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Influence of Kex1p and Kex2p proteases on the function of *Saccharomyces cerevisiae* K2 preprotoxin

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Studies of dependence of *Saccharomyces cerevisiae* K2 killing and immunity properties modulated by the action of Kex1p and Kex2p enzymes showed that the lack of Kex1p carboxypeptidase 10 times decreased toxin activity, and the deficiency of Kex2p endopeptidase completely removed killing ability. The obtained data confirm requirement of both Kex1p and Kex2p peptidases for functional K2 toxin production. The immunity of mutated strains bearing the K2 killer gene was investigated by evaluating the amount of viable cells subjected to the externally applied K2 toxin. K2-specific immunity was found to be completely independent of the action of Kex1p and slightly dependent on Kex2p functioning, suggesting not only the importance of precursor in resistance formation, but also of toxin involvement.

Key words: *Saccharomyces cerevisiae*, killer toxin, immunity, Kex1p, Kex2p proteases

INTRODUCTION

In *Saccharomyces cerevisiae*, the killer phenotype is caused by an infection with cytoplasmic-persisting dsRNA viruses of the family *Totiviridae*, a member of the constantly growing class of mycoviruses which are widely distributed among yeast and higher fungi [1]. Until now, three killer types (K1, K2 and K28) have been identified, of which K1 and K28 have been studied most extensively [2]. Members of all these groups are capable of killing non-killer as well as killer yeast of the opposite killer class, remaining immune to their own toxin and to the strains belonging to the same killer group [1, 2]. Each killer toxin is translated as a preprotoxin precursor (pptox) consisting of an N-terminal signal sequence which is necessary for pptox import into the lumen of the ER, followed by the α- and β-subunits of the mature toxin separated from each other by a potentially N-glycosylated γ-sequence [3]. During passage through the yeast secretory pathway, the toxin precursor is enzymatically processed to the biologically active α / β heterodymer [4, 5]. The N-glycosylated γ-sequence is removed by the action of the furin-like endopeptidase Kex2p, and the C terminus of the β-subunit is trimmed by the carboxypeptidase Kex1p [1, 5, 6]. In case of K28 toxin precursor, the C terminus of the β-subunit contains four amino acid-epitope that represent a classical ER-retention sygnal (HDELR) marked by a carboxyterminal arginine residue and uncovered after Kex1p cleavage [7].

Yeast killer toxins exert their lethal effect in a receptor-mediated fashion. K1 disturpts cytoplasmic membrane function by forming cation-selective ion channels [8], whereas K28 enters its target cell by receptor-mediated endocytosis and blocks DNA synthesis, leading to both G1 / S cell-cycle arrest and caspase-mediated apoptosis [9]. The question of how immunity is realized remains to be answered and is among the most intriguing aspects of the killer phenomenon. K1-toxin immmunity might be conferred by the preprotoxin itself acting as a competitive inhibitor of the mature toxin by saturating / eliminating the plasma membrane receptors [10], or by inhibition of TOK1 channels by internal toxin and suppresion of external toxin action [11]. K28--toxin immunity occurs via formation of complexes between reinternalized toxin and unprocessed precursor moieties that are subsequently ubiquitinated and proteosomally degraded [3].

The K1 and K2 killers are very similar in their mode of action, nevertheless, they are proteins differing in the primary sequence level, preprotoxin organization (γ--subunit most likely not present) [5], and the pH optimum of the killing activity [5, 12]. The K2 killer preprotoxin is synthesized as a 362-amino acid precursor consisting of the N-terminal secretion signal followed by the α- and β-subunits (172 and 140 amino acids, respectivelly). Expression of the K2 preprotoxin gene cDNA on a multicopy plasmid proved the encoding of both the immunity and toxin activities by a single precursor, ORF [13]. Site-specific mutagenesis of the K2 toxin gene identified one of the two potential Kex2p cleavage sites to be critical for toxin action [5]. Changes

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in both signal and subunit sequences have been demonstrated to compromise or abolish the immunity function of the resulting protein [14]. Fifteen years ago Dignard et al. [5] determined that efficient K2-specific killing was dependent on the action of the Kex2p endopeptydase and the Kex1p carboxypeptidase, while K2-immunity was independent of these proteins. New data on K1 and K28 toxin lethality and self-protection [1, 3] as well as discovery of different modes of action of K1 and K2 killer toxins on the plasma membrane and the cell wall [15] put us on the way to look deeper into the functioning of the K2 killer system.

The objective of the present work was analysis of dependence of K2 killing and immunity function on the action of Kex1p and Kex2p enzymes and comparison of the obtained data with M471 line K2 cDNA expression.

MATERIALS AND METHODS

The plasmid pYEX12, coding LEU2 marker and *S. cerevisiae* K2 preprotoxin gene was used in this study [13]. The *S. cerevisiae* strains α'1 (MATα leu2-2 (KIL-0)) [16], BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) as well as kex1 and kex2 deletions strains (BY4741 background) (Open Biosystems, Huntsville AL, USA) sensitive to K2 killer toxin were used for K2 preprotoxin gene expression studies. For immunity tests we used *S. cerevisiae* killer strains K7 (*MATa arg9 [KIL-K1]*), Rom K-100 (*wt*, *HM/HM [KIL-K2]*), M437 (wild type, HM/HM (KIL-K2) MS300 (*MATa leu2 ura3-52 SKI2-2 [KIL-K28]* [17]. Media for the propagation of *S. cerevisiae* yeast have been described in Ausubel et al. [18].

Each yeast strain was transformed with pYEX12 plasmid by using the lithium acetate / ssDNA / polyethylene glycol method [19]. The transformants were selected by complementation of LEU2 auxotrophy, and the clones were verified for toxin production by replica plating to the lawn of α'1 strain. Killer activity was detected in a plate and well agar diffusion assay [17] by measuring the growth-free zones aroud the wells. The sensitivity / immunity tests were performed by patching colonies of killer strains onto MB plates with an overlay of the transformed cells of interest (approximately 10^6 cells per plate). Yeast strains incapable of conferring functional immunity to K2 killers show a sensitive phenotype, resulting in a cell-free growth inhibition zone, whereas resistant yeast do not. Also, the immunity of the tested transformants was estimated by evaluating the amount of viable cells in media containing 42 U/ml of the killer toxin (treatment at 18 °C for 24 h) versus the total number of killer-free media.

RESULTS AND DISCUSSION

A cDNA copy of the K2 killer preprotoxin gene (line Rom K-100) was expressed in yeast under control of the constitutive alkoholdehydrogenase (ADH1) promoter. First, this construct was introduced into strains α'1 and BY4741. Parental yeast and transformants were tested for immunity and killing capabilities using the indicative media (MB) with glucose as a carbon source. It is known that the α'1 strain is sensitive to all *S. cerevisiae* toxins and lacks killer phenotype [16]. We have determined that strain BY4741 also belongs to non-killer yeast but showed twice as low sensitivity to K2 toxin as compared to strain α'1 (Table, lines 1, 2). Plate tests indicate that α'1 [pYEX12] and BY4741 [pYEX12] transformants produce intact K2 killer phenotype and resistance to killer toxins of the same type. To determine the transformant-produced toxin pH-optimum, we tested the killer activity and immunity function at various pH values ranging from 4.0 to 4.8. The toxin activity was maximal at a lower pH value (4.0): strain α'1[pYEX12] showed a 6-mm and BY4741[pYEX12] a 5-mm growth-free zone around the colonies (Table). For comparison, the wild type K2 killer-expressing strains Rom K-100 and M437 produced 1.5–2 times higher level of killer toxin than transformant yeasts (lysis zones about 7mm and 9mm, respectively) (Table).

Table. **Comparison of K2 toxin activity in Kex1p and Kex2p protease deletion yeast along with wild type strains Rom K-100 and M437.**

N.	Strains / Inhibition zone mm	Overlay of α'1 strain, with different pH values			Overlay of BY4741 strain, with different pH values		
		4.0	**4.4**	**4.8**	**4.0**	**4.4**	**4.8**
1.	Rom K-100	7	4	2	3.5	3	2
2.	M437	9	5	4	4.5	3.5	3
3.	α'1	-	-	-	-	-	-
4.	BY4741	-	-	-	-	-	-
5.	α'1 [pYEX12]	6	4	2	3	2	1.5
6.	BY4741 [pYEX12]	5	2.5	1.5	2	1.5	1
7.	Δkex1	-	-	-	-	-	-
8.	Δkex1 [pYEX12]	0.5	-	-	-	-	-
9.	Δkex2	-	-	-	-	-	-
10.	Δkex2 [pYEX12]	-	-	-	-	-	-

In order to analyse the dependence of K2 killing and immunity functions on the action of Kex1p and Kex2p enzymes, we transformed the single-gene deletion strains Δkex1 and Δkex2 with pYEX12 plasmid and performed a killing zone plate assay. The obtained K2 preprotoxin gene expression level in carboxypeptidase (Kex1p) defficient strain was low (the growth-free zone reached only 0.5mm) (Table, lane 8). Thus, it was shown that the lack of Kex1p enzyme 10 times decreased toxin activity. At the same time we observed that deficiency in Kex2p enzyme completely removed K2 killing ability. We didn't notice any lysis zones around Δkex2 [pYEX12] colonies plated on the lawn of strain α'1 (Table, lane 10). Performing these experiments we investigated the requirement for the Kex1p and Kex2p functions in the extracellular production of the active K2 toxin. It was noted that K2 toxin activity was enhanced by the action of Kex1p, but this was not an absolute requirement for the enzyme.

Parallelly, the immunity of deletion strains bearing the K2 preprotoxin gene was tested. Analysis of Δkex1 [pYEX12] transformants allowed to point out that deletion of Kex1p enzyme didn't influence preprotoxin production and the transformants retained complete immunity to K2 killer toxins (Fig. 1, A). However, deficiency in Kex2p protease compromised the protein-dependent immunity function, and Δkex2 [pYEX12] transformants failed to display full resistance. They were sensitive to K2 toxins produced by wt K2 killer strains and only resistant to their own toxin (Fig. 1, B).

A more sensitive method for estimation of the immunity of transformants bearing the K2 preprotoxin gene but lacking Kex2p endopeptidase or Kex1p carboxypeptidase was used. We evaluated the amount of survived cells of mutant strains subjected to the externally applied K2 toxin. The survival of Δkex1 [pYEX12] was found not to depend on Kex1p protein action: 90.9 ± 0.8% of cells survived in a carboxypeptidase-lacking strain versus 89.4 ± 2.9% in enzyme-producing yeast (Fig. 2). However, in the Kex2p defficient strain the survival of yeast cells dropped by about 15% and reached only 77.7 ± 1.3% (Fig. 2). These results are in line with the plate test data and confirm the dependence of K2-specific immunity on Kex2p protease action. It is known that this enzyme is involved in the K1 and K2 processing pathway and are essential for active toxin production, but not important for preprotoxin formation. Following one of the hypotheses, immunity to K1 toxin is determined by a preprotoxin that can act as a competitive inhibitor saturating cell membrane receptors that normally mediate the mature toxin action [10]. At the same time, data are available regarding the K1 killer system dependence on the supression of external toxin action by the internal toxin [11]. We have confirmed that in case of the K2 killer system, preprotoxin is a major component in K2-immunity formation and in this respect shows similarity to K1 killers. Nevertheless, it was demonstrated that for complete K2-self-protection both preprotoxin and toxin are necessary. These data are different for M471 line K2 killer toxin gene expression analysis. However, it is necessary to point out that both *wt* killer strains Rom K-100 (the donor of M2 dsRNA, used for cDNA synthesis in our experiments) and M471 (analysed in Dignard's laboratory) produce K2 type killer toxins which have distinctions at the M2 dsRNA level. The discrepancies had been previously observed at 31, 68, 180, 475, 689 and 781 positions of the coding sequence [5, 13]. Nonmatching nucleotides determine changes in a protein sequence and therefore can result in altered properties of killer toxins.

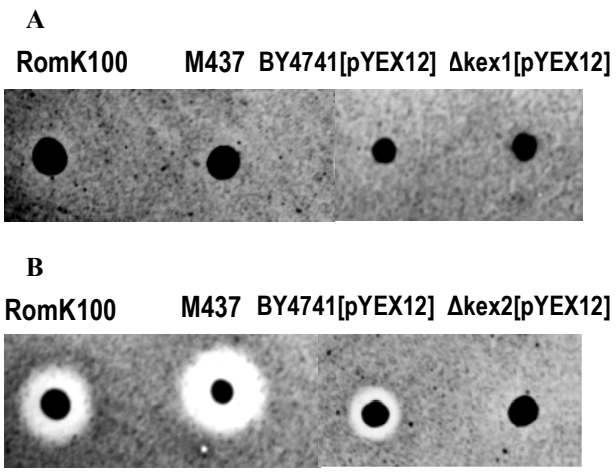

Fig. 1. Immunity function of deletion strains Δkex1 [pYEX12] (**A**) and Δkex2 [pYEX12] (**B**) bearing K2 killer gene

The transformants were tested for immunity using indicative MB media. Similar amounts of Δkex1 [pYEX12] (**A**) and Δkex2 [pYEX12] (**B**) cells were seeded into the plates. K2 killer producing *wt* strains Rom K-100 and M437, control transformant BY4741 [pYEX12] expressing intact killer phenotype and full immunity as well as transformants of interest were patched onto the plates.

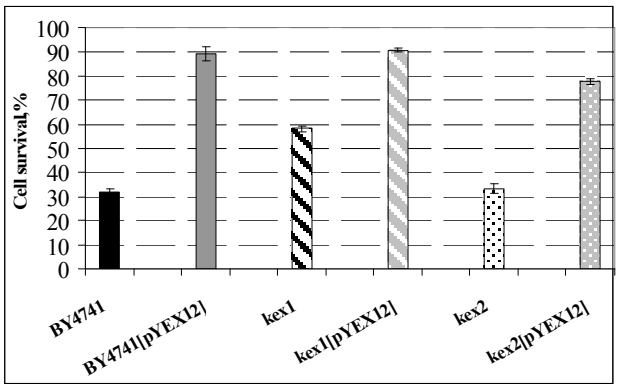

Fig. 2. Survival of mutant strains in presence of externally applied K2 killer toxin

Strains are indicated on the abscisse. Cell survival estimated in % as a ratio of cells grown in presence (42 U/ml) and absence of externally applied K2 toxin.

In summary, it could be concluded that both Kex1p and Kex2p peptidases are required for functional K2 toxin production. Our data indicate not only the importance of precursor in resistance formation, but also toxin involvement.

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KEX1P IR KEX2P PROTEAZIŲ POVEIKIS *SACCHAROMYCES CEREVISIAE* K2 KILERINIO PREPROTOKSINO FUNKCINIAM PASIREIŠKIMUI

S a n t r a u k a

Šiame darbe atskleista Kex1p karboksipeptidazės ir Kex2p endopeptidazės svarba K2 kilerinio preprotoksino brendimui ir funkciniam pasireiškimui. Nustatyta, kad Kex1p proteazės nebuvimas 10 kartų sumažina sekretuojamo toksino aktyvumą, o Kex2p pašalinimas visiškai eliminuoja toksino produkavimą. Taigi atskleista abiejų fermentų reikšmė funkcionalaus toksino susidaryme. Atlikus rezistentiškumo tyrimus nustatyta, kad K2-specifinis imuniškumas nepriklauso nuo Kex1p veikimo, bet yra iš dalies inhibuojamas mutantiniame Kex2p kamiene. Gauti duomenys leidžia manyti, kad ne tik preprotoksinas, bet ir subrendęs toksinas yra įtraukti į rezistentiškumo formavimo procesą.