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A novel yeast gene, *RHK1*, is involved in the synthesis of the cell wall receptor for the HM-1 killer toxin that inhibits β-1,3-glucan synthesis

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Abstract The HM-1 killer toxin from *Hansenula mrakii* is known to inhibit cell wall β-1,3-glucan synthase of *Saccharomyces cerevisiae* and other sensitive strains of yeast. A number of mutants of *Saccharomyces cerevisiae* that show resistance to this toxin were isolated in order to clarify the killing mechanism of the toxin. These mutants, designated *rhk* (resistant to *Hansenula* killer), were classified into three complementation groups. A novel gene *RHK1*, which complements the killer-resistant phenotype of the largest complementation group *rhk1*, was isolated. DNA sequence analysis revealed an open reading frame that encodes a hydrophobic protein composed of 458 amino acids. Gene disruption followed by tetrad analysis showed that *RHK1* is not essential and loss of *RHK1* function endowed *S. cerevisiae* cells with complete killer resistance. A biochemical analysis suggested that *RHK1* does not participate directly in the synthesis of β-1,3-glucan but is involved in the synthesis of the receptor for the HM-1 killer toxin.

Key words β-1,3-Glucan · Cell wall · *Hansenula mrakii* · Killer toxin · *Saccharomyces cerevisiae*

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Introduction

Yeast cells of many genera secrete protein toxins that can be lethal to other species of yeast. Among these toxins, the best understood are the *Saccharomyces cerevisiae* K1 and *Kluyveromyces lactis* toxins. The killing mechanisms of these toxins have been extensively studied by Bussey (1991) and Stark et al. (1990). The action of the killer toxin of K1 starts with the adsorption of the toxin to β-1,6-glucan in the cell wall of a susceptible yeast strain. Although the precise structure of the toxin receptor remains unknown, the killer toxin alters the cell membrane, which becomes permeable to protons and metabolites. On the other hand, the toxin produced by *K. lactis* has been shown to act by arresting sensitive cells at the G1 stage of the cell cycle, which leads to loss of viability. The interaction of the killer toxin with cell wall chitin is the first step in toxin action, and then the toxin is internalized into the cell. Finally, the γ subunit of the toxin causes G1 arrest. Understanding the mechanisms of the killing action of these toxins addresses many intrinsically interesting cell biological questions, especially that of the mechanism that ensures the orderly assembly of cell wall components. Selection for mutants resistant to these toxins and analysis of these mutants has defined a series of genes whose products are required for the assembly of cell wall components such as glucan, chitin and mannan, and which act as the receptors of killer toxins. Bussey's group has described several *KRE* genes, the products of which are involved in synthesis of β-1,6-glucan which itself is thought to be the receptor to which K1 killer toxin first binds (Boone et al. 1990; Maeden et al. 1990; Rommer and Bussey 1991; Hill et al. 1992; Brown and Bussey 1993; Brown et al. 1993). Analysis of the resistance of mutants to *K. lactis* toxin also lead to the isolation of several genes responsible for the sensitivity to this toxin. Among these, at least two genes are involved in the synthesis of the cell wall receptor; one of them appears to be the chitin synthase gene (Takita and

Castilho-valanicius 1993) and the other is essential for the regeneration of cell wall materials (Kawamoto et al. 1992), though its precise function is unclear. These reports show that identification and characterization of toxin-resistant mutants can provide insights into the synthesis of the cell wall receptor for the specific toxin. Recent advances have focused considerable attention on the process of assembley of cell wall components during proliferation of yeast. However, not all the genes involved have been isolated since many proteins are required to carry out this process. Studies of the synthesis of cell wall glucan, mannan and chitin may facilitate the development of specific antifungal agents as well as the development of new polymers.

Killer strains of the yeast genus *Hansenula* secrete a protein toxin that kills *S. cerevisiae* and other sensitive species of yeasts (Nomoto et al. 1984). We have previously reported the nucleotide sequences of genes encoding two of these toxins (Kimura et al. 1993) and showed that some toxins from different strains of *Hansenula* have similar structures (Kimura et al. 1995a). Among these toxins, HM-1 secreted from *H. mrakii* IFO 0895 has been well characterized. It consists of 88 amino acids, 10 of which are cysteine and, as a unique feature, is stable over a wide range of pH (pH 2 to 11) and at high temperature (100° C for 10 min), but is inactivated by reducing reagents, demonstrating that S—S bonds are essential for biological activity (Yamamoto et al. 1986a). It has also been demonstrated that the toxin inhibits the synthesis of β-1,3-glucan in vivo and in vitro, whereas the synthesis of other cell components, such as chitin, mannan and alkali-soluble glucan, is unaffected (Yamamoto et al. 1986b). Recently, purified β-1,3-glucan synthase was shown to be inhibited by HM-1 (Takasuka et al. 1995). These results imply that the toxin kills sensitive cells by interfering with synthesis of β-1,3-glucan. Two genes of *S. cerevisiae* that are confer sensitivity to HM-1 toxin have been cloned and both genes were found to be involved in the synthesis of β-1,3-glucan. One is *KNR4*, which complements one of the killer-resistant mutations that confer hypersensitivity to cercosporamide, an inhibitor of β-1,3-glucan synthesis (Hong et al. 1994). Disruption of this gene resulted in the complete killer-resistant phenotype and also resulted in a reduced level of β-1,3-glucan synthase activity and a low content of β-1,3-glucan. The other gene is *HKR1*, overexpression of which gives rise to a HM-1 resistant-phenotype (Kasahara et al. 1994a). This gene encodes a high-molecular-weight protein that has the profile of type I membrane proteins, which are rich in serine and threonine, and contains an EF-hand motif characteristic of calcium-binding proteins. Overexpression of *HKR1* increased the β-glucan content, though the β-glucan synthesis activity was unaffected. Recently, this protein was reported to regulate β-glucan synthesis and budding pattern (Yabe et al. 1996). Although HM-1 appeared to inhibit glucan synthase, the precise structure of the primary receptor for the toxin in the cell wall remains unclear. Analysis of HM-1 resistant mutants can provide information about the cell wall receptor for the toxin and contribute to our understanding of the biosynthesis and functional interaction of cell wall components.

In this study, to gain further insight into the mechanism of HM-1 killer toxin and the mechanism of synthesis of the fungal cell wall, we screened HM-1 resistant mutants of *S. cerevisiae* and characterized a gene which complements the HM-1 resistance phenotype.

Materials and methods

Strains and media

The *S. cerevisiae* strains used in this study are described in Table 1. The killer strain of *H. mrakii* IFO 0895 was obtained from the Institute of Fermentation, Osaka. General methods for the genetic manipulation of yeast cells were carried out as described by Rose et al. (1990). Yeast cultures were routinely grown in YPD medium (1% Bacto yeast extract, 2% Bacto Peptone, 2% glucose). Plasmid-containing cells were grown in synthetic medium (0.67% Difco yeast nitrogen base without amino acids, 2% glucose) containing the appropriate nutrients for maintaining plasmids. Yeast transformation was carried out by lithium acetate method (Ito et al. 1983).

DNA and RNA manipulations

General recombinant DNA procedures were carried out as described by Sambrook et al. (1989). Subcloning of *RHK1* for DNA sequence analysis was made using the Bluescript KS vector. *Escherichia coli* strain MV1184, with helper phage M13K07, was used for transformation of plasmid-containing subclones and production of single-stranded DNA. Sequencing was done by the

Table 1 Yeast strains used in this study

Strain	Description	Source
Bj1824	a, *ura3*, *leu2*, *trp1*, pep4	E.W. Jones
SEY6210	α, *ura3*, *leu2*, *his3 trp1*, *lys2*, *suc2*	S.D. Emr
RAY3A-D	a/α, *ura3/ura3*, *leu2/leu2*, *his3/his3*, *trp1/trp1*	This study
rhk1	a *rhk1-1*, *ura3*, *leu2*, *trp1*, *pep4*	This study
kre1	*kre1Δ:::HIS3* in SEY6210	H. Bussey
kre6	*kre6Δ:::HIS3* in SEY6210	H. Bussey
TK101a	a, *rhk1Δ:::URA3*, isogenic to RAY3A-D	This study
TK101b	α, isogenic to RAY3A-D	This study
TK101c	a, isogenic to RAY3A-D	This study
TK101d	α, *rhk1Δ:::URA3*, isogenic to RAY3A-D	This study
TK201	*rhk1Δ:::URA3* in BJ1824	This study
TK301	*rhk1Δ:::URA3* in SEY6210	This study

dideoxy method of Sanger et al. (1977), with a BcaBest sequencing kit (Takara) using [α-^{32}P]dCTP as a substrate.

Preparation of HM-1 killer toxin and HM-1 killer plates

H. mrakii IFO 0895 was grown in synthetic medium at 30° C for 24 h. The culture supernatant containing HM-1 was collected by centrifugation and concentrated by evaporation at 40° C. The concentrated medium was extensively dialyzed against distilled water using Spectra-pore 1000 dialysis tubing. Finally, the toxin was concentrated to 1/200th of the original volume by lyophilization. The concentrated toxin was sterilized by passing it through a 0.2-μm pore size membrane and the final preparation of HM-1 was kept at −80° C until use. This toxin solution was added to YPD or synthetic medium at a ratio of 1 ml/200 ml after the medium was autoclaved.

Measurement of β-1,3-glucan synthase activity

Yeast cells were cultured in YPD medium up to the logarithmic phase and collected by centrifugation. The resultant pellets were disrupted with a Bead-Beater. Preparation of the membrane fraction and the activity assay for β-1,3-glucan synthase were carried out according to the method of Kang and Cabib (1986), except that amylase and bovine serum albumin were omitted from the reaction mixture. The reaction was started by adding UDP [^{14}C]-glucose and incorporation of the radioactivity into the trichloroacetic acid-insoluble fraction was measured with a scintillation counter.

Scanning electron microscopy

Morphological changes in the yeast after HM-1 toxin treatment were observed with a scanning electron microscope. Log-phase cells were incubated with the HM-1 toxin in YPD medium at 30° C for 30 min. The treated cells were dropped onto a glass slide previously treated with 0.2% poly-L-lysine and fixed with 1% paraformaldehyde 92% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h. The specimens were dehydrated in a graded series of ethanols, critical-point dried in CO_2, and spatter-coated with gold. Then, the specimens were observed with a scanning electron microscope (JSM-820, JEOL) operating at an accelerating voltage at 15 kV.

Results

Isolation and characterization of mutants resistant to HM-1

To search for genes that are required for killer sensitivity, we selected for toxin-resistant mutants. Several yeast strains were first tested for HM-1 sensitivity in order to choose a strain for mutagenesis. BJ1824 was selected because it was the most sensitive to HM-1. BJ1824 was treated with ethyl methanesulfonate, or UV irradiation, or untreated, and then inoculated onto plates containing HM-1. Twenty spontaneous by arising surviving colonies, were recovered and 36 surviving colonies were obtained from ethyl methansulfonate-treated cells and 44 surviving colonies from UV-treated cells. We designated these mutant strains as *rhk* (for resistant to *Hansenula* killer). They were tested for complementation among themselves and fell into three complementation groups: *rhk1*, *rhk2* and *rhk3*, with 82, 14 and 4 members, respectively. All of these groups were recessive for HM-1 resistance. We characterized these mutants for cell wall-defects, such as glucanase sensitivity, detergent resistance, regeneration efficiency or osmo-sensitivity. However, no abnormalities other than HM-1 resistance were observed. Therefore, we decided to analyze the largest complementation group, *rhk1*, especially since all of the spontaneous mutants fell into this group.

Isolation of the *RHK1* gene

The wild-type *RHK1* gene locus was isolated by complementation of the *rhk1-1* allele. The *rhk1-1* strain was transformed with a yeast genomic DNA library prepared using the centromeric vector YCp50, which carries the *URA3* gene as a selectable marker. Approximately 20 000 uracil prototrophs were screened for the HM-sensitive phenotype. Eight transformants were found to have restored killer sensitivity and the toxin sensitivity was shown to be due to transformation with the plasmids. The resistant mutants harboring these plasmids turned pale blue on killer media containing methylene blue. These plasmids contained yeast DNA inserts of 13.4 kb. All of the inserts had the same restriction maps. A 3.9 kb *Sal*I-*Hind*III fragment of the insert DNA was found to complement the killer-resistant phenotype of the *rhk1-1* mutation. Smaller subclones of this fragment were cloned into YCp50 and tested for their ability to do the same. A detailed restriction map of the insert DNA and the results of assays for the complementing activity of each subclone are shown in Fig. 1. The smallest subclone capable of complementing *rhk1-1* was the *Kpn*I-*Sca*I fragment of 2.0 kb, indicating that this fragment includes the gene encoding killer sensitivity. The nucleotide sequence of the 2.3-kb *Kpn*I-*Hind*III fragment was determined.

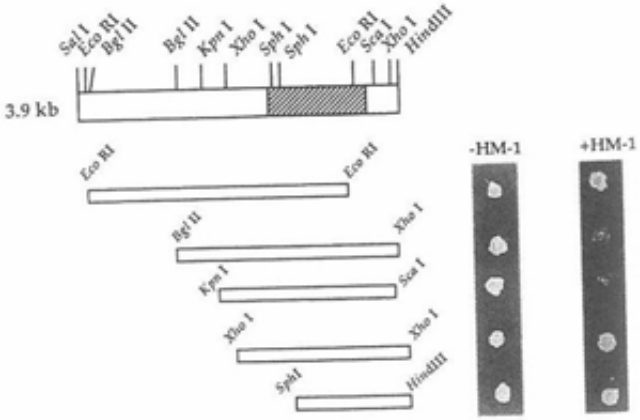

Fig. 1 Restriction map and subcloning of *RHK1*. The ORF of *RHK1* deduced from the nucleotide sequence is shown as a *hatched box*. The complementing activity of various fragments is shown on the right. *rhk1-1* cells were transformed with each of the subclones and assayed for growth on complete medium with and without HM-1 toxin

Nucleotide sequence of the *RHK1* gene

The nucleotide sequence of the 2.3-kb fragment revealed a large uninterrupted ORF encoding a polypeptide composed of 458 amino acids. A DNA homology search using the BLAST program on the NCBI mail server showed a striking match to a yeast genome DNA sequence deposited in Genbank (accession M89908), which maps to the left arm of chromosome II between the *CDC27* and *ILS1* (Cusick 1994). Our sequence showed some disagreement with the previous sequence in the N-terminal region of the ORF. Recently, the yeast genomic DNA sequence of chromosome II was reported (Obermair et al. 1995) and *RHK1* was found to be identical to the mannosyl transferase gene *ALG3*.

Though the ORF contains several in-frame start codons, the nucleotide sequence around the first initiation codon showed good agreement with the Kozak canonical sequence (A/CNNATGPu) (Kozak 1981). We concluded *RHK1* encodes a polypeptide of 458 amino acid residues with a predicted molecular mass of 52959. Neither a TATA box sequence nor a characteristic yeast promoter were noted in the region immediately upstream of the ORF, the promoter of this gene should reside in the 600 bp of the 5′ flanking region found in the *Kpn*I-*Sca*I fragment, since this was the smallest one which could complement the resistance phenotype. The region downstream of the ORF lacks a classical polyadenylation signal (AATAAA) and transcription termination signal but has analogous AT-rich sequences downstream of the stop codon.

RHK1 protein sequence analysis

The sequence encoded by the ORF is extraordinary in that 57% of the amino acid residues are hydrophobic. Leucine accounts for 16% of the residues. A hydrophobicity analysis predicted that the amino acid sequence of the protein specified by *RHK1* (Rhk1p) has very hydrophobic profile with 10 membrane-spanning domains (Fig. 2). These characteristics suggest that Rhk1p is an integral membrane protein, although no obvious signal sequence for secretion was detected in the N-terminal region. No potential N-linked glycosylation sites were identified.

Comparison of the amino acid sequence of Rhk1p with the FASTA program to the PIR and SwissPlot databases, and with the BLAST program showed significant similarity with the product of the *Drosophila* gene *neighbor of l(2) tid* (*l(2)not*), Not56, which is expressed in the embryo (Fig. 3). The products of the two genes (Rhk1p and Not56) exhibit 36% identity over all. Also, the amino acid sequences specified by several human cDNAs and one *Arabidopsis thaliana* cDNA characterized in the EST cDNA sequence project showed strong similarity to the amino acid sequence of Rhk1p (Fig. 3).

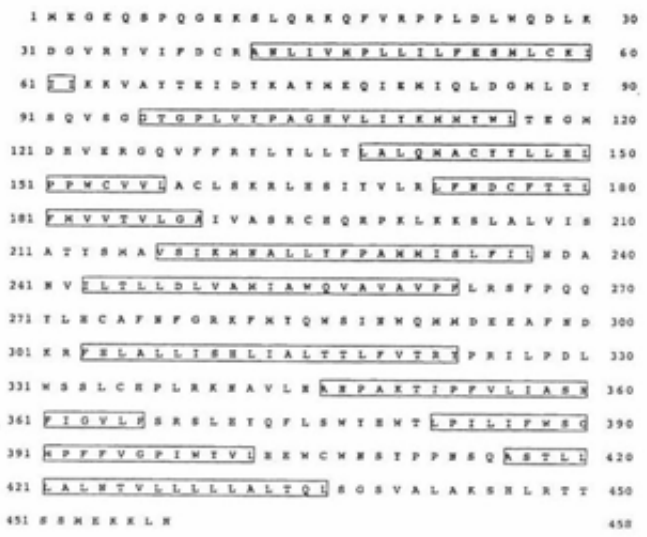

Fig. 2 Predicted amino acid sequence of the *RHK1* gene. Ten putative membrane-spanning regions are *boxed*. The nucleotide sequence was deposited in Genbank under the accession number X79489. The nucleotide sequence of the ORF is identical to YBL0720, which is located on the left arm of chromosome II between the nucleotide coordinates 63784 and 65160 (Obermaier et al. 1995)

RHK1 gene expression

To characterize the expression of *RHK1* mRNA, total RNA from the wild-type strain was isolated at mid log and stationary phases and subjected to Northern analysis using the *RHK1* ORF as a probe. The result demonstrated that the *RHK1* mRNA is expressed more strongly in the log phase than in the stationary phase (Fig. 4). Furthermore, the level of expression of this gene was relatively low, since a large amount of total RNA (about 150 μg) was necessary to allow detection of this mRNA. The codon adaptation index of *RHK1* is 0.11. The low expression levels of *RHK1* in the Northern blot reflects this value.

Disruption of *RHK1* and killer resistance of the disruptant

In order to determine whether the *RHK1* is an essential gene, an insertion mutation was generated at the *Sph*I site of *RHK1* by using *URA3* as a selectable marker (Fig. 5A). A homozygous diploid Ura$^-$ strain, RAY3A-D, was transformed with *rhk1*Δ::*URA3*, and selected for Ura$^+$ prototrophy. Integration and disruption at the *RHK1* locus was confirmed by a Southern blot analysis (data not shown). Ura$^+$ diploids were sporulated and dissected. Analysis of 12 tetrads revealed that all four spore clones grew on YPD medium. In each case, uracil prototrophy segregated 2:2, and all Ura$^+$ spore clones displayed resistance to HM-1. Therefore, inactivation of *RHK1* leads to HM-1 resistance and *RHK1* is not essential for cell viability.

Fig. 3 Comparison of *Drosophila*, human and plant homologues of *RHK1*. Sequence identies between the amino acid sequence of Rhk1p and the deduced polypeptide encoded by the *Drosophila* gene *l(2)not*, Not 56 (accession no. X77820), human ESTs (accession nos. R86657 for human 1, and R23327, R76440 and H26854 for human 2; the nucleotide sequences of these three clones overlap) and a plant (*Arabidopsis thaliana*) EST (accession no. T43898). Amino acid identities present in all of the sequences are highlighted. In several cases, frameshifting allows alignments with significant sequence conservations, possibly reflecting sequence errors in the ESTs. Therefore, only the conserved sequences are shown

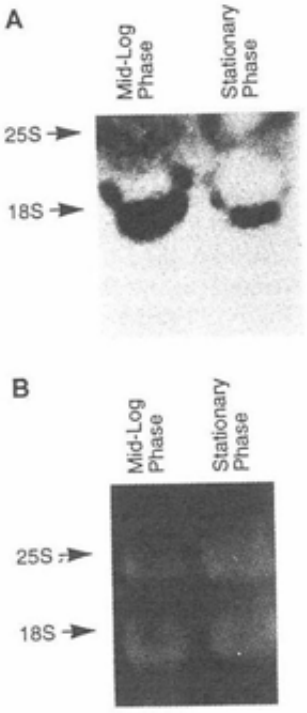

Fig. 4A, B Northern blot analysis of *RHK1* mRNA. Total RNA was extracted from *S. cerevisiae* BJ1824 and subjected to Northern blot analysis (A). The integrity of RNAs and the amounts loaded were assessed by ethidium bromide staining of 25S and 18S rRNAs (B)

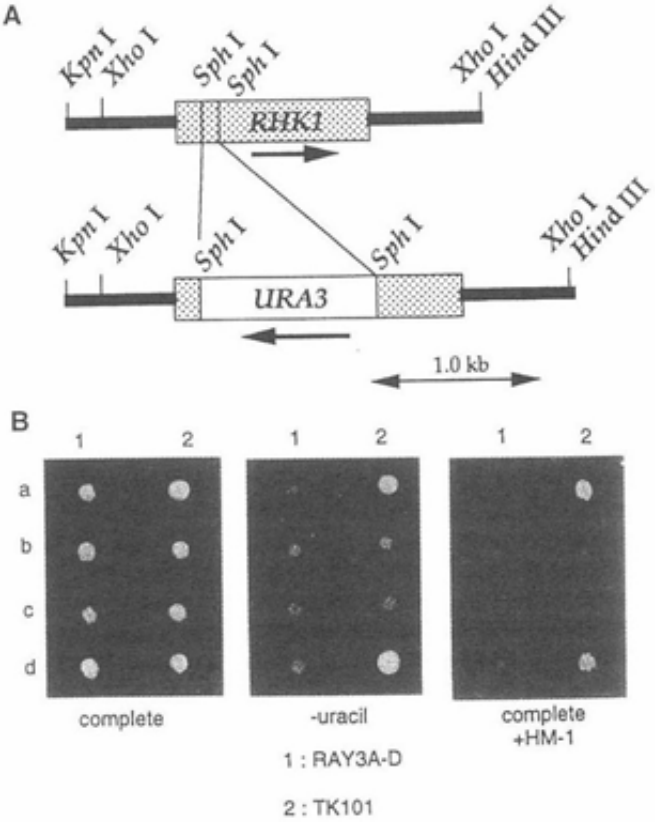

Fig. 5A, B Disruption of the *RHK1* gene. A *Sph*I fragment in the ORF (*dotted box*) of *RHK1* was replaced by the *URA3* gene to disrupt *RHK1*. The directions of transcription of *RHK1* and *URA3* are indicated by arrows. The *rhk1Δ::URA3* construct was used to transform the diploid strain RAY3A-D and a Ura$^+$ transformant (TK101) was sporulated and dissected. B Growth of individual spore clones from transformants on complete medium, complete medium without uracil and complete medium containing HM-1

We examined the HM-1 sensitivity of *rhk1Δ::URA3* mutant cells (TK301) and that of three other strains (SEY6210, kre1 and kre6) by treating them with various concentrations of HM-1 in a microtiter well assay (Fig. 6). Some 10^6 cells of each strain were cultivated in microtiter wells in the presence of different concentrations of HM-1 toxin. Complete growth inhibition of the wild strain SEY6210 was observed at a 1/100 dilution of the concentrated HM-1. The *kre1* and *kre6* mutants, which show resistance to K1 killer toxin, also showed

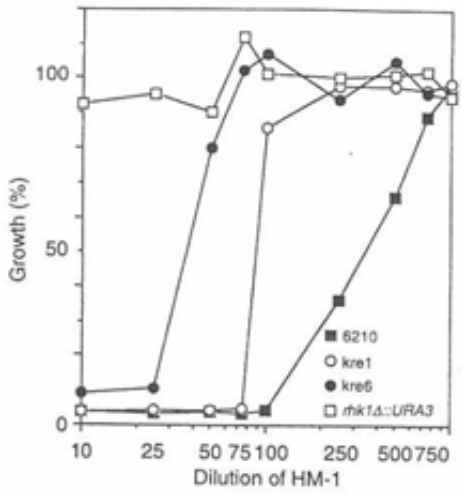

Fig. 6 Cytocidal effect of HM-1 on SEY6210, kre1, kre6 and TK301 (*rhk1Δ::URA3* mutant) strains. SEY6210 (■), kre1(○), kre6(●) and TK301(□) were tested for HM-1 sensitivity in a microtiter well assay which monitors the effect of the toxin on cell accumulation over a fixed period (48 h at 25° C) at each HM-1 dilution. Growth was calculated relative to control wells lacking toxin

partial resistance to HM-1, as described previously (Kasahara et al. 1994b). On the other hand, the *rhk1Δ::URA3* mutant strain TK301, which is isogenic with SEY6210, showed completely normal growth, even at HM-1 dilutions of less than 1:10.

Characterization of *RHK1* disruptant cells

Since *RHK* had been isolated and shown to complement the *rhk1* mutation on the basis of its resistance to HM-1, and since HM-1 had been shown to inhibit β-1,3-glucan synthase activity, we tested the in vitro activity of β-1,3-glucan synthase in spore clones from the same tetrad. However, the clones with the disrupted *RHK1* (TK101a and TK101d) displayed the same specific activity of β-1,3-glucan synthase as the wild-type clones (TK101b, TK101c) (data not shown).

Recently, Ram et al. (1994) reported a general method for isolating cell wall-defective mutants based on their hypersensitivity to Calcofluor White (CFW). In this screen, several mutants showed HM-1 resistance (Ram et al. 1995). Therefore, we compared the CFW hypersensitivity of our *rhk1Δ::URA3* mutant cells (TK301) with that of *kre1* and *kre6* strains, which have the same genetic background. As shown in Fig. 7 (top), all of the strains tested showed similar growth rates on YPD plates. On the other hand, in the presence of CFW growth was restricted to different extents (Fig. 7, bottom). The wild-type strain (SEY6210) was most resistant to CFW, and *kre6*, which has a defect in the synthesis of β-1,6-glucan, showed hypersensitivity to CFW. The *rhk1Δ::URA3* mutant (TK301) showed moderate CFW hypersensitivity. This sensitivity was almost same as that shown by *kre1* which also has a defect in β-1,6-glucan

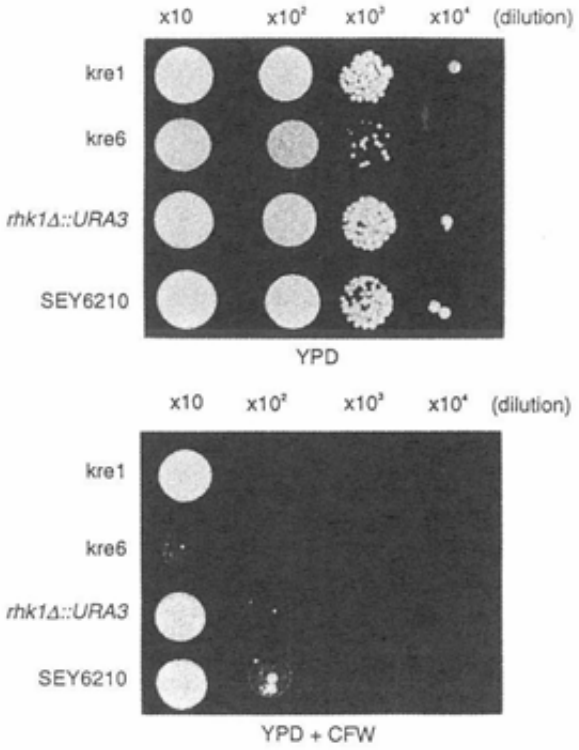

Fig. 7 Calcofluor White (CFW) sensitivity of strains SEY6210, kre1, kre6 and TK301. Cell densities of the strains pre-grown in liquid YPD medium were adjusted to an optical density of 8.0 at 530 nm drops; (5 μl) of individual diluted cell suspension were placed on YPD medium and YPD containing 50 μg/ml Calcofluor White. Growth was scored after 2 days incubation at 28° C. Dilutions are indicated above each panel

synthesis. This suggests that *rhk1Δ::URA3* mutant cells have a cell wall defect.

Effects of HM-1 on regeneration of *rhk1Δ::URA3* mutant cells from protoplasts

To examine the role of Rhk1p in cell wall synthesis, the frequencies of protoplast regeneration of wild-type (BJ1824) and *rhk1Δ::URA3* mutant cells (TK201) were compared. As shown in Fig. 8 (top), regeneration frequencies of these cells were not obviously different in the absence of HM-1 toxin. However, in the presence of HM-1 toxin, a much lower regeneration frequency was observed for the wild-type cells (Fig. 8, bottom left). In contrast, almost the same regeneration frequency was observed for the *rhk1Δ::URA3* mutant cells (TK201) in the absence and presence of HM-1 (Fig. 8, bottom right).

Morphological changes in cells after treatment with HM-1

HM-1 destroys the structure of log-phase cells of the sensitive strain BJ1824. Some cells appear to be lysed at

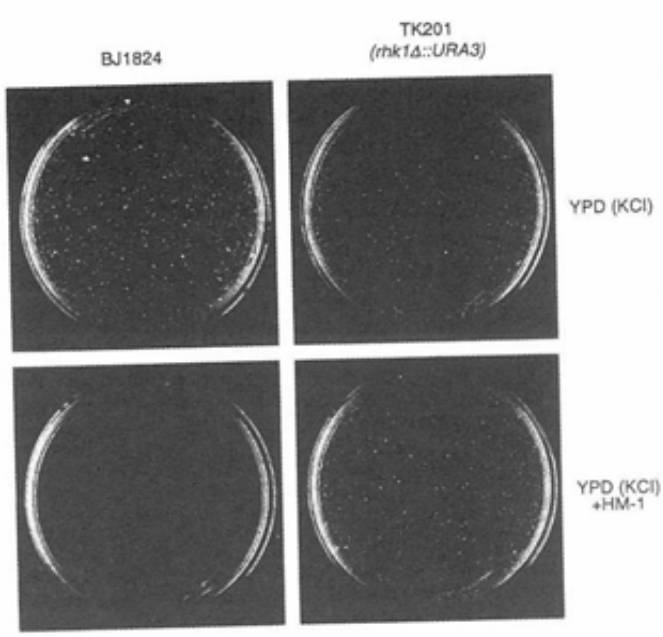

Fig. 8 Effects of HM-1 on protoplast regeneration. YPD agar plates containing 0.7 M KCl, with or without HM-1 (1/200 dilution at final concentration), were seeded with 10^6 protoplasts of wild-type (BJ1824) and TK201 (*rhk1iΔ::URA3*) cells. The seeded plates were incubated at 25° C for 7 days

the bud-neck, resulting in discharge of cellular components (Fig. 9, left). A recent report showed that one of the components released is nucleic acid (Komiyama et al. 1996). The surfaces of the control cells appeared to be rough, suggesting some disorganization of cell surface structure. These observations are entirely compatible with the fact that HM-1 toxin inhibits β-1,3-glucan synthesis. On the other hand, *rhk1Δ::URA3* mutant cells (TK201), which showed complete HM-1 resistance, showed scarcely any morphological changes following HM-1 treatment, and no unusual morphological features were detectable with the scanning electron microscope.

Expression of HM-1 in *rhk1Δ::URA3* mutant cells

Since *rhk1Δ::URA3* mutant cells showed complete resistance to HM-1, regardless of whether they were assayed as intact cells or protoplasts, we expressed the gene encoding HM-1, under the control of the yeast *GAL10* promoter on the YEp51 plasmid, in *rhk1Δ::URA3* mutant cells (TK201). The BJ1824 cells harboring the HM-1 gene showed the suicidal phenotype (stained pale blue with methylene blue) on medium containing galactose (YPGal) (Fig. 10, bottom left), indicating that the secreted HM-1 killed the host cells. On the other hand, *rhk1Δ::URA3* mutant cells secreted HM-1 on YPGal plates and killed only the sensitive cells around them (Fig. 10, top left). In the absence of galactose, neither type of cell death was observed (Fig. 10, right).

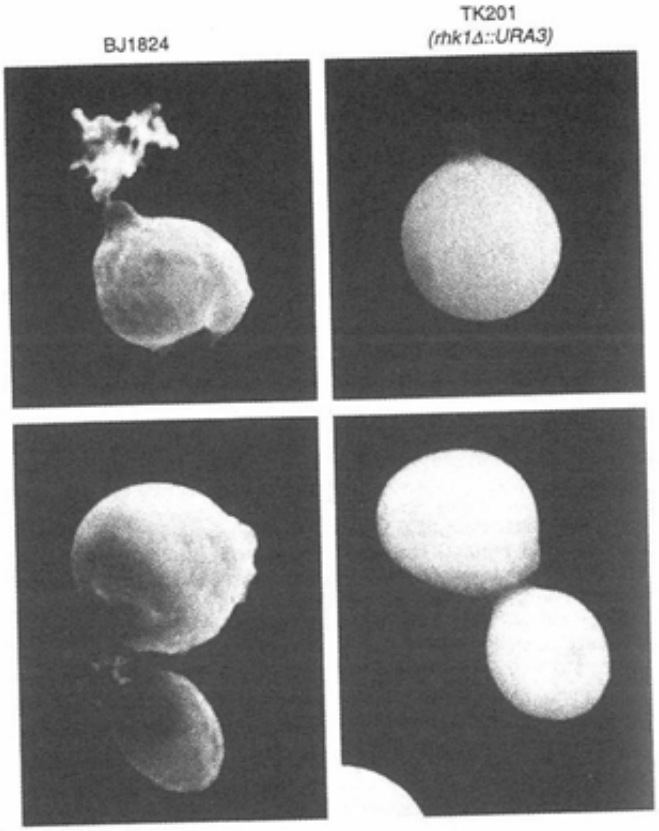

Fig. 9 Morphological changes in cells treated with HM-1. Cells of wild-type (BJ1824) and TK201 (*rhk1Δ::URA3*) strains were treated with HM-1 and observed with a scanning electron microscope

Discussion

In this report, we have described the isolation of *S. cerevisiae* mutants that are resistant to HM-1 toxin produced by *H. mrakii*. These mutants will provide insight into the cellular processes that are inhibited by HM-1. Previously, Hong et al. (1994) isolated HM-1 resistant mutants which fell into nine complementation groups and isolated the *KNR4* gene by complementation of the *knr4-1* mutant. The *knr4-1* mutant showed secondary cell wall defects that resulted in significant resistance to the treatment with Zymolyase and was more susceptible than parental cells to lysis at 30° C by SDS and cercosporamide, an antifungal agent isolated from *Cercosporidium henningsii*. Hong et al. (1994) suggested that these features were a reflection of changes in the carbohydrate linkages of β-1,3-glucan. Indeed, disruption of the *KNR4* led to reductions in both β-1,3-glucan synthase activity and cell wall β-1,3-glucan content, indicating that the product of *KNR4* plays a regulatory role in β-1,3-glucan synthesis. On the other hand, *HKR1*, which confers HM-1 resistance on *S. cerevisiae* cells, was found to increase β-1,3-glucan content without affecting β-1,3-glucan synthase activity, when it was overexpressed (Kasahara et al. 1994a). Recently, partial

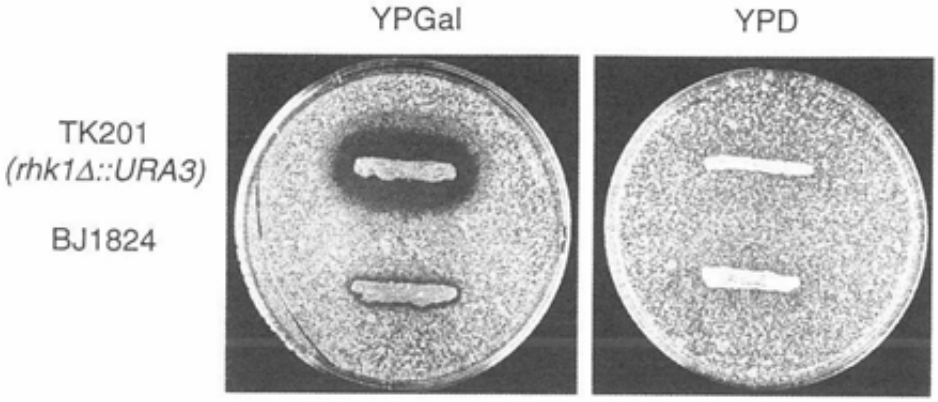

Fig. 10 Expression of *HMK* (the gene that encodes HM-1) in wild-type (BJ1824) and TK201 (*rhk1Δ::URA3*) cells. *HMK* was expressed from YEp51 in both cell types (streched on a lawn of sensitive cells), under the control of the yeast *GAL10* promoter, and killer activity was monitored as previously reported (Kimura et al. 1995b). Bottom left, BJ1824 cells harboring *HMK* showed the suicidal phenotype (staining pale blue with methylene blue) on medium containing galactose. Top left, *rhk1Δ::URA3* mutant cells (TK201) secreted HM-1 and killed the sensitive cells around them, producing the prominent halo Right, cells grown on YPD medium

disruption of this gene has been shown to cause a decrease in both the β-1,3-glucan synthase activity and the level of β-1,3-glucan content in the cell wall (Yabe et al. 1996). It was also reported that *HKR1* affects bud site selection, suggesting that this gene is involved in the assembly of cell wall β-1,3-glucan. Loss of the function of *KNR4* or overexpression of *HKR1* would therefore seem to confer HM-1 resistance on the cells as the result of various cell wall-associated defects.

In our screen, we identified three genes which, when mutated, abolish the sensitivity of yeast cells to HM-1, and we checked all of them for their sensitivity to Zymolyase and SDS. However, there was no obvious difference between the *rhk* mutants and the parental strain in these traits. Our mutants therefore differ from highly defective cell wall mutants such as *knr4-1*. Resistance to HM-1 is expected to reflect changes not only in β-1,3-glucan levels but also in cell wall structures recognized by HM-1. Among the mutants isolated in our study, *rhk1* comprises the largest complementation group and *rhk1* cells did not differ from parental cells in any way other than their HM-1 resistance. Our data suggest that Rhk1p is involved in the cross-linkage of cell wall polysaccharides and/or mannoproteins which are recognized by HM-1, rather than in β-1,3-glucan synthesis, since (i) cells harboring a defect at the *rhk1* locus did not have reduced β-1,3-glucan synthase activity when compared with wild-type cells (data not shown); (ii) *rhk1Δ::URA3* mutant and wild-type cells showed no differences in susceptibility to Zymolyase digestion, suggesting that β-1,3-glucan content was not substantially changed; (iii) protoplasts of *rhk1Δ::URA3* mutant cells regenerated with the same efficiency as wild-type cells, and (iv) β-1,3-glucan synthase in both *rhk1Δ::URA3* mutant and wild-type cells was inhibited by HM-1 to the same extent (data not shown). The dominant component of cell wall architecture consisting of glucan or chitin may be normal in *rhk1Δ::URA3* mutant cells as no changes in morphology or budding pattern were observed with the scanning electron microscope (Fig. 9, left). Ram et al. (1995) screened for CFW-hypersensitive mutants and found several HM-1 resistant mutants among them. As shown in Fig. 7, *rhk1Δ:::URA3* mutant cells show slight hypersensitivity to CFW, suggesting that they have a defect in their cell wall structure. The *kre1* and *kre6* mutant cells, which have defects in the synthesis of β-1,6-glucan, also showed CFW hypersensitivity. These cells also showed partial killer resistance. However, there is no obvious relationship between the extent of CFW hypersensitivity and HM-1 resistance (Figs. 6 and 7). It is likely that a drastic change in cell wall structure will lead to pleiotropic phenotypes, including HM-1 resistance. Susceptibility to HM-1 would be expected to be decreased by altered cell wall integrity.

Although the ultimate target of HM-1 is thought to be a β-1,3-glucan synthase, the mechanism by which HM-1 first recognizes one of the components in the cell wall remains to be determined. An analysis of HM-1 binding to cell wall components did not identify the receptor to which HM-1 first binds because of the lower affinity of HM-1 for the isolated cell wall (Kasahara et al. 1994b). The results obtained here suggest that one of the receptor structures resides in the internal region of the cell wall, since regeneration from protoplasts was not inhibited by HM-1 in *rhk1Δ::URA3* mutant cells (Fig. 8) and since *rhk1Δ::URA3* cells could secrete HM-1 (Fig. 9). How the toxin recognizes the target cell and why it can kill only some species of yeast are interesting questions. HM-1 may recognize a complex structure composed of several oligosaccharides since addition of β-1,3-glucan, β-1,6-glucan and mannan, which are similar to components of the cell wall, suppressed the action of HM-1. While this manuscript was in preparation, *RHK1* was shown to be identical to the *ALG3* gene, which encodes a mannosyl transferase for N-linked protein glycosylation (Obermair et al. 1995). It is thus conceivable that the product of *RHK1/ALG3* is involved

in the glycosylation of mannoproteins which are recognized by HM-1 in the first step of toxin action, since a significant component of cell wall architecture is composed of mannoproteins that are cross-linked to glucan via their glycosyl side chains. A mutational analysis of HM-1 resistance will provide further insights into understanding fungal cell wall structure and into developing a new anti-fungal agent.

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