Yeast Kre1p is GPI-anchored and involved in both cell wall assembly and architecture

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Kre1p is a cell surface O-glycoprotein involved in a late stage of 1,6- β -glucan formation in the yeast *Saccharomyces cerevisiae*. Disruption of *KRE1* leads to a 40 % reduction in the overall 1,6- β -glucan content of the cell wall. This paper shows that in a yeast $\Delta kre1$ null mutant, neither an N-terminal-truncated Kre1p nor Kre1p variants lacking a C-terminal glycosylphosphatidylinositol (GPI) attachment site are capable of achieving normal function in glucan assembly, while full-length Kre1p completely complements a $\Delta kre1$ null mutation and restores cell wall 1,6- β -glucan content up to wild-type level. In a yeast *gpi1* mutant, a green-fluorescent-protein-tagged Kre1p derivative is secreted into the medium, indicating an at least transient GPI-anchoring stage of Kre1p during its processing within the yeast secretory pathway. In contrast to the severe defect in cell wall β -D-glucan, the amount of cell wall mannoproteins is not significantly decreased in a $\Delta kre1$ disruptant, as could be confirmed in competition assays by investigating toxin binding to isolated cell wall mannoproteins. Since the yeast $\Delta kre1$ mutant differed in its sensitivity to zygocin and K28, two killer viral protein toxins that use different cell wall mannoprotein populations as a primary toxin receptor, it can be concluded that in a yeast $\Delta kre1$ background, mannoproteins do not differ significantly in total amount from a Kre1⁺ wild-type but rather in their expression and distribution at the cell surface. Taken together, these data favour and suggest a structural, rather than enzymic, function of Kre1p in yeast cell wall assembly.

Received 18 March 2004

Revised 25 June 2004

Accepted 1 July 2004

INTRODUCTION

Binding of yeast killer toxins to defined components of the *Saccharomyces cerevisiae* cell wall provides a valuable means of identifying genes that are involved in the biosynthesis and/or structure of particular cell wall components (Cid *et al.*, 1995). In particular, binding of yeast killer toxin K1 to cell wall 1,6- β -glucans has allowed the identification of a number of genes that are essential for glucan synthesis, while the localization of their gene products within the secretory pathway provided further insight into the synthesis and assembly of this important yeast cell 'organelle' (Brown *et al.*, 1993). In yeast *kre* mutants (indicating killer resistant), the binding of the K1 toxin to the cell wall is blocked either by disturbing the synthesis or by altering the structure of cell wall 1,6- β -glucans (recently reviewed by Schmitt & Breinig, 2002). Just as K1 has been successfully employed for the selection of toxin-resistant mutants defective in

various steps of cell wall 1,6- β -D-glucan biosynthesis, the viral K28 toxin is a powerful tool for investigating cell wall mannoprotein structure, synthesis and function since it uses a high-molecular-mass cell wall mannoprotein as a primary receptor (Schmitt & Breinig, 2002). Thus, mutations in different chromosomal *MNN* genes, whose gene products are directly or indirectly involved in mannoprotein synthesis and/or side-chain structure, lead to a K28 toxin resistance at the cell wall level (Tipper & Schmitt, 1991).

Yeast *KRE1* encodes a serine/threonine-rich, highly O-glycosylated protein of the cell surface that is involved in a late stage of the synthesis and/or assembly of yeast cell wall 1,6- β -D-glucan (Boone *et al.*, 1990). In a $\Delta kre1$ null mutant, the total amount of cell wall 1,6- β -glucan is 40 % lower than in the wild-type and the resulting glucan polymer shows a lower degree of polymerization and possesses significantly fewer β -1,3-glycosidic branches. It has been speculated that during its secretion Kre1p is glycosylphosphatidylinositol (GPI)-anchored via its C-terminal hydrophobic part, but so far this hypothesis has not clearly been proven. Recently, the membrane-bound form of Kre1p has been shown to function as a plasma membrane receptor for the yeast K1 killer toxin (Breinig *et al.*, 2002). Here, we extend our previous finding by investigating Kre1p with

This paper is dedicated to Professor Dr Ferdinand Radler on the occasion of his 75th birthday.

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Abbreviations: GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol.

respect to its function in cell wall assembly and composition. The presented data imply a structural, rather than enzymic, function of yeast Kre1p within cell wall β -1-6-glucan assembly and architecture.

METHODS

Yeast strains and culture conditions. *S. cerevisiae* Kre1⁺ and Kre1⁻ strains SEY6210 (*MAT α leu2-3 his3- Δ 200 lys2-801 trp1- Δ 901 suc2- Δ 9*) and SEY6210 [Δ kre1] (*MAT α leu2-3 his3- Δ 200 lys2-801 trp1- Δ 901 suc2- Δ 9 kre1::HIS3*) were kindly provided by Howard Bussey (McGill University, Montreal, Canada). The *gpi* mutant strains 2A (*MAT α ade2-101 ura3-52 his4 gpi1*), 27-5D (*MAT α ade2-101 ura3-52 gpi2*), 53-12C (*MAT α lys2 gpi3*) and its parental *GPI1* wild-type strain 37-10C (*MAT α ade2-101 ura3-52*) were kindly provided by Peter Orlean (University of Illinois, Urbana, USA). The *mn2* mutant strain X2180-m2-1a (*MAT α suc2 mal gal2 CUP1 mn2*) was kindly provided by Clinton Ballou (University of California, Berkeley, USA). Cells were grown in YEPD medium at 30 °C. Transformation was carried out by the lithium acetate method described by Ito *et al.* (1983) and transformants were selected on synthetic complete (YNB) medium lacking uracil.

The K1 and K28 toxins were prepared as described by Pfeiffer & Radler (1982) and Schmitt & Tipper (1990) using *S. cerevisiae* strains T158C and MS300b, respectively, as toxin producer. Zygocin was prepared from the *Zygosaccharomyces bailii* killer strain 412 essentially as previously described (Radler *et al.*, 1993; Weiler & Schmitt, 2003). In brief, the corresponding killer yeast was grown at 20 °C in synthetic B-medium (pH 4.7 for K1 and K28, pH 4.0 for zygocin). After entering the stationary growth phase, cells were pelleted by centrifugation and the cell-free culture supernatant was concentrated 1000-fold by ultrafiltration using a Sartorius ultrafiltration system (SM 16525) and trifluoroacetate membranes with a molecular mass cut-off of 10 kDa. The biologically active toxins were filter-sterilized and kept frozen at -20 °C. Toxin activity was determined in a standard killing-zone assay as described previously (Breinig *et al.*, 2002) and toxin activity is expressed in arbitrary units (Schmitt & Tipper, 1990).

For spheroplast preparation, the appropriate yeast was cultivated at 30 °C in YEPD medium until late exponential growth phase (5×10^7 cells ml⁻¹), harvested and washed twice with sterile water. Cells were resuspended in Tris/HCl buffer (0.1 M Tris/HCl, pH 8.0, 5 mM DTT, 5 mM EDTA), incubated at 30 °C for 30 min and washed twice in 1.2 M sorbitol. Washed cells were finally resuspended in 1.2 M sorbitol containing 0.5 M Na₂HPO₄ and yeast cell spheroplasts were generated by zymolyase 20T treatment (Schmitt & Tipper, 1990).

Escherichia coli strains, plasmids and DNA manipulations. Standard molecular manipulations were performed as described by Sambrook *et al.* (1989). For cloning, *E. coli* strain DH5 α [*F*⁻ *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ (argF-lacZYA)U169 (ϕ 80dllaZ Δ M15) λ* ⁻] was used. All Kre1p expression plasmids used in this study are derivatives of the episomal 2 μ vector pPGK-M28-I (Schmitt & Tipper, 1995) in which the entire K28 ppto open reading frame (M28) was replaced by the indicated *KRE1* gene fusion and placed under transcriptional control of the constitutive *PGK1* promoter (Breinig *et al.*, 2002).

Extraction of cell wall mannoproteins. Crude cell wall mannoproteins were extracted using a method according to Nakajima & Ballou (1974a, b) modified as previously described (Schmitt & Radler, 1987). Briefly, cells were cultivated at 30 °C in YEPD containing 5 % glucose until late exponential growth phase, harvested and washed three times with sterile water. Cells were resuspended in 20 mM citrate/phosphate buffer (pH 7.0; 1 ml cell suspension per g

wet weight of cells) and autoclaved for 90 min at 121 °C. After centrifugation the pellet was resuspended in 20 mM citrate/phosphate buffer and autoclaved again. This procedure was repeated and the supernatants were pooled. After the addition of 3 vols ice-cold ethanol, the obtained crude mannoprotein precipitate was pelleted at 4 °C, resuspended in 70 ml extraction buffer and dialysed overnight against deionized water. The amount of mannoprotein was determined after lyophilization. For partial purification of the crude mannoproteins, a cetavlon fractionation was carried out by the method of Lloyd (1970). The crude mannoprotein was dissolved in double-distilled water (25 ml g⁻¹) and combined with a solution of 1 g cetavlon (cetyl-N,N,N-trimethyl-ammonium bromide) in 10 ml double-distilled water. After stirring for 1 h at 20 °C, the precipitate was pelleted and washed with 50 ml distilled water. In order to precipitate cell wall mannoproteins as a boric acid complex, the pooled supernatants were acidified by the addition of 100 ml boric acid (1 %) and the pH was adjusted to pH 8.8 by the addition of 2 M NaOH. The resulting complex was pelleted, washed twice with sodium acetate (0.5 %, pH 8.8) and resuspended in acetate (0.5 %). Another precipitation was performed by the addition of 1 g solid sodium acetate and 3 vols ethanol. The precipitate was pelleted, washed twice with acetate (2 % in ethanol), and resuspended in double-distilled water. After neutralization with 2 M NaOH and subsequent dialysis against 200 vols of distilled water, the cetavlon mannoprotein was maintained and lyophilized for performing further studies.

Adsorption of killer toxins K1 and K28 to cetavlon mannoprotein – in vivo competition assay. Cetavlon mannoproteins were resuspended at concentrations from 1 to 10 mg ml⁻¹ in 880 μ l B-medium, pH 4.7 (Pfeiffer & Radler, 1982). Each sample also contained 20 μ l of the appropriate killer toxin (about 10^4 U ml⁻¹) and 100 μ l of *S. cerevisiae* SEY6210 with a cell concentration of about 10^7 cells ml⁻¹. After incubation of the samples for 24 h with gentle shaking at 20 °C, colony-forming units of the sensitive strain were determined by plating a range of dilutions onto YEPD agar, pH 7.0. The remaining toxin in the samples was rapidly inactivated due to the high pH of the agar. Competition assays were performed according to the methods of Hutchins & Bussey (1983) and Schmitt & Radler (1987), with *S. cerevisiae* 518 as sensitive test strain.

Immunoblot analysis. After transformation with the indicated Kre1p expression construct, approximately 2×10^7 yeast cells were grown either at the permissive (20 °C) or at the semi-restrictive (25 °C) temperature in minimal medium under conditions of plasmid selection. Cells were harvested and the extracellular proteins secreted into the cell-free culture medium were concentrated by ethanol precipitation and incubated overnight at -20 °C. Protein samples were resuspended in water and separated by Tricine-SDS-PAGE according to the method of Schagger & von Jagow (1987). After electrotransfer of the proteins onto a nitrocellulose membrane, blots were incubated with a monoclonal anti-GFP (green-fluorescent-protein) antibody (Roche; diluted 1/10 000) followed by treatment with an alkaline phosphatase-coupled anti-rabbit antibody (Sigma; diluted 1/3000), and developed with NBT/BCIP (Roche).

Preparation of cell wall 1,6- β -D-glucan. 1,6- β -D-Glucan was isolated and quantified as described by Boone *et al.* (1990). In brief, yeast cells were cultivated in SC medium (or the appropriate D/O-medium if plasmid selection was required) until stationary growth phase. Cells were harvested, washed twice with distilled water and then extracted three times with 0.5 ml 3 % NaOH at 75 °C (1 h per extraction) to remove alkali-soluble glucan and mannoproteins. After alkali extraction, cells were neutralized and washed by the addition of 1 ml 0.1 mM Tris/HCl (pH 7.5) and 1 ml 10 mM Tris/HCl (pH 7.5). Washed cells were resuspended in 1 ml 10 mM Tris/HCl (pH 7.5) containing 5 mg zymolyase 20T (ICN Biomedicals)

and incubated overnight at 37 °C. After digestion of the cell wall, the insoluble cell pellet was removed by centrifugation (13 000 r.p.m., 15 min) and the supernatant was dialysed against distilled water. Analysis of the carbohydrate retained after dialysis yielded the proportion of alkali-insoluble 1,6- β -glucan. Total carbohydrate in each fraction was measured as hexose by the phenol/sulfuric acid method of Dubois *et al.* (1956). Each experiment was repeated at least three times.

RESULTS

The length of Kre1p affects the total amount of alkali-insoluble cell wall 1,6- β -glucan

By expressing an N-terminal-truncated derivative of Kre1p in a yeast $\Delta kre1$ null mutant we recently showed that Kre1p functions as a plasma membrane receptor for the yeast K1 virus toxin (Breinig *et al.*, 2002). Here, we investigated the influence of this truncation on its *in vivo* function in late-stage 1,6- β -D-glucan assembly.

We and others have previously shown that K1 toxin resistance of a yeast $\Delta kre1$ mutant is due to a block in the initial interaction of the toxin with the yeast cell surface, and that susceptibility can be restored by retransformation with the wild-type *KRE1* gene. This observation implies that expression of wild-type Kre1p not only increases the 1,6- β -glucan content of the disruption mutant up to wild-type level, but also restores toxin binding to the cytoplasmic membrane. In order to investigate the effect of Kre1p on cell wall glucans in more detail, we determined the amount of alkali-insoluble 1,6- β -D-glucan in a $\Delta kre1$ null mutant before and after expression of either full-length Kre1p or its N-terminally truncated derivative Δ_{1-225} Kre1p. As summarized in Table 1, the 1,6- β -glucan level in the null mutant compared to the wild-type was diminished by 33 %, thus confirming earlier reports of Boone *et al.* (1990). As

expected, expression of Δ_{1-225} Kre1p did not restore 1,6- β -glucan levels, while expression of full-length Kre1p did, indicating that the N-terminal part of Kre1p is responsible for its function in 1,6- β -glucan assembly. The slightly increased 1,6- β -glucan content seen in the $\Delta kre1$ mutant after expression of the full-length protein Kre1p is likely to be caused by its multi-copy expression from the strong *PGK1* promoter.

Deletion of *KRE1* affects cell wall mannoprotein composition

Since deletion of *KRE1* results not only in K1- but also in K28-toxin resistance, we investigated the response of a $\Delta kre1$ knock-out mutant to K28 in more detail. Killing-zone assays showed that intact cells of the $\Delta kre1$ mutant were likewise K1 and K28 resistant (Table 2). However, at the plasma membrane level, studied using spheroplasts, the mutant remained K1 toxin-resistant but showed pronounced sensitivity to K28 (Table 2). This unique phenotypic response of $\Delta kre1$ spheroplasts to K1 and K28 implies the existence of two different toxin receptor populations for K1 and K28 within the cytoplasmic membrane. Interestingly, intact cells of the $\Delta kre1$ mutant remained sensitive to the *Z. bailii* virus toxin zygocin, although this toxin – like K28 – specifically binds to cell wall mannoproteins as the primary zygocin receptor (see Table 3; Radler *et al.*, 1993; Weiler & Schmitt, 2003). This observation indicates that the reduced amount of 1,6- β -glucan seen in the $\Delta kre1$ mutant might also affect the expression of certain mannoproteins at the cell surface, in particular those cell wall mannoproteins that contain 1,3- α -mannotriose outer side-chains. In the case of K28 it is known that toxin binding to the cell wall receptor critically depends on the length of the outer mannoprotein side-chains and particularly requires the presence of α -1,3-linked mannotriose side-chains

Table 1. Effect of the length of Kre1p on K1 toxin sensitivity and cell surface 1,6- β -glucan levels in Kre1⁺ and Kre1[−] derivatives of *S. cerevisiae* SEY6210

Strain (allele at <i>KRE1</i> locus)	Plasmid	K1 sensitivity (mm)*		Alkali-insoluble 1,6- β -glucan†
		Intact cells	Spheroplasts	
SEY6210 (<i>KRE1</i>)	–	15	21	83.6 ± 7
	pPGK-M28-I	15	20	83.9 ± 7
	pPGK-[Δ_{1-225} KRE1]	16	20	81.3 ± 14
	pPGK-[<i>KRE1</i>]	16	20	90.5 ± 10
SEY6210 [$\Delta kre1$] ($\Delta kre1$)	–	0	0	56.2 ± 6
	pPGK-M28-I	0	0	51.3 ± 5
	pPGK-[Δ_{1-225} KRE1]	0	23	53.6 ± 5
	pPGK-[<i>KRE1</i>]	18	20	107.7 ± 5

*After transformation with the specified plasmid, K1 sensitivity was determined by the killing-zone assay (see Methods) and is expressed as diameter of growth inhibition zone detected with 10⁴ units K1 toxin on methylene blue agar plates (pH 4.7) seeded with a lawn of the indicated yeast strain.

†The amount of 1,6- β -glucan was measured as μ g alkali-insoluble glucan per mg dry weight cell wall as described in Methods.

Table 2. Sensitivity of the *S. cerevisiae* Kre1⁺ wild-type SEY6210 and its isogenic Kre1[−] mutant SEY6210 [Δ *kre1*] to viral killer toxins of *S. cerevisiae* (K1, K28) and *Z. bailii* (zygocin)

Strain	K1 sensitivity		K28 sensitivity		Zygocin sensitivity	
	Cells	Spheroplasts	Cells	Spheroplasts	Cells	Spheroplasts
SEY6210	+	+	+	+	+	+
SEY6210 [Δ <i>kre1</i>]	−	−	−	+	+	+

+, Susceptible; −, resistant.

(Schmitt & Radler, 1988). In contrast, the exact manno-protein binding site for the *Z. bailii* virus toxin zygocin has not yet been identified. Thus, in a yeast Δ *kre1* mutant, whole-cell sensitivity to zygocin and resistance to K28 might be caused by the absence of or reduction in certain 1,3- α -mannotriose-containing cell surface mannoproteins which in turn prevent K28 toxin binding without affecting cell wall binding of zygocin.

To investigate K28 toxin resistance in intact cells of the Δ *kre1* knock-out mutant, mannoproteins were isolated and partially purified from cell walls of Kre1[−] and Kre1⁺ strains. A K28-resistant mannoprotein mutant (*mn2*) was included as a control. In comparison to the *KRE1* wild-type, the Δ *kre1* mutant showed only a slight decrease in the overall content of cell wall mannoproteins (33 and 28 mg per g wet weight, respectively) while in the yeast *mn2* mutant, mannoproteins were more severely reduced (23 mg per g wet weight). However, the moderate decrease in cell wall mannoprotein content observed in the Δ *kre1* mutant could not be seen by SDS-PAGE and periodic acid-Schiff staining: both electrophoretic mobility and glycoprotein staining intensity of the isolated high-molecular-mass cell wall mannoproteins (>180 kDa) were indistinguishable in the Δ *kre1* mutant and its isogenic Kre1⁺ wild-type (data not shown). This indicates a more qualitative change in mannoprotein composition, rather than a simple reduction in the overall mannoprotein content.

The capacity of the isolated mannoproteins to adsorb K1 as well as K28 was also determined. Mannoprotein fractions of the Δ *kre1* disruption mutant showed a decreased adsorption of K28 compared to wild-type, confirming the

above-mentioned assumption that cell surface manno-proteins containing 1,3- α -mannotriose outer side-chains, which are responsible for K28 toxin binding *in vivo* and *in vitro*, are either missing or somehow under-represented in the genetic background of a Δ *kre1* null mutation (Fig. 1). Interestingly, isolated and partially purified cell wall mannoproteins could also adsorb significant amounts of K1 toxin, indicating that the mannoprotein fraction still contains 1,6- β -D-glucans. Thus, our data are consistent with observations of Kapteyn *et al.* (1996) and Kollar *et al.* (1997) that cell wall mannoproteins are embedded in a heteropolymeric matrix consisting of 1,3- β - and 1,6- β -glucans. Whereas isolated mannoproteins from an *mn2* mutant did not bind significant amounts of K28, they were still capable of binding to K1 toxin in a similar manner as Mnn2⁺ cells; this again implies that 1,6- β -glucan synthesis is not negatively affected in the *mn2* mutant.

Function of the C-terminal hydrophobic stretch of Kre1p

In Kre1p, the 21 amino acid hydrophobic tract at the C-terminus either serves as a recognition sequence for the attachment of a GPI anchor or simply acts as a trans-membrane domain. Roemer & Bussey (1995) failed to demonstrate a possible GPI-anchoring of Kre1p by labelling the GPI moiety with *myo*-[³H]inositol or by releasing Kre1p from the membrane by treatment with phosphatidylinositol-phospholipase C. To investigate the membrane anchorage of Kre1p in more detail, we phenotypically characterized a set of yeast *gpi* mutants (*gpi1*, *gpi2*, *gpi3*) which are defective in GPI anchor biosynthesis of various yeast plasma membrane and cell wall proteins due to an early block in

Table 3. Properties of yeast killer toxins K1, K28 and zygocin

Killer toxin	Structure	Receptor		Lethal effect
		Cell wall	Plasma membrane	
K1	α/β heterodimer	1,6- β -Glucan	Kre1p	Ionophore
K28	α/β heterodimer	Mannoprotein*	Unknown	Cell cycle arrest
Zygocin	Monomer	Mannoprotein*	Unknown	Ionophore

*Note that the cell wall receptors for K28 and zygocin represent different mannoproteins (adapted from Schmitt & Breinig, 2002; Weiler & Schmitt, 2003).

Fig. 1. Effect of partially purified cell wall mannoproteins (CMP) on toxin-mediated cell death of *S. cerevisiae* strain 518 after treatment with killer toxin K1 (a) or K28 (b). C.f.u. were determined in the presence of varying concentrations of CMP from the *S. cerevisiae* *KRE1* wild-type SEY6210 (■), its isogenic $\Delta kre1$ null mutant (●) and from the *mnn2* mutant X2180-m2-1a (▲).

N-acetylglucosaminyl-phosphatidylinositol (GlcNAc-PI) synthesis, a central metabolic intermediate within the first step of GPI anchor assembly (Leidich *et al.*, 1994, 1995; Leidich & Orlean, 1996). If GPI-anchoring of Kre1p was dependent on Gpi1p, Gpi2p and/or Gpi3p, then K1 sensitivity of the corresponding *gpi* mutant should decrease when mutant cells are shifted from the permissive to the semi-restrictive temperature, due to a diminished incorporation of Kre1p into the plasma membrane and – because of the function of Kre1p in cell wall 1,6- β -D-glucan assembly – a decrease in cell wall 1,6- β -D-glucan content. The *gpi* mutants and the isogenic *GPI* wild-type were incubated at the permissive (20 °C) and at the semi-restrictive (25 °C) temperatures and subsequently tested for K1 sensitivity. To check that the toxin remained stable throughout the experiment, the sensitive tester strain SEY6210 was also included as control. As shown in Fig. 2, the *GPI* wild-type strain 37-10C and its isogenic *gpi2* and *gpi3* mutants as

Fig. 2. K1 toxin sensitivity in yeast *gpi* mutants and the isogenic *Gpi1*⁺ wild-type after growth under permissive (20 °C) or semi-restrictive (25 °C) culture conditions. *S. cerevisiae* *gpi* mutant strains 2A (*gpi1*), 27-5D (*gpi2*) and 53-12C (*gpi3*) as well as the parental *Gpi1*⁺ wild-type strain 37-10C were incubated at the indicated temperature and K1 sensitivity was determined by the killing-zone assay as described in the Methods. *S. cerevisiae* SEY6210 (*Gpi1*⁺) was used as a positive control to check for K1 toxin stability at 25 °C. K1 sensitivity, determined by pipetting 10⁴ units K1 toxin into wells of a methylene blue agar plate (pH 4–7) and incubating for 3 days at 20 °C, is expressed as percentage of K1 sensitivity at 25 °C in comparison to K1 sensitivity at the permissive temperature (100 %).

well as the positive control strain SEY6210 were equally sensitive to K1 at both temperatures (20 °C and 25 °C) and showed only a moderate loss in K1 sensitivity at the elevated temperature; thus, temperature stability of K1 at 25 °C is confirmed. In contrast, *gpi1* mutant cells showed a significant decrease in K1 sensitivity after shift to the semi-restrictive temperature (25 °C), strongly indicating an essential involvement of *GPI1* in GPI-anchoring of Kre1p *in vivo*. The fact that such a phenotype was not detectable in the *gpi2* nor in the *gpi3* mutant is likely to be caused by differences in the temperature-sensitive (ts) phenotype of the corresponding *gpi1,2,3^{ts}* alleles. While *gpi1^{ts}* mutant cells showed the expected block in GPI-anchoring already at the semi-restrictive temperature of 25 °C, this temperature was too low to see a phenotype in the *gpi2/3^{ts}* mutants. Since cell growth and viability of both mutants (*gpi2/3^{ts}*) was severely impaired at temperatures above 25 °C, and K1 activity is greatly reduced by prolonged incubation at elevated temperatures, K1 toxin sensitivity could not be determined under the restrictive culture conditions at which *gpi2/3^{ts}* mutants are blocked in GPI-anchoring.

Interestingly, the amount of alkali-insoluble cell wall 1,6- β -glucan was equal at both temperatures in the *gpi1* mutant (data not shown). It therefore can be concluded that the function of Kre1p in 1,6- β -glucan synthesis and/or assembly is not negatively affected when GPI anchoring of Kre1p is at least partially functional, although its essential role in K1

Fig. 3. A GFP-tagged derivative of Kre1p is secreted into the medium after expression in a *gpi1* mutant and cultivation at the semi-restrictive temperature. The *S. cerevisiae gpi1* mutant was cultivated at the permissive (20 °C) and the semi-restrictive (25 °C) temperature and aliquots of the cell-free culture supernatant of equal cell concentrations were ethanol precipitated. The resulting proteins were separated by SDS-PAGE and, after electrotransfer to a nitrocellulose membrane, the K28/GFP/Kre1p fusion was probed with a monoclonal anti-GFP antibody. Positions of the secreted fusion protein K28/GFP/Kre1p and of the recombinant marker protein rGFP (positive control) are indicated. The low-molecular-mass protein bands seen in the culture supernatant of *gpi1* mutant cells grown at 25 °C most likely represent C-terminal degradation products of the K28/GFP/Kre1p protein fusion.

lethality of mediating toxin binding to the plasma membrane is completely lost.

To further confirm that GPI-anchoring is necessary for the *in vivo* targeting of Kre1p and its subsequent incorporation into the wall, the *gpi1* mutant was transformed with a plasmid expressing a GFP-tagged derivative of Kre1p and subsequently examined for secretion of the K28/GFP/Kre1p fusion into the culture medium before and after shifting the cells to the semi-restrictive temperature. As illustrated in Fig. 3, a signal for the secreted K28/GFP/Kre1p fusion was detectable in the culture supernatant of cells grown at 25 °C, but not at the permissive temperature, confirming that *GPI1* is involved in the transient GPI-anchoring of Kre1p.

Kre1p variants lacking a GPI-anchoring signal cannot complement a $\Delta kre1$ mutation

In yeast, GPI-anchored plasma membrane and cell wall proteins contain a conserved ω -site near their C-terminal end which signals GPI anchor addition in the endoplasmic reticulum (Caro *et al.*, 1997; de Groot *et al.*, 2003). In Kre1p, such a motif is located at amino acid position 286 (Fig. 4). To further demonstrate that the *in vivo* GPI-anchoring of Kre1p is necessary for both K1 toxin binding to the yeast cell surface and cell wall 1,6- β -glucan assembly, four different C-terminally truncated Kre1p derivatives were constructed

Fig. 4. Schematic drawing of wild-type Kre1p and the various C- and N-terminally truncated derivatives studied and their functional complementation of K1 toxin resistance and cell wall 1,6- β -D-glucan in a yeast $\Delta kre1$ null mutant. Full-length Kre1p contains an N-terminal signal peptide (SP) that ensures protein import into the yeast secretory pathway, followed by a serine/threonine-rich spacer domain and a C-terminal GPI attachment signal (ω -site). Each C-terminally truncated Kre1p variant lacks a GPI-anchoring signal and contains progressive internal deletions within its intramolecular serine/threonine-rich domain. Data for the N-terminally truncated derivatives of Kre1p were taken from Boone *et al.* (1991). The box on the left shows the phenotypes of the indicated Kre1p derivatives which were determined on the basis of cell wall 1,6- β -D-glucan and whole-cell K1 toxin sensitivity after expression in a yeast $\Delta kre1$ null mutant. + + +, wild-type 1,6- β -D-glucan (corresponding to 84 μ g alkali-insoluble glucan per mg dry weight cell wall; see also Table 1); -, significantly reduced cell wall 1,6- β -D-glucan (40–50% of Kre1⁺ wild-type); + + +, wild-type K1 toxin sensitivity; ++, reduced K1 sensitivity; +, greatly reduced K1 sensitivity; -, K1 toxin resistance; ND, not determined.

which all lacked the GPI attachment site and only differed in the length of their intramolecular serine/threonine-rich spacer sequence (Fig. 4). Interestingly, after *in vivo* expression in a yeast $\Delta kre1$ mutant, only wild-type Kre1p but none of its C-terminally truncated derivatives was capable of complementing the $\Delta kre1$ null mutation and restoring cell wall 1,6- β -D-glucan (Fig. 4). Thus, although the truncated Kre1p derivatives still possess a serine/threonine-rich spacer domain of varying length, cell wall 1,6- β -D-glucan in the $\Delta kre1$ mutant could not be restored in the absence of a C-terminal GPI attachment site. In contrast, if the intramolecular serine/threonine-rich region of Kre1p is

progressively shortened without altering or deleting its C-terminal GPI attachment site, the corresponding N-terminally truncated Kre1p derivative retains considerable partial function in complementing a $\Delta kre1$ null mutation (Boone *et al.*, 1991; Fig. 4). It therefore can be concluded that Kre1p, in its GPI-anchored and plasma membrane-bound forms, is not only essential for its *in vivo* function as a K1 membrane receptor but also for ensuring wild-type cell wall 1,6- β -D-glucan content, indicating a more structural role of Kre1p in yeast cell wall assembly and architecture.

DISCUSSION

Recently, the involvement of Kre1p in the initial binding of the K1 virus toxin to the plasma membrane of a sensitive target cell was demonstrated, and it was shown that K1 toxin binding to the cell wall of a yeast $\Delta kre1$ null mutant can only be restored by expressing the full-length Kre1p protein, not the N-terminally truncated derivative Δ_{1-225} Kre1p (Breinig *et al.*, 2002). We now extend these findings by showing that in a $\Delta kre1$ mutant, expression of wild-type Kre1p (in contrast to its N-terminally truncated derivative) enhances the amount of cell wall 1,6- β -glucans up to wild-type level. The observation of Boone *et al.* (1990) that the *kre1* disruption mutant shows a 40 % diminished amount of alkali-insoluble 1,6- β -glucan in the cell wall could be confirmed in our studies. In agreement with the phenotype observed in the toxin sensitivity/resistance assays, expression of Δ_{1-225} Kre1p did not increase the overall 1,6- β -glucan content of the cell wall, whereas expression of wild-type Kre1p did. This indicates that the N-terminal part of Kre1p (including its serine/threonine-rich segment) is responsible for the observation of Boone *et al.* (1990) that the 1,6- β -glucan fraction isolated from cell walls of a $\Delta kre1$ mutant has an altered structure with a lower mean polymer size and fewer β -1,6-linked side-chains than the corresponding glucan of a Kre1⁺ wild-type. It has been speculated that Kre1p mediates the addition of linear side-chains of β -1,6-linked glucose units to a highly branched β -1,6- and β -1,3-linked glucan backbone (Boone *et al.*, 1990). However, so far it is not known how this addition occurs, and the question arises whether this function of Kre1p is a structural or an enzymic one. Boone *et al.* (1991) have shown that deletions in the non-conserved serine/threonine-rich region of Kre1p led to a progressive loss in K1 sensitivity as the deletion increased in size, ending up with complete K1 resistance when the conserved coding region was absent. In the N-terminally truncated protein (Δ_{1-225} Kre1p), this conserved region is completely removed, leading to the inability of the fusion to restore its function in 1,6- β -glucan assembly and consequently causing a severe defect in K1 toxin binding to the outer surface of a $\Delta kre1$ mutant cell.

Moreover, the observed K1/K28 cross-resistance at the cell wall level in a yeast $\Delta kre1$ mutant suggests that resistance to K28 toxin can be attributed to the decreased amount of cell wall mannoproteins which represent the primary toxin

receptors for K28 (Schmitt & Radler, 1988). This finding is consistent with data from Kapteyn *et al.* (1997) and Kollar *et al.* (1997) showing that a major group of mannoproteins is covalently anchored in the cell wall by 1,6- β -glucans. Furthermore, it has been reported that severe 1,6- β -glucan defects in several *kre* mutants lead to the secretion of certain cell wall mannoproteins, e.g. α -agglutinin (Lu *et al.*, 1995). This result is somewhat contradictory to that of Hutchins (1982), who found that *kre1* mutants do not show a significant reduction in total cell wall mannoproteins. Our data confirm the latter observation since deletion of *KRE1* did not significantly decrease the overall amount of mannoproteins. The regained K28 toxin sensitivity of the $\Delta kre1$ mutant after removal of the cell wall demonstrates the existence of two different receptor populations for the toxins K1 and K28 at the level of the cytoplasmic membrane, as formerly speculated by Schmitt & Compain (1995). This is also confirmed by more recent studies in which it was shown that soon after K28 has bound to the cell wall, the toxin is taken up by receptor-mediated endocytosis and traverses the secretion pathway in reverse, and finally dislocates into the cytosol, where K28- α transmits the cytotoxic signal into the nucleus (Schmitt & Eisfeld, 1999; Eisfeld *et al.*, 2000).

However, the cell wall receptor of the *Z. bailii* virus toxin zygocin, which is also a cell wall mannoprotein (see Table 3), is still present in the $\Delta kre1$ mutant, indicating that the normal composition of cell wall mannoproteins is somehow altered in a Kre1⁻ genetic background. Since isolated mannoproteins from a $\Delta kre1$ mutant showed a significantly decreased K28 toxin binding when tested in an *in vivo* competition experiment, it can be speculated that the serine/threonine-rich region of Kre1p is responsible for ensuring wild-type levels of those cell wall mannoproteins which contain 1,3- α -mannotriose outer side-chains that function as primary K28 toxin receptors (Schmitt & Radler, 1988). This would suggest a more structural function of Kre1p in linking certain mannoproteins of the cell surface to cell wall glucans. The observation that a haemagglutinin-tagged derivative of Kre1p mainly localizes to the cell wall likewise supports a more structural role of Kre1p, but an enzymic function of Kre1p after its transfer to and incorporation into the wall is unlikely (Roemer & Bussey, 1995). This speculation is further supported by recent work of Terashima *et al.* (2003), who showed that changing the normal localization of a GPI-associated yeast protein from the plasma membrane to the cell wall greatly affects its cellular function.

Taken together, the data presented in this study argue against an enzymic function of Kre1p and rather suggest a more structural role within the formation of the cell wall network, possibly by being somehow involved in covalently cross-linking 1,6- β -glucans to other cell wall components such as 1,3- β -glucan, chitin and certain mannoproteins (Fig. 5). Roemer & Bussey (1995) could not detect any covalently attached 1,6- β -glucan by using a 1,6- β -glucan

in cross-linking of certain mannoproteins and cell wall 1,6- β -glucan. Additional experiments will be required to show if our current model of Kre1p *in vivo* function (as presented in Fig. 5) holds true; we intend to address this aspect in the near future.

ACKNOWLEDGEMENTS

We are grateful to Howard Bussey, Peter Orlean and Clinton Ballou for providing strains and to Beate Schmitt for technical assistance. This work was kindly supported by a grant from the Deutsche Forschungsgemeinschaft (Schm 541/11-1).

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