to kill sensitive yeast strains. The relationship between toxin concentration and killing is indicated in Fig. 7. Toxin at 5×10^{-9} M will kill 2.3×10^7 cells of sensitive strain S14a per ml with a multiplicity of 1 (corresponding to 2.8×10^4 molecules per cell), and killing was essentially complete at 2.3×10^{-7} M.

DISCUSSION

The use of radioactive killer toxin has enabled us to directly measure its interaction with cell wall receptors on sensitive yeast cells. A major portion of the binding appears to be biologically relevant, because it is reduced in resistant mutants *krel*-1 and *kre2*-1, findings which suggest that the site is necessary for killing to occur. Competition experiments with pure non-radioactive killer toxin provide evidence that the radioactive toxin was representative of cold killer toxin. Quantitation of the binding of toxin to the

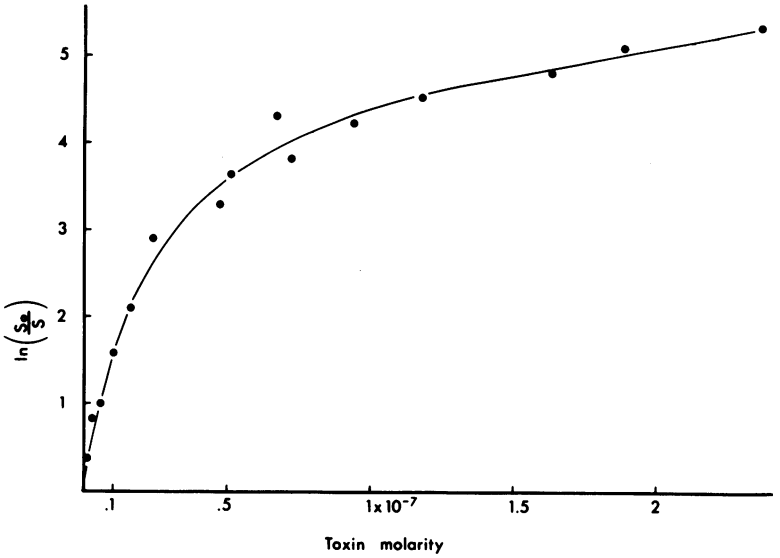

FIG. 7. *Killing of S14a in presence of increasing concentrations of killer toxin. Initial cell concentration (S_0) was 2.3 × 10⁷ cells per ml in YEPD. Cell concentrations of survivors (S) were determined by plating onto YEPD agar after 3 h in toxin and counting colonies after 2 days at 30°C.*

receptor present in S14a but missing from *kre1*-1 gave a K_a of 2.9×10^6 M^{-1} and a receptor concentration of 1.1×10^7 sites per cell. Scatchard analysis of this binding was consistent with one receptor species.

Soluble wall digests of sensitive strain S14a contain components that can compete for killer toxin binding. These components are greatly reduced in wall digests from *kre1*-1. This suggests that we have solubilized the *kre1*-dependent cell wall receptor for killer toxin. Previous work indicated that this receptor was removed from sensitive cells by glusulase digestion, was periodate sensitive, but was not affected by protease treatment of boiling (1). It should now be possible to purify and characterize this cell wall receptor.

Although the cell wall receptor may be necessary for toxin action, the kinetics of killing of sensitive cells show that some 2.8×10^4 toxin molecules can kill a sensitive cell, and they suggest that some other component is saturated with toxin at a concentration 50-fold lower than that necessary to saturate the cell wall receptor. We do not know the nature of the component, but it could be involved in either the energy-dependent event in toxin action (7), or the *kre3*-dependent event. *kre3* is a nuclear gene mutation that leads to toxin resistance with normal binding to the cell wall receptor (1).

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