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Yeast β-1,6-Glucan Is a Primary Target for the *Saccharomyces cerevisiae* K2 Toxin

Juliana Lukša,[a] Monika Podoliankaitė,[a] Iglė Vepštaitė,[a] Živilė Strazdaitė-Žielienė,[a] Jaunius Urbonavičius,[a,b] Elena Servienė[a]

Laboratory of Genetics, Institute of Botany, Nature Research Centre, Vilnius, Lithuania[a]; Department of Molecular Microbiology and Biotechnology, Institute of Biochemistry, Vilnius University, Vilnius, Lithuania[b]

Certain *Saccharomyces cerevisiae* strains secrete different killer proteins of double-stranded-RNA origin. These proteins confer a growth advantage to their host by increasing its survival. K2 toxin affects the target cell by binding to the cell surface, disrupting the plasma membrane integrity, and inducing ion leakage. In this study, we determined that K2 toxin saturates the yeast cell surface receptors in 10 min. The apparent amount of K2 toxin, bound to a single cell of wild type yeast under saturating conditions, was estimated to be 435 to 460 molecules. It was found that an increased level of β-1,6-glucan directly correlates with the number of toxin molecules bound, thereby impacting the morphology and determining the fate of the yeast cell. We observed that the binding of K2 toxin to the yeast surface receptors proceeds in a similar manner as in case of the related K1 killer protein. It was demonstrated that the externally supplied pustulan, a poly-β-1,6-glucan, but not the glucans bearing other linkage types (such as laminarin, chitin, and pullulan) efficiently inhibits the K2 toxin killing activity. In addition, the analysis of toxin binding to the intact cells and spheroplasts confirmed that majority of K2 protein molecules attach to the β-1,6-glucan, rather than the plasma membrane-localized receptors. Taken together, our results reveal that β-1,6-glucan is a primary target of K2 toxin and is important for the execution of its killing property.

The production of antimycotic killer toxins has been observed in several yeast genera and proved to be a widespread phenomenon (1, 2). Killer strains of *Saccharomyces cerevisiae* secrete protein toxins derived from a family of double-stranded RNAs (dsRNAs). The toxins have been grouped into four types (K1, K2, K28, and Klus) based on their killing profiles and lack of cross-immunity (3, 4). Such proteins are able to kill the nonkiller yeast, as well as yeast of other killer types, while the toxin-producing cells remain immune to their own or to the same type of killers (4, 5). K1 toxin disrupts the regulated ion flux across the plasma membrane, leading to the death of sensitive yeast strains (6, 7). The killing action of K1 toxin involves at least two steps. During the first step, the toxin binds to the cell wall, whereas the second step leads to the translocation and insertion of the toxin into the plasma membrane (6). Beta-1,6-glucan was originally proposed to be a cell wall receptor for K1 (8). Analysis of several *kre* mutants demonstrated that decrease of the cell wall β-1,6-glucan level leads to K1 resistance, thus confirming the involvement of this type of glucan in toxin binding (9). During the second step, K1 toxin interacts with plasma membrane receptors and disrupts the functional integrity of the plasma membrane either by inducing the formation of new ion channels (7) or through the activation of existing potassium channels (10). Products of *TOK1* (protein, forming the potassium ion channel) and *KRE1* (glycoprotein, involved in β-glucan assembly) have been suggested as membrane receptors for K1 (10, 11). K1 toxin is thought to increase the permeability of Tok1p, but the corresponding mutant did not show the increased resistance to K1. At the same time, Kre1p was shown to be necessary for the action of the K1 toxin on target cells and was regarded as a plasma membrane-localized receptor. However, the discovery of numerous yeast factors involved in K1 resistance and their connection to cell wall and plasma membrane biogenesis suggests that other targets of K1 cannot be excluded (9, 11).

While dominant in vineyard/winery ecosystems, K2 toxin was studied less extensively (5, 12–14). It was generally assumed that K1 and K2 toxins act in a similar fashion (11, 15, 16). However, there are several lines of evidence pointing to prominent differences between these toxins. Yeasts producing K1 toxins and those producing K2 toxins are able to kill each other while remaining resistant to their own toxin. Also, the primary sequence of K2 is unrelated to that of K1. K2 toxin differs from K1 in preprotoxin organization (K2 lacks the γ-subunit) (12, 13). The optimal pH for the action of the K2 toxin is 4.0 to 4.3 (17), somewhat lower than that of the K1 toxin, which reaches 4.6 (15, 16, 18). Distinct sets of host factors affecting resistance and sensitivity toward K1 and K2 killer toxins were identified, revealing functional discrepancies between these toxins as well (9, 19). Analysis of the sensitivity of a Δ*kre1* mutant to either K1 or K2 toxin demonstrated total resistance in both cases, suggesting that Kre1p serves as plasma membrane receptor (19, 20). Also, it was shown that both K1 and K2 bind less efficiently to a set of mutants featuring a decreased level of β-1,6-glucan, implying that it serves as a cell wall receptor for both proteins (9, 19, 20). Despite the similarities between the K1 and K2 toxins and taking into account the above-mentioned differences among them, the details of killer protein interactions with the target cells and immunity mechanisms remain to be uncovered.

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Address correspondence to Elena Servienė, elena.serviene@botanika.lt.

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In this study, we analyzed the K2 toxin binding dynamics and calculated the apparent number of toxin molecules bound to a yeast cell under saturating conditions, both in the wild-type (wt) strain and in mutants with altered levels of cell wall glucan content. In addition, we performed similar binding experiments with K1 toxin and demonstrated that both toxins (K1 and K2) bind to the yeast in a similar manner. Using both *in vivo* and *in vitro* approaches, we experimentally confirmed the previous assumptions regarding the role of β-1,6-glucan as primary target for the K2 toxin. We also demonstrated a direct correlation between the cellular level of β-1,6-glucan and K2 toxin binding, as well as the importance of such interactions for the killing process. Finally, we showed that changes of yeast cell morphology upon the treatment with K2 toxin are linked to the increased cellular level of β-1,6-glucan.

MATERIALS AND METHODS

Yeast strains and culture media. *S. cerevisiae* strains M437 (wt *HM/HM* [*kil-K2*]) and K7 (*MAT***a** *arg9* [*kil-K1*]) were used to isolate K2 and K1 toxins, respectively (21). BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and several isogenic mutant strains from the *S. cerevisiae* nonessential gene deletion collection, i.e., Δ*smi1*, Δ*aim26*, Δ*kre6*, Δ*kre1*, Δ*map1*, Δ*mnn9*, and Δ*tok1* mutants, purchased from Thermo Scientific Molecular Biology (Lafayette, CO, USA) were used to test the activity of the K2 and K1 toxins. Strains from the yeast overexpression library, containing plasmid-borne *SMI1*, *AIM26*, *KRE6*, *KRE1*, *MAP1*, or *MNN9* genes, expressed under the control of GAL1/10 promoter (Dharmacon, Lafayette, CO, USA), were used in K2 survival assays. Yeast strain α'1 (MATα *leu2-2* [*Kil-0*]) was used as the sensitive strain in the well assay (21).

Yeast cells were grown in standard YPD medium (1% yeast extract, 2% peptone, 2% dextrose). To test the K2 activity, MBA medium (0.5% yeast extract, 0.5% peptone, 2% dextrose, 2% agar), adjusted to pH 4 with 75 mM phosphate-citrate buffer and supplemented with 0.002% methylene blue dye, was used. For the isolation of K2 toxin, synthetic SC medium [2% glucose, 6 mM K_2HPO_4, 8 mM $MgSO_4$, 8 mM $(NH_4)_2SO_4$], adjusted to pH 4 with the 75 mM phosphate-citrate buffer and containing 5% glycerol, was used. In the experiments using K1 toxin, the pH of MBA or SC medium was adjusted to 4.6. For cultivation of yeast overexpression library strains, SC-Gal medium (pH 4.0), containing 2% galactose as a carbon source, was used.

Preparation of the K2 toxin. *S. cerevisiae* strain M437, producing the K2 toxin, was grown in synthetic SC medium for 4 days at 18°C. Yeast cells were sedimented by centrifugation at 3,000 × *g* for 10 min, and the supernatant was filtered through a 0.22-μm sterile polyvinylidene fluoride membrane and concentrated by ultrafiltration through an Amicon PM-10 membrane. This toxin isolate was used for activity measurement, yeast binding, and yeast survival assays (19, 21). Toxin activity was expressed in the arbitrary units (U) based on well assays (21, 22). The amount of K2 protein was calculated according to the SDS-PAGE using bovine serum albumin (BSA) as a concentration standard and quantifying the intensity of bands by the densitometric analysis. The presence of K2 toxin was confirmed by Western blotting using K2-specific polyclonal antibodies (23). The correlation between the concentration of K2 killer protein and strength of killing action was analyzed. It was determined that 10^4 units of toxin activity corresponds to about 1 ng of K2 protein.

The extracellular K1 toxin was prepared similarly to K2 by concentrating and partially purifying supernatant after cultivation of *S. cerevisiae* strain K7. Toxin activity was measured by the well assay. The amount of K1 protein was estimated based on the previous observation that 10^4 units of K1 corresponds to about 1 ng of toxin (11).

Dynamics of K2 toxin binding to yeast cells. Strain BY4741 was cultivated at 30°C in YPD medium to the late exponential growth phase (about 1×10^8 cells per ml). The appropriate amounts of cells (1×10^6, 2×10^6, 3×10^6, and 1×10^8, respectively) were collected by centrifugation (3,000 × *g*, 3 min) and washed with 1 ml of SC medium (pH 4.0). The supernatant was removed by centrifugation (3,000 × *g*, 3 min), the cells were mixed with 1 ml (0.1 ng to 25 ng) of K2 toxin preparation and incubated at 4°C with gentle agitation (40 rpm) for different periods of time (from 1 to 180 min). The supernatant was then collected by centrifugation (10,000 × *g*, 1 min), and the remaining K2 killing activity was tested using the well test bioassay: 100 μl of the supernatant was deposited in 10-mm-diameter cut-in wells of MBA medium (pH 4.0), which contains α'1 yeast cells. The plates were incubated for 2 days at 25°C, and the diameter of the lysis zones was measured (21). Binding level was expressed as the percentage of toxin activity obtained by the subtraction of the remaining activity from the total activity. To evaluate the binding of K2 toxin to different mutant strains, a similar procedure was followed, but all time points were after 60 min under saturation conditions. When 0.1 to 1 ng of K2 toxin was used for the binding assay, 2×10^6 yeast cells were used; when the amount of K2 was increased to 5 to 25 ng, the amount of cells was raised to 1×10^8. All data presented are averages from at least five independent experiments, conducted on different days with distinct batches of toxin production. Analysis of K1 binding to the target cells was performed as for K2, but 0.1 ng of toxin was used and the pH of the washing solution and MBA medium was adjusted to 4.6.

Since toxin binding was evaluated indirectly by testing the remaining killing activity, only the apparent number of toxin molecules bound could be estimated. Such amount of K2 toxin molecules bound to a single cell at saturation conditions was calculated as follows. According to the obtained data, 2×10^6 of BY4741 cells bind about 55 pg of K2 toxin. Assuming that the molecular mass of mature K2 is 36 kDa (calculated based on data provided in reference 12), division of the total number of toxin molecules bound to the number of yeast cells results in 460 molecules of the toxin bound to a single cell. In the case of K1 toxin, calculation was performed as for K2, but taking into account that at saturation, 2×10^6 BY4741 cells bind about 35 pg of K1 and the molecular mass of K1 is about 19 kDa (24). Thus, the apparent number of K1 toxin molecules bound to a single cell reached about 555.

The spheroplasts were prepared as described in reference 25, and the K2 toxin binding assays were performed similarly to the experiments using intact cells, except that 1.2 M sorbitol was added as an osmotic stabilizer.

Determination of K2 killing activity in the presence of different polysaccharides. Toxin-sensitive *S. cerevisiae* BY4741 cells were grown in liquid YPD medium at 30°C until the cell density reached about 1×10^8 cells per ml. Then 5×10^5 of the yeast cells were collected by centrifugation (3,000 × *g*, 3 min), washed with 1 ml of SC medium, and treated with 1 ml of K2 toxin (10^3 U) in the presence of 9 mg of one of the following polysaccharides: chitin, laminarin, pullulan, or pustulan (purchased from Sigma). After 1 h or 24 h of incubation at 20°C with gentle agitation, the diluted cells were plated on YPD-agar plates, plates were incubated for 2 days at 30°C, and the number of viable cells was determined and compared to the controls to which no polysaccharide was added. Simultaneously, after incubation with each polysaccharide and toxin, yeast cells were spotted after serial dilutions onto YPD-agar plates and incubated for 1 to 2 days at 30°C, and the numbers of viable cells of differently treated yeast were calculated. Three independent experiments with two replicates in parallel were conducted, and the means and standard errors of the means were determined.

Evaluation of K2 toxin binding to the different polysaccharides *in vitro*. Nine milligrams of each polysaccharide (chitin, laminarin, pullulan, or pustulan) in 1 ml of K2 toxin (10^3 U) preparation was incubated at 20°C for different periods of time, and the residual toxin activity was analyzed in the well test bioassay.

Preparation of cell wall β-1,6-glucan. Yeast cells were cultivated overnight in YPD medium at 30°C until the late exponential growth phase. Cells were collected by centrifugation (3,000 × *g*, 3 min), washed twice with 1 ml of distilled water, and then treated 1 h with 0.5 ml 3% NaOH at 75°C; extraction was repeated three times. After alkali extrac-

tion, cells were washed with 1 ml 100 mM Tris-HCl (pH 7.5) and 1 ml 10 mM Tris-HCl (pH 7.5), subsequently resuspended in 1 ml 10 mM Tris-HCl (pH 7.5) containing 5 mg Zymolyase 20T, and incubated 16 h at 37°C. The insoluble pellet was removed by centrifugation (13,000 × *g*, 15 min), and the supernatant was dialyzed for 16 h against distilled water. The fraction before dialysis included both low-molecular-weight (MW) β-1,3-glucan, constituting approximately 65% of the cell wall β-1,3-glucan, and high-MW β-1,6-glucan, constituting about 60% of the cell wall β-1,6-glucan (26). After dialysis, only alkali-insoluble β-1,6-glucan was retained. An estimation of the carbohydrate content in each fraction was performed by the phenol-sulfuric acid method described in reference 27. Each experiment was repeated at least five times.

Survival of the toxin-treated cells. Wild-type (wt) *S. cerevisiae* BY4741 and isogenic mutant strains were grown in liquid YPD medium at 30°C to an optical density at 600 nm (OD_{600}) of about 1. Each yeast overexpression strain was cultivated for different time intervals under inducing conditions in liquid SC-Gal medium at 30°C, until the OD_{600} was ~1. Then 3×10^6 cells were washed with SC medium (pH 4.0), mixed with a sample of K2 killer toxin (50 U), and incubated for 2 h at 20°C with gentle agitation. Viability was determined by plating cells on YPD agar plates followed by colony counting, and results are expressed as the percentage of control cells, incubated in the absence of toxin. Data are presented as averages and standard deviations of the results determined with 5 biological replicates. Pairwise comparison with wt strain BY4741 was conducted with the Student *t* test to calculate a *P* value, and the results indicate significance of differences.

Transmission electron microscopy. Yeast cells were grown in YPD medium at 30°C for 16 h and washed with SC medium. The collected cells (5×10^5) were treated with K2 toxin (10^3 U) for 2 h at 20°C and were carefully pelleted by centrifugation at 1,200 × *g* for 5 min. The cells were subjected to fixation with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, at room temperature for 1 h. After the cells had been washed with the same buffer without glutaraldehyde for 45 min at room temperature, they were treated with four drops of 2% osmium tetroxide in s-collidine buffer, pH 7.4, and incubated at room temperature for 1 h. The samples were serially dehydrated with ethanol (50%, 70%, and 96%) at room temperature, each step lasting about 10 min. In addition, the samples were washed twice with isopropyl alcohol for 20 min each, followed by treatment with propylene oxide for 20 min at room temperature. The samples were embedded in Spurr resin by treatment with propylene oxide at different ratios (1:2, 1:1, and 2:1, as well as resin without propylene oxide). Finally, the samples were hardened (for 24 h at 70°C), sectioned with an ultramicrotome (EM UC6; Leica, Germany) using a diamond knife, and placed on a 200-mesh copper grid. Samples were observed in an FEI Morgagni 268(D) transmission electron microscope operating at 80 kV.

RESULTS

Dynamics of K2 toxin binding to yeast cells. To investigate the kinetics of K2 binding to sensitive yeast cells, different amounts (0.1 to 25 ng) of K2 protein were incubated with an increasing number of yeast cells (1×10^6 to 1×10^8). The K2 toxin was preincubated with the wt (BY4741) strain of *S. cerevisiae* for different intervals of time, and its residual activity was estimated by ability to form the inhibition zones. It was found that, when the amount of cells was 1×10^6 to 3×10^6, changes of killing activity were detected using only 0.1 ng of toxin. In contrast, when the amount of cells was increased to 10^8, the assay required more (5 ng) K2. We found that about half of the maximum binding level is achieved already after 1 min of incubation, whereas the plateau of binding is reached after about 10 min (Fig. 1A). Based on the results for residual toxin activity, the apparent number of K2 molecules bound to a single yeast cell under saturation conditions was calculated. We found that using 1×10^6 to 3×10^6 cells/ml (Fig. 1A), this amount was about 460 molecules. When a higher cell concentration (1×10^8/ml) and more toxin (5 ng) were used for the assay, a longer time (about 30 min) was required to reach saturation, but a similar number of K2 molecules (about 435) was bound per cell (Fig. 1B).

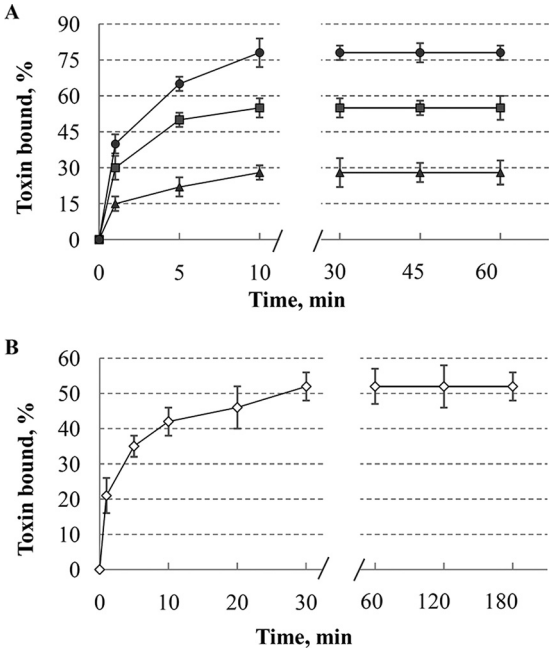
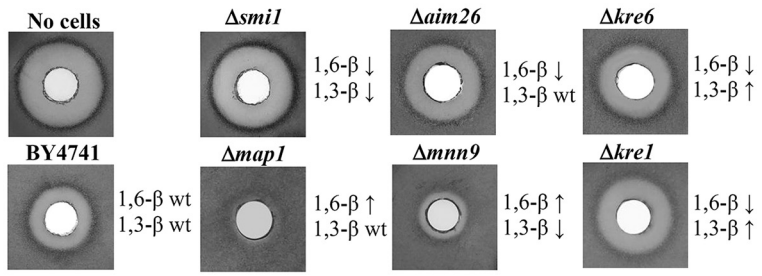


FIG 1 Dynamics of K2 toxin binding to yeast BY4741 cells. Different amounts of cells (1×10^6 [▲], 2×10^6 [■], 3×10^6 [●], and 1×10^8 [◇]) were incubated at 4°C for various periods of time in the presence of 0.1 ng (A) or 5 ng (B) of K2. Yeast cells were pelleted by centrifugation, and the remaining toxin activity was determined by the well assay. Toxin binding was estimated as the percentage of toxin activity obtained by subtracting the remaining activity from the total activity. Average values from five independent experiments are provided.

To compare the binding efficiencies of K1 and K2, we also performed the K1 binding assay under conditions similar to those used for K2. We incubated 2×10^6 of BY4741 cells with 10^3 U (0.1 ng) of concentrated K1. We observed that saturation of cell by the K1 toxin was achieved in 10 min, when about 35 pg of the toxin was bound, and the apparent number of K1 molecules bound to a single cell was calculated as being about 555 (see Fig. S1A in the supplemental material).

Binding of K2 toxin to yeast mutants with altered levels of β-glucans. To correlate K2 toxin binding with the composition of the cell wall and to calculate the amount of toxin bound to β-glucans present at different levels in various yeast mutants, 6 strains with altered ratios of β-1,3-/β-1,6 linkages were used. These mutants were shown to display altered resistance to K2 toxin (19). The primary information on glucan content was based on that reported in reference 9. In the present work, alkali-insoluble cell wall β-1,3- and β-1,6-glucans were isolated from each yeast strain tested, and the actual amount of glucans was estimated (Fig. 2). To this end, 2×10^6 cells/ml of each mutant (Δ*smi1*, Δ*aim26*, Δ*kre6*, and Δ*kre1* mutants) were incubated with 0.1 ng of K2 toxin under saturation conditions. In addition, the Δ*map1* and Δ*mnn9* mutants, which bind toxin more extensively, were incubated with 1 ng of K2. The residual toxin activity was measured by performing the well assay (Fig. 2A; see also Materials and Methods). The values obtained were used to calculate the apparent number of toxin

A



B

Genotype	Alkali-insoluble glucans, (μg mg^{-1} of cell mass)		K2 toxin molecules bound per cell	
	β-1,6-glucan	β-1,3-glucan	0.1-1 ng toxin used	5-25 ng toxin used
BY4741	38.2±1.2	142.3±2.0	460±25	435±22
Δ*smi1*	14.2±2.2	71.1±2.1	107±11	117±16
Δ*aim26*	16.9±3.2	133.6±2.1	171 ±8	184±19
Δ*kre6*	20.5±1.3	157.2±3.7	214 ±18	217±26
Δ*kre1*	21.8±3.0	156.5±2.9	201±33	208±29
Δ*map1*	63.7±1.3	137.6±3.0	1371 ±38	1254±35
Δ*mnn9*	62.4±1.4	84.5±2.4	1285 ±35	1045±31

FIG 2 Binding of K2 toxin to yeast mutants with altered levels of β-glucans. (A) Cells of different yeast mutants (2×10^6 each) were incubated with 0.1 to 1 ng of K2, and the remaining toxin activity was measured by the well assay as described in Materials and Methods. After incubation with the indicated yeast strain, the unbound K2 toxin is able to kill sensitive tester strain α'1, seeded in the MBA plates. The size of the lysis zones formed was converted to the relative toxin activity and subtracted from the total activity to calculate the binding efficiency. The level of the β-1,3- and β-1,6-glucans (increased [↑], decreased [↓], or wt) is indicated for each mutant. (B) The level of alkali-insoluble β-1,6- and β-1,3-glucans in each mutant was estimated by the phenol-sulfuric acid method. The apparent number of K2 molecules bound to a single mutant cell using a small (0.1 to 1 ng) or large (5 to 25 ng) amount of toxin was calculated as described in Materials and Methods. Numbers for both measurements are averages from five independent experiments.

molecules bound to a single yeast cell (Fig. 2B). When the levels of β-1,3- and β-1,6-glucans were decreased by, respectively, 50% and 63%, compared to the wt, as in the Δ*smi1* mutant, only 107 molecules (compared to 460 molecules binding to the wt cells) were attached (Fig. 2). In comparison, when the amount of β-1,6-glucan was decreased by 55% without a change in the β-1,3-glucan level, as in the Δ*aim26* mutant, 171 molecules of K2 protein were bound. When the amount of β-1,6-glucan was decreased by 43 to 47% with a concomitant increase of the β-1,3-glucan up to 10%, as in the Δ*kre6* and Δ*kre1* mutants, the number of K2 molecules bound to a single yeast cell reached 201 to 214. Taken together, these observations demonstrate that a decrease of β-1,6-glucan concentration in the yeast cell wall correlates with the low-level binding of K2, whereas the cellular amount of β-1,3-glucan is of minor importance under such conditions. In contrast, when the amount of β-1,6-glucan is increased by 63 to 67%, and the level of β-1,3-glucan is at the wt level or decreased by 40%, as in the respective Δ*map1* and Δ*mnn9* mutants, the amount of bound K2 toxin increased and reached about 1,285 to 1,371 molecules per yeast cell. These results confirm previous observations that β-1,6-glucan, but not β-1,3-glucan, has a major impact on efficiency of K2 binding to the yeast cell. Similar levels of binding for each mutant were achieved when elevated amounts of both cells (1×10^8) and toxin (5 to 25 ng) were used for the assays (Fig. 2B), thus confirming our statement regarding importance of cell wall-localized β-1,6-glucan in the action of K2 toxin.

To compare the binding efficiency of either K2 or K1 to yeast with different glucan content, we incubated 2×10^6 cells/ml of the different mutants (Δ*smi1*, Δ*aim26*, Δ*kre6*, Δ*kre1*, Δ*map1*, and Δ*mnn9* mutants) with about 0.1 ng of K1 toxin under saturation conditions. The residual toxin activity was measured by the well assay and used for the calculation of the K1 binding efficiency, expressed as a percentage (see Fig. S1B in the supplemental material), which was used for the estimation of the apparent number of K1 toxin molecules bound to a single yeast cell (see Materials and Methods). It was observed that in mutants with decreased level of β-1,6-glucan (Δ*smi1*, Δ*aim26*, Δ*kre6*, and Δ*kre1* mutants), the binding efficiency was 2- to 3.5-fold lower than that of the wt (BY4741); thus, the amounts of K1 bound to a single cell reached about 160, 190, 240, and 205 molecules, respectively (see Fig. S1B in the supplemental material). In contrast, when the level of β-1,6-glucan was elevated, as in the Δ*map1* and Δ*mnn9* mutants, binding of K1 increased about 2-fold, reaching about 1,100 or 1,300 molecules per cell. Taken together, the obtained results demonstrate that yeast binds K1 and K2 toxins in a similar manner, which depends on the cellular content of β-1,6-glucan.

Survival rate of yeast mutants with altered levels of β-glucans after treatment with K2 toxin. To investigate the survival of yeast upon the action of K2, we chose several mutants, which were shown previously to exhibit altered susceptibility to K2 (19) as well as changed β-glucan levels. We optimized the experimental conditions to a K2/yeast ratio that results in 50% of the wt (BY4741) cells surviving the toxin treatment after 2 h (Fig. 3). It was demonstrated that depending on the β-glucan level in different mutants, the survival of corresponding cells was altered. When the amount of β-1,6-glucan was decreased 1.8- to 2.7-fold, as in

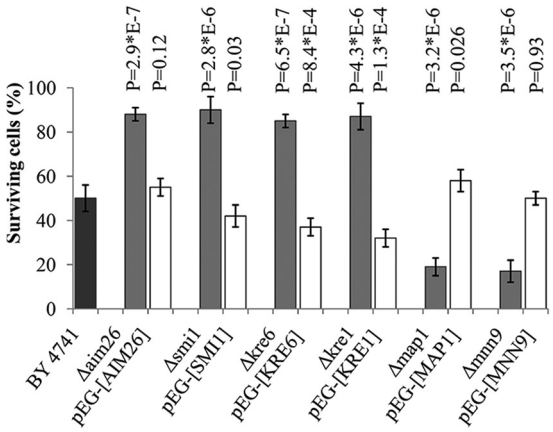


FIG 3 Survival of toxin K2-treated yeast cells with altered levels of β-glucans. K2 (50 U) was incubated with 3×10^6 cells of different mutants or corresponding overexpression strains for 2 h at 20°C. The cells were plated on YPD-agar. The percentage of surviving cells is expressed as the ratio between toxin-treated and untreated control cells of each strain. The data are averages ± standard deviations (SD) ($n = 5$). *P* values were acquired with Student's *t* test (two-tailed with equal variance) and indicate the significance of the differences in survival rate of the mutant versus the parental strain (BY4741).

the Δ*aim26*, Δ*kre1*, Δ*kre6*, and Δ*smi1* mutants, K2 binding was decreased 2- to 4-fold (Fig. 2), and survival increased to about 85% (Fig. 3). In contrast, when the level of β-1,6-glucan was increased 1.7-fold, as in the Δ*map1* and Δ*mnn9* mutants, K2 binding was increased about 3-fold (Fig. 2), and the cell survival dropped to less than 20% (Fig. 3). We observed the correlation of the increased level of the β-1,6-glucan with both higher binding of the K2 toxin (Fig. 2) and decreased survival of yeast cells (Fig. 3), and vice versa; however, it remains possible that in addition to the altered β-1,6-glucan level, the other cellular processes may impact the response to the action of the toxin. In order to confirm the importance of the tested gene products for survival of the yeast treated with K2 toxin, we performed similar survival assays using the corresponding overexpressed genes. These experiments showed that in contrast to the increased viability of the Δ*aim26*, Δ*kre1*, Δ*kre6*, and Δ*smi1* mutants, the overexpression of corresponding genes leads to the survival at the wt (for *AIM26*) or even lower levels (for *SMI1*, *KRE6*, and *KRE1*) (Fig. 3). At the same time, the sensitive Δ*map1* and Δ*mnn9* mutants became more resistant to the K2 toxin when the respective genes were overexpressed. Concurrently, the survival of these cells rose to the wt level (for *MNN9*) or even higher (for *MAP1*). Taken together, these findings confirm the involvement of the tested proteins in the response to the action of the K2.

Competitive inhibition of K2 killing activity by polysaccharides. Binding of K2 toxin to primary components of the yeast cell wall was tested via competitive inhibition of the killing process by certain glucans: laminarin (consisting of β-1,3 and β-1,6 linkages), pustulan (β-1,6 linkage), pullulan (α-1,4 and α-1,6 linkages), and chitin (β-1,4 linkage). The number of viable CFU after incubation of yeast with both K2 toxin and different polysaccharides was counted by depositing cells on YPD-agar plates and performing titration of diluted samples in parallel (Table 1). When the toxin-sensitive *S. cerevisiae* strain BY4741 (5×10^5 cells/ml) was incubated for either 1 h or 24 h in the absence of both the K2 toxin and the polysaccharide (control experiment), the number of viable cells was estimated to remain at about 4.2×10^5 to 4.3×10^5/ml. In the presence of K2 toxin without any polysaccharide added, the number of viable cells decreased 32- to 36-fold (to 0.12×10^5 to 0.13×10^5 cells/ml). Among the polysaccharides tested, the presence of pullulan, chitin, or laminarin increased the number of viable cells 4-fold after 1 h of incubation, whereas a 7- to 12-fold increase was observed after much longer (24 h) incubation (compared to the control with K2 toxin but without any polysaccharide) (Table 1). The effect of pustulan was most pronounced, increasing viability of yeast 28- to 30-fold independently of the incubation time. In the presence of this polysaccharide, the number of yeast cells surviving the effect of K2 toxin reached 3.4×10^5 to 4.0×10^5 cells/ml, close to the number of CFU obtained in the control experiment (without K2 toxin or any polysaccharide). The competitive inhibition of the action of the killer toxin *in vivo* demonstrated that the β-1,6-glucan present in pustulan provides binding sites for the K2 toxin. Other polysaccharides with different linkages (such as β-1,4- and β-1,3-glucans as well as α-1,6- and α-1,4-glucans) showed a considerably lower ability to augment the viability of yeast cells. The viability was somewhat pronounced after long (24 h) incubation, probably due to nonspecific binding of K2.

Pustulan inhibits K2 toxin *in vitro*. To confirm the hypothesis that pustulan binds the K2 toxin most efficiently of all the polysaccharides tested and so inhibits its action, K2 was preincubated for different periods of time with the respective compound, and the residual activity was tested by the well test assay. After 5 min of preincubation with pustulan, almost all the toxin was already bound and therefore unable to kill yeast cells (Fig. 4). At the same time, after preincubation with laminarin, pullulan, or chitin, even for several hours, the residual K2 toxin was able to kill the yeast. Some effect of the neutralization of K2 toxin activity was observed only after 24 h of incubation with these polysaccharides, leading to diminished sizes of the lysis zones. These observations are consistent with the findings obtained in the competition experiments, demonstrating some unspecific binding of K2 to different glucans but not to the pustulan.

Binding of K2 toxin to Δ*kre1* and Δ*tok1* yeast cells and respective spheroplasts. In order to confirm that a majority of K2 toxin binds to the β-1,6-glucan of the yeast cell wall, rather than the plasma membrane-localized receptors, we investigated the binding of K2 to the intact cells or respective spheroplasts. For this purpose, the wild-type yeast BY4741 and isogenic Δ*kre1* mutant,

TABLE 1 Competition of different polysaccharides with the cell surface receptors for the binding of K2 toxin[a]

Polysaccharide	No. of viable cells (10^5 CFU/ml)[b]	
	1 h	24 h
Pullulan	0.44 ± 0.04	1.50 ± 0.08
Pustulan	3.97 ± 0.16	3.40 ± 0.10
Chitin	0.52 ± 0.08	1.20 ± 0.06
Laminarin	0.58 ± 0.08	0.90 ± 0.02
None (controls)		
With toxin	0.13 ± 0.08	0.12 ± 0.01
Without toxin	4.16 ± 0.11	4.30 ± 0.11

[a] Yeast BY4741 cells (5×10^5) were incubated for either 1 h or 24 h with 9 mg of the respective polysaccharide in the presence of 10^3 U/ml of K2 toxin.
[b] Values are averages ± SEM from three independent experiments.

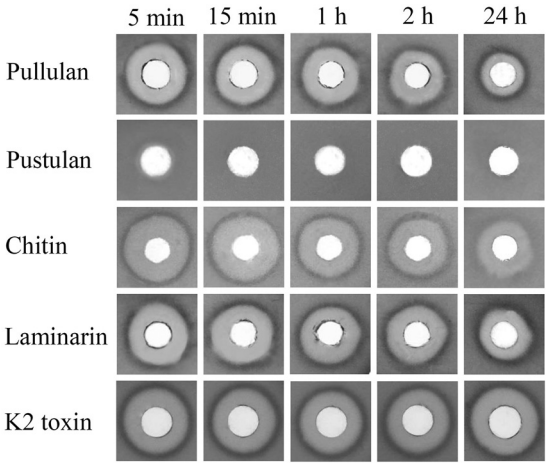


FIG 4 Binding of K2 toxin to different polysaccharides *in vitro*. K2 (10^3 U/ml) was preincubated with 9 mg of various polysaccharides for different periods of time, and the remaining activity was estimated by the well assay. The absence of a lysis zone reflects the strong binding of K2 toxin to pustulan. The diminished size of the lysis zones after prolonged incubation demonstrates the nonspecific binding of K2 to the respective polysaccharides.

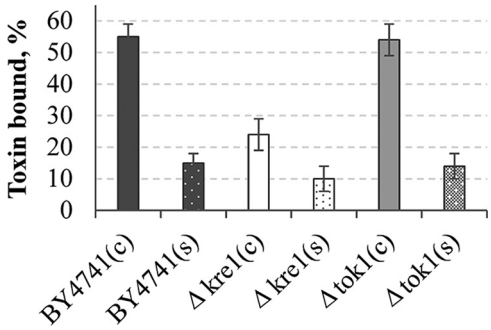


FIG 5 Binding of K2 toxin to the intact cells and spheroplasts. (A) K2 (10^3 U) was incubated with 2×10^6 of either wt (BY4741), Δ*kre1*, or Δ*tok1* cells (c) or spheroplasts (s) for 1 h at 4°C, and the remaining activity was estimated by the well assay. The percentage of the toxin bound was calculated by subtracting the relative activity of the unbound toxin from that obtained without preincubation with the respective cells. Average values from three independent experiments are shown.

which has about a 50% decreased level of β-1,6-glucan and lacks the Kre1p receptor, were used. When K2 was incubated with 2×10^6 of wt cells, around 55% of toxin was bound, whereas up to 15% of it was attached to the wt spheroplasts (Fig. 5). However, in the case of the Δ*kre1* mutant, about 24% of K2 was attached to the intact cells, whereas about 10% of toxin was bound to the spheroplasts. Taken together, the results of toxin binding to Δ*kre1* versus wt cells and respective spheroplasts demonstrate once again that the decreased cellular level of the β-1,6-glucan correlates with the reduced efficiency of K2 toxin binding to the intact cells. The reduction in toxin binding by about 40% when cell walls of BY4741 cells were removed indicated that the major part of toxin molecules bound to cell wall receptors. In comparison, only about 15% of toxin could be identified as being attached to Kre1p or other plasma membrane-localized receptors. The ability of Δ*kre1* spheroplasts to still bind a small amount (~10%) of K2 toxin could be due to incomplete removal of cell wall and/or the presence of another, not-yet-identified plasma membrane receptor(s).

Since Tok1p has been implicated as being the receptor for K1 toxin in addition to Kre1p (10, 11), we investigated the efficiency of K2 binding to the Δ*tok1* strain. The binding ability was very similar to that of strain BY4741: about 50% of toxin bound to Δ*tok1* cells, whereas about 13% bound to Δ*tok1* spheroplasts. The results suggest that Tok1p is not the plasma membrane receptor for K2. However, since K2 was still bound by Kre1p in the Δ*tok1* strain, additional experiments are required to establish the exact role of Tok1p in K2 binding.

Competitive inhibition of K2 killing activity by pustulan in Δ*tok1* and Δ*kre1* mutants. In the K2 and pustulan competitive inhibition experiments using the Δ*kre1* cells, the high viability of the tested mutant relative to that of wt yeast was demonstrated (Fig. 6). This high viability is likely because of the reduced level of β-1,6-glucan and/or the absence of the Kre1p receptor. However, when the amount of β-1,6-glucan is at the wt level in the Δ*tok1* mutant, pustulan provides the binding sites for K2, thus protecting the mutant from the action of the toxin. In the presence of K2 toxin, but without pustulan, the number of viable cells decreased in the case of the Δ*tok1* but not the Δ*kre1* mutant. A similar effect of the pustulan was observed also for the K1 toxin: in the presence of this polysaccharide, the viability of cells (BY4741 and Δ*tok1*) was increased upon the action of toxin compared to the control experiment, where the pustulan was absent (see Fig. S2 in the supplemental material). However, in the case of Δ*kre1* cells, a high level of resistance to K1 toxin, not dependent on the presence of pustulan, was observed. Our data demonstrate that for efficient action of either K1 or K2, both β-1,6-glucan and Kre1p are required.

Damage of yeast cells upon the action of the K2 toxin. To investigate the integrity of the yeast cells after the treatment with the K2 toxin, the representatives of groups of mutants with decreased (Δ*kre1*) and increased (Δ*mnn9*) amounts of β-1,6-glucan, leading to resistant and sensitive phenotypes, were used. The respective mutant cells were incubated with 10^3 U of K2 for 2 h, and cells were fixed with osmium tetroxide, sectioned, and analyzed using transmission electron microscopy (TEM). When the untreated cells were investigated (control experiment), the cells had a normal shape with clearly distinguishable smooth cell surfaces, nuclei, vacuoles, and mitochondria (Fig. 7A). In contrast, when the resistant Δ*kre1* mutant was incubated with K2 toxin, intact cells, similar to the untreated controls, were observed (Fig. 7B). The integrity of yeast did not change either in the mature or in the dividing cells. In contrast, when sensitive Δ*mnn9* cells were affected by K2 toxin, rough cell walls were observed, and no distinguishing nuclei, vacuoles, or other organelles were visible (Fig. 7B). The effect was even more pronounced in the dividing cells, with clear ruptures and visible fragments of the cell wall. The data obtained by TEM analysis are consistent with those from the killing experiments, demonstrating that the cells with increased levels of β-1,6-glucan are more susceptible to K2 toxin, and vice versa.

DISCUSSION

In this study, we investigated the dynamics of K2 binding to yeast cells, estimated the apparent number of toxin molecules bound to a single cell, and evaluated the dependence of K2 binding and killing activities on the presence of different glucans *in vitro* and *in vivo*. One of the objectives of our experiments was to compare

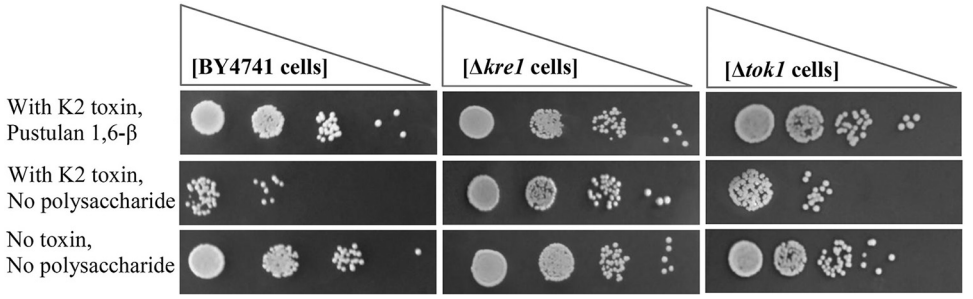


FIG 6 Competition of pustulan with the cell wall receptors for binding of K2 toxin in BY4741, Δ*kre1* or Δ*tok1* cells. Yeast cells (5×10^5) were incubated for 1 h with 9 mg of pustulan in the presence of 10^3 U/ml of K2 toxin. Surviving cells were serially diluted and spotted onto YPD-agar to demonstrate the effect of pustulan on K2 inactivation.

such parameters with the ones obtained with similar toxins, especially K1.

In the present work, we observed relatively fast binding of the K2 toxin to yeast cells, with a half-time of about 1 min, whereas binding saturation was reached in about 10 min. Under the similar conditions, K1 demonstrated comparable dynamics of binding. In our hands, the binding was somewhat slower than previously shown for K1 (28), when the half-time of adsorption was less than 1 min, and the saturation was completed within 5 min. These variations could be attributed to the different test strains and approaches used. In addition, we have observed that the higher (10^8/ml) cell concentration increases the time needed to reach satura-

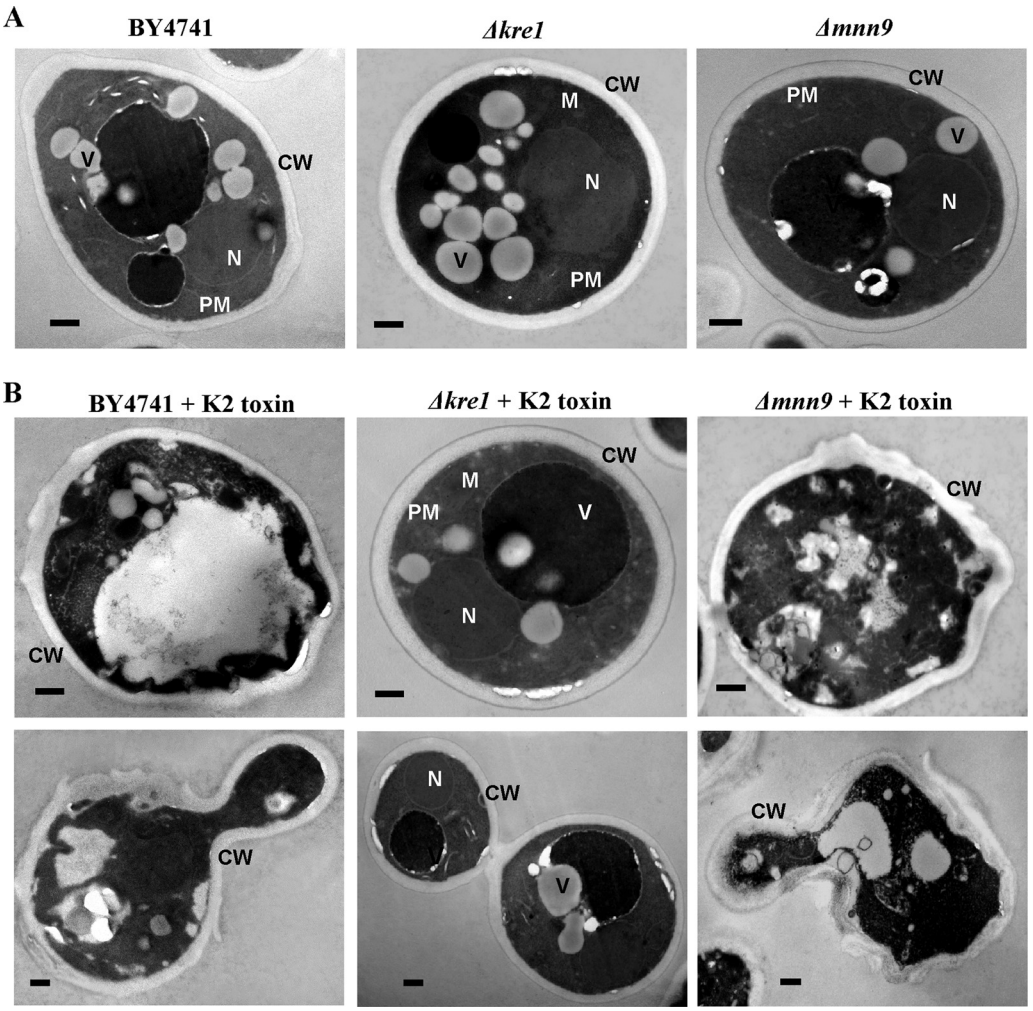


FIG 7 Transmission electron microscopy (TEM) images of BY4741, Δ*kre1*, and Δ*mnn9* cells before (A) and after (B) treatment with the K2 toxin. Representative cells of each type are shown. Abbreviations: CW, cell wall; M, mitochondria; N, nucleus; PM, plasma membrane; V, vacuole. Bars, 500 nm.

tion of the binding by K2. A possible reason for this could be the reduced accessibility of toxin to the entire surface of each yeast cell.

Our previous study (19) revealed that in many cases, the increase in the level of β-1,6- but not of β-1,3-glucan could be related to increased binding of K2 toxin and sensitivity of yeast to it, and vice versa. However, in that work we did not perform quantitative studies regarding K2 binding. This time, we calculated the apparent number of both K1 and K2 toxin molecules bound to wt or different mutant cells under the saturation conditions. We found that about 460 molecules of K2 and about 555 of K1 bound to a single wt yeast cell. During this study, we determined that mutants with decreased level of β-1,6-glucan bind 2- to 3-fold fewer K2 toxin molecules than the parental strain with the regular glucan content. On the other hand, an increased level of β-1,6-glucan augmented the binding efficiency about 3-fold. The competition experiments involving various polysaccharides demonstrated that only pustulan (poly-β-1,6-glucan) was efficiently bound by K2 toxin *in vitro*. A similar dependence of toxin binding on β-1,6-glucan level and inhibition of killing activity by pustulan was observed by us in the case of K1. The additional argument supporting the importance of cell wall receptors for the binding of K2 comes from the experiments using yeast spheroplasts, which bind less toxin than intact cells. In summary, our results confirm the major role of β-1,6-glucan as a primary receptor for both K2 and K1 toxins.

It was previously estimated that the total number of β-1,6-glucan molecules in a haploid parent cell of *S. cerevisiae* at exponential growth phase is 6.6×10^6 (29). At the same time, it was also demonstrated that cell wall structure and physical properties can vary depending on temperature, pH, as well as growth conditions and phase (29). At the end of late-exponential stage and by the entering stationary phase, cell wall becomes thicker, more resistant to various agents, and less permeable to macromolecules (30). Since our experiments were performed using the late-exponential-phase grown yeast, the accessibility of toxins to the receptors could be diminished, thus affecting the binding efficiency. Alternatively, the discrepancy between the estimated number of β-1,6-glucan molecules per cell and the actual number of bound K2 toxin molecules might indicate that in live cells the accessibility of β-1,6-glucan to the toxin is limited, possibly, mainly to areas of active growth (31). To measure the toxin binding, we used the killing assay, which is rather indirect. In addition, binding and killing processes may have different levels of dependence on toxin concentration. Altogether, the above-mentioned factors may influence the estimation of the number of yeast-bound toxin molecules, which is different from the amount of β-1,6-glucan receptors available. Nonetheless, we believe that our method is useful for comparison of binding efficiencies between different toxins, in this particular case (i.e., between K1 and K2), as well as for the evaluation of relationship between the binding efficiency and glucan content.

In this study, we showed that increased binding of the K2 toxin to cells with elevated amounts of β-1,6-glucan leads to decreased survival of yeast, thus demonstrating that the binding level affects the killing property. Similar observations were made previously for several *kre* mutants with increased resistance to K2 toxin and therefore decreased survival (20), but this phenomenon was not correlated with changes in β-1,6-glucan level. Still, we cannot exclude the possibility that in addition to the altered level of this glucan in the tested mutants, the survival of cells may be modulated by other cellular processes due to changed cellular levels of the tested proteins.

To directly investigate the effect of K2 on yeast morphology, we observed mutant cells with either increased or decreased amounts of β-1,6-glucan by TEM. Changes in the structures of cells subjected to K2 were concomitant with an increased level of β-1,6-glucan, whereas treated cells remained intact when this level was lowered.

In summary, all the data obtained in the course of this study demonstrate the importance of β-1,6-glucan for the action of the K2 toxin. The *in vitro* and *in vivo* approaches, used here to measure the K2 binding and the survival of the target cell, strongly suggest that this type of glucan is a primary cell surface receptor. Although this was postulated previously (11, 15, 24), it had not been confirmed experimentally. Presently, it is not clear whether the β-1,6-glucan facilitates the passage of the K2 toxin across the cell wall to reach the plasma membrane or additional factors also contribute to this process. Recently, we found numerous specific modulators conferring cell resistance to K2 toxin (19). Some of these modulators could be involved in the process of the K2 entry into the target cell and are under investigation.

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