The protein kinase Kic1 affects 1,6- β -glucan levels in the cell wall of *Saccharomyces cerevisiae*

Edwin Vink,[¶]† Jack H. Vossen,[¶]‡ Arthur F. J. Ram,[§]
Herman van den Ende, Stephan Brekelmans, Hans de Nobel||
and Frans M. Klis

Author for correspondence: Frans M. Klis. Tel: +31 20 525 7834. Fax: +31 20 525 7056.
e-mail: klis@science.uva.nl

Swammerdam Institute for Life Sciences, University of Amsterdam, Nieuwe Achtergracht 166, 1018 WV, Amsterdam, The Netherlands

KIC1 encodes a PAK kinase that is involved in morphogenesis and cell integrity. Both over- and underexpressing conditions of KIC1 affected cell wall composition. Kic1-deficient cells were hypersensitive to the cell wall perturbing agent calcofluor white and had less 1,6- β -glucan. When Kic1-deficient cells were crossed with various *kre* mutants, which also have less 1,6- β -glucan in their wall, the double mutants displayed synthetic growth defects. However, when crossed with the 1,3- β -glucan-deficient strain *fks1 Δ* , no synthetic growth defect was observed, supporting a specific role for KIC1 in regulating 1,6- β -glucan levels. Kic1-deficient cells also became highly resistant to the cell wall-degrading enzyme mixture Zymolyase, and exhibited higher transcript levels of the cell wall protein-encoding genes *CWP2* and *SED1*. Conversely, overexpression of KIC1 resulted in increased sensitivity to Zymolyase and in a higher level of 1,6- β -glucan. Multicopy suppressor analysis of a Kic1-deficient strain identified *RHO3*. Consistent with this, expression levels of *RHO3* correlated with 1,6- β -glucan levels in the cell wall. Interestingly, expression levels of KIC1 and the MAP kinase kinase *PBS2* had opposite effects on Zymolyase sensitivity of the cells and on cell wall 1,6- β -glucan levels in the wall. It is proposed that Kic1 affects cell wall construction in multiple ways and in particular in regulating 1,6- β -glucan levels in the wall.

Keywords: KIC1/CWH30, PBS2, RHO3, cell wall synthesis

INTRODUCTION

Saccharomyces cerevisiae is protected from extracellular challenges by its cell wall. These challenges can vary from hypo-osmotic stress to mechanical damage and

toxic compounds from other organisms. The yeast cell wall consists of 1,3- β -glucan, 1,6- β -glucan, chitin and mannoproteins, which are interconnected in an ordered manner (Klis *et al.*, 2002). Cell wall construction and composition are highly dynamic: the composition and structure of the newly formed cell wall are continuously adjusted in response to extracellular conditions, and even to progress in the cell cycle. This indicates that cell wall construction is highly regulated.

The cell wall perturbing agent calcofluor white has been a valuable tool in identifying mutants with a defective cell wall (Roncero *et al.* 1988; Ram *et al.* 1994; Lussier *et al.* 1997; De Groot *et al.*, 2001). Analysis of mutants hypersensitive to calcofluor white has resulted in the identification of numerous genes involved in different aspects of cell wall biogenesis (Ram *et al.*, 1995; Jiang *et al.*, 1995, 1996; Vossen *et al.*, 1995; Van Berkel *et al.*, 1999). In addition, screening for mutants resistant to this

† Present address: AMC Liver Center, Meibergdreef 69-71, 1105 BK, Amsterdam, The Netherlands.

‡ Present address: Section of Plant Pathology, Swammerdam Institute for Life Sciences, University of Amsterdam, Kruislaan 318, 1098 SM, Amsterdam, The Netherlands.

§ Present address: Institute of Molecular Plant Sciences, Leiden University, Wassenaarseweg 64, 2333 AL, Leiden, The Netherlands.

|| Present address: Genencor International B. V., PO Box 218, 2300 AE, Leiden, The Netherlands.

¶ These authors contributed equally to this work.

Abbreviations: HOG, high osmolarity glycerol response; MAPK, mitogen-activated protein kinase; SPB, spindle pole body.

compound has led to the identification of several genes involved in chitin biosynthesis (Roncero *et al.*, 1988).

The *KIC1* gene encodes an essential protein kinase, which is involved in cell integrity and morphogenesis. This kinase was identified in a two-hybrid screen with the yeast centrin *CDC31*. The *in vitro* kinase activity of Kic1 was found to be dependent on *CDC31*. However, *KIC1* did not share the *CDC31* functions in spindle pole body (SPB) duplication, but rather revealed a novel function for *CDC31* (Sullivan *et al.*, 1998). This was further supported by a mutational analysis of *CDC31*, which resulted in dissection of the SPB-related functions and *KIC1*-related functions (Ivanovska & Rose, 2001).

The PKC mitogen-activated protein kinase (MAPK) pathway is commonly known as the cell (wall) integrity pathway, although this is certainly not the only MAPK pathway that has an effect on cell wall biosynthesis and composition (Klis *et al.*, 2002). For instance, activation of the pheromone response pathway results in the formation of a mating projection and considerable alterations in the cell wall. In this case, at least part of this may be coordinated through the PKC MAPK pathway (Buehrer & Errede, 1997). Evidence is also accumulating that the HOG (high osmolarity glycerol response) MAPK pathway plays a role in cell wall assembly. Overexpression of *PBS2* – the MAP kinase kinase of this pathway – results in resistance to the K1 killer toxin (Jiang *et al.*, 1995) and to laminarinase (Lai *et al.*, 1997), an enzyme preparation that contains 1,3- β -glucanase as its main activity. In addition, mutations in either *PBS2* or *HOG1* results in calcofluor white resistance (García-Rodríguez *et al.*, 2000).

K1 killer toxin is a powerful tool for identifying mutants with defects in 1,6- β -glucan synthesis and/or assembly (Al-Aidroos & Bussey, 1978; Boone *et al.*, 1990; Brown *et al.*, 1993). This pore-forming protein needs to bind its acceptors (1,6- β -glucan and Kre1) to perform its lethal action (Bussey, 1991; Breinig *et al.*, 2002). Mutants defective in the biogenesis of 1,6- β -glucan hold less of this acceptor, and therefore are resistant to this killer toxin. Several genes have been identified whose gene products mainly localize throughout the secretory pathway (reviewed by Shahinian & Bussey, 2000). How the synthesis of 1,6- β -glucan is regulated is largely unknown.

Here we describe the identification and characterization of *CWH30*, which is allelic to the previously described *KIC1* gene. A Kic1-deficient strain is not only hypersensitive to calcofluor white, but is also resistant to Zymolyase, a cell wall degrading enzyme mixture, indicating that its cell wall is affected. We show that mutation of *KIC1* results in K1 killer toxin resistance and decreased levels of 1,6- β -glucan. Furthermore, *KIC1* expression levels were found to correlate with 1,6- β -glucan levels in the cell wall. Multicopy suppressor analysis of a Kic1-deficient strain identified *RHO3* which itself was found to strongly affect 1,6- β -glucan levels. We propose that *KIC1* is involved in regulating the 1,6- β -glucan levels in the cell.

METHODS

Strains and media. The yeast strains used in this study are listed in Table 1. The strains were grown in YPD [1% (w/v) yeast extract, 1% (w/v) Bacto Peptone, 3% (w/v) glucose], YPGal [1% (w/v) yeast extract, 1% (w/v) Bacto Peptone, 3% (w/v) galactose] or in SD [0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate, 2% (w/v) glucose, 0.5% (w/v) ammonium sulfate, buffered at pH 6.0 with 1% (w/v) MES] supported with the necessary amino acids, at 28 °C or 37 °C. For solid media, 2% (w/v) Bacto Agar was added. The K1 killer assays were performed in either YPD or SD media, which for this purpose were buffered at pH 4.7 using 3% (w/v) sodium citrate and supplemented with 0.003% methylene blue. Yeast genetics, sporulation and transformation followed established procedures (Sherman & Hicks, 1991). *Escherichia coli* strain DH5 α was used for propagation of all plasmids and was grown in LB medium [1% (w/v) Bacto Tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract]. Yeast extract, Bacto Peptone, Bacto Tryptone, yeast nitrogen base and Bacto Agar were all from Difco Laboratories.

Strain construction. Strains JV67 and JV68 were constructed by transforming strains HAB251-15B and AR835 with *KIC1* disruption constructs, using the *HIS3* and *TRP1* markers, respectively. The disruption constructs were created according to Berben *et al.* (1991). Primers used for this purpose are listed in Table 2. Correct integration of the disruption constructs was confirmed by Southern analysis, using the 4 kb *HindIII* fragment from plasmid p14 as a probe (see below). JV80 and JV83 were haploid offspring of JV68 and JV67, respectively. JV142 and JV143 were haploid offspring of JV67 transformed with the *P_{GALI}:KIC1* plasmid (see below). JV144 and JV145 were offspring of JV68 transformed with the *P_{GALI}:KIC1* plasmid. These four haploid strains propagated the *P_{GALI}:KIC1* plasmid even without selective pressure.

JV202 was the progeny of the JV143 $\times$ HAB813 diploid. JV215 was a haploid offspring of the JV143 $\times$ TR95 diploid. JV220 was a haploid descendant from the JV143 $\times$ HAB637-1A diploid. The JV168 strain was a haploid descendant from the JV145 $\times$ AR100 diploid. JV264 was constructed in the FY833 background, using a *PBS2* disruption construct with a *HIS3* marker. This construct was created as described by Berben *et al.* (1991), and correct integration was confirmed by PCR. Primers are all listed in Table 2. JV268 resulted from the JV144 $\times$ JV264 diploid.

Strains EV116 and EV077 were constructed in the FY834 background, using PCR-generated disruption constructs with the *HIS3* marker (Berben *et al.*, 1991). Primers are listed in Table 2. Correct integration was confirmed by PCR.

Plasmids, oligonucleotides and recombinant DNA techniques. For the cloning of the *KIC1/CWH30* gene, a YCp50-based genomic library was used (Rose *et al.* 1987). The plasmid that could complement the *cwh30-1* mutant was named p14. The 4 kb *HindIII* fragment containing the complete *KIC1* ORF, was subcloned into YEplac195 (Gietz & Sugino, 1988) and named p61. Plasmid p62 was isolated from a YEp13-based genomic library based on its ability to complement the *cwh30-1* mutant.

DNA handling and manipulation were carried out according to Sambrook *et al.* (1989). DNA sequencing was performed as described by Sanger *et al.* (1977), using T7 DNA polymerase (Pharmacia). Restriction enzymes, nucleotides, Klenow fragment and alkaline phosphatase were all from Pharmacia. DNA ligase was purchased from Gibco-BRL, SuperTaq polymerase was from HT Biotechnology, Expand high fidelity

Table 1. Yeast strains used in this study

Strain	Genotype	Source/reference
SEY6210	MAT α <i>leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9</i>	S. D. Emr (University of California, San Diego, CA, USA)
HAB251-15B	MAT α / α SEY6210 autodiploid	Roemer & Bussey (1991)
FY833	MAT α <i>his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63</i>	Winston <i>et al.</i> (1995)
FY834	MAT α <i>his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63</i>	Winston <i>et al.</i> (1995)
AR835	MAT α / α FY833 $\times$ FY834	A. F. J. Ram
T158C/S14a	Killer toxin producing strain	Bussey <i>et al.</i> (1979)
<i>cwh30-1</i>	MAT α <i>ura3-52 cwh30-1</i>	Ram <i>et al.</i> (1994)
JV67	HAB251-15B <i>kic1::HIS3</i>	This study
JV68	AR835 <i>kic1::TRP1</i>	This study
JV80	MAT α <i>his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 kic1::HIS3</i>	This study
JV83	MAT α <i>leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kic1::HIS3</i>	This study
JV141	MAT α <i>leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kic1::TRP1 + P_{GAL1}:KIC1</i>	This study
JV142	MAT α <i>leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kic1::HIS3 + P_{GAL1}:KIC1</i>	This study
JV143	MAT α <i>leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kic1::HIS3 + P_{GAL1}:KIC1</i>	This study
JV144	MAT α <i>his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 kic1::TRP1 + P_{GAL1}:KIC1</i>	This study
JV145	MAT α <i>his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 kic1::TRP1 + P_{GAL1}:KIC1</i>	This study
AR100	MAT α <i>his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 fks1::HIS3</i>	Ram <i>et al.</i> (1998)
HAB637-1A	MAT α <i>leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kre1::HIS3</i>	Boone <i>et al.</i> (1990)
HAB813	MAT α <i>leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kre9::HIS3</i>	Brown & Bussey (1993)
TR95	MAT α <i>leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kre6::HIS3</i>	Roemer & Bussey (1991)
JV168	MAT α <i>his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 fks1::HIS3 kic1::TRP1 + P_{GAL1}:KIC1</i>	This study
JV202	MAT α <i>leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kre9::HIS3 kic1::HIS3 + P_{GAL1}:KIC1</i>	This study
JV215	MAT α <i>leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kre6::HIS3 kic1::HIS3 + P_{GAL1}:KIC1</i>	This study
JV220	MAT α <i>leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kre1::HIS3 kic1::HIS3 + P_{GAL1}:KIC1</i>	This study
JV264	MAT α <i>his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 pbs2::HIS3</i>	This study
JV268	MAT α <i>his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 kic1::TRP1 pbs2::HIS3 + P_{GAL1}:KIC1</i>	This study
EV116	MAT α <i>his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 rho3::HIS3</i>	This study
EV077	MAT α <i>his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 rho4::HIS3</i>	This study

polymerase was from Boehringer Mannheim and oligo-nucleotides were from Eurogentec.

Cloning of KIC1 behind the GAL1 promoter. The Expand high fidelity polymerase was used to create a KIC1 fragment with a 5' *Xba*I restriction site, followed by three bases of the 5' untranslated region (UTR) of KIC1 and then the KIC1 ORF. The first 500 bases of the 3' UTR were included in this fragment, which was followed by an *Xho*I restriction site. The primers used for this are listed in Table 2. This PCR generated fragment was digested with *Xba*I and *Xho*I, and subsequently cloned into the corresponding sites of the pYEura3 plasmid (Clontech), resulting in the P_{GAL1}:KIC1 plasmid.

Multicopy suppressor screen. A high-copy pMA3a-based genomic library [kindly provided by M. Crouzet (University of Bordeaux II, France) & M. F. Tuite (University of Kent, UK)] with a mean insert size of 5–10 kb was transformed to strain JV141, which contains *kic1::TRP1* and carries the P_{GAL1}:KIC1 plasmid. A total of 3500 transformants were

replica-plated on selective SD medium containing 100 μ g calcofluor white ml⁻¹. Viable colonies were isolated, library plasmids were recovered and retransformed into the JV141 strain. Serial dilutions of transformants of these strains were spotted on selective SD medium containing 50 μ g calcofluor white ml⁻¹. The seven plasmids that showed the best suppression were selected for further analysis, and were found to contain five unique inserts (Table 3). Clone 2 contained amongst others the *RHO3* gene, which proved to be the gene responsible for the suppression. A 2.2 kb *Sall*–*Xho*I fragment containing the *RHO3* ORF was removed from clone 2 and cloned into a YEplac181 vector (Gietz & Sugino, 1988) resulting in the pEV021 plasmid. The remaining part of clone 2 was religated, and the resulting plasmid lost the ability to suppress the *kic1* mutant, whereas plasmid pEV021 retained the ability to suppress the *kic1* mutant. Clones 11 and 13 contained an identical insert, and clone 11 was subjected to further analysis. A 2.4 kb *Xho*I–*Sall* fragment (the *Sall* restriction site was located in the pMA3a plasmid, 0.3 kb from

Table 2. Oligonucleotide primers used in this study

Name	Sequence (5'–3')
<i>KIC1</i> forward disruption	CAGTAATGACGACGAAGCCACAAAATAGTAAGCAGGGTTTAGCCGAAGGAGAATTCCCGGGGATCCG
<i>KIC1</i> reverse disruption	CAGAACCTGGGGTTTCCTGTCTACCCGCCCTCTGTTTTCTGGCAGAAACGAAGCTAGCTTGGCTGCAG
P_{GAL1} : <i>KIC1</i> forward	ACTCTAGATAATGACGACGAAGCCAC
P_{GAL1} : <i>KIC1</i> reverse	TTCTCGAGTCTAGGCGCGTTTATAAG
<i>PBS2</i> forward disruption	AAGATGGAAGACAAGTTTGCTAACCTCAGTCTCCATGAGAAAACTGGTAAGAATTCCCGGGGATCCG
<i>PBS2</i> reverse disruption	ACGCTATAAACCACCCATATGTAATGCCGGTACATTTTTAGATAAACCATAAGCTAGCTT7GGCTGCAG
<i>PBS2</i> forward control	CAGATCGAGACGTTAATTTCTCAA
<i>PBS2</i> reverse control	TCACGTGCCTGTTTGCTTTT
<i>RHO3</i> forward disruption	AACATGTCATTTCTATGTGGGTCAGCTTCAACGTCAAATAAACCGATCGAGAATTCCCGGGGATCCG
<i>RHO3</i> reverse disruption	ATATTACATAATGGTACAGCTGGATCCACTGTCACTTTTCACTTCGGTTGAAGCTAGCTTGGCTGCAG
<i>RHO4</i> forward disruption	TTCATGAATACACTATTATTTAAGCGAAAAGGTGGCAATTGTGGGAACGAGAATTCCCGGGGATCCG
<i>RHO4</i> reverse disruption	TTCTTACATTATAATACACTTGTTTTTTCTTAATCTTTTCGTTCCGGAAGCTAGCTTGGCTGCAG

Table 3. High copy suppressors of *KIC1*

Clone no.	Chromosome	Coordinates	Complete ORFs*
2	9	135225–144766	YIL120w, <i>RPI1</i> , <i>RHO3</i> , YIL117c, <i>HIS5</i> , <i>NUP159</i>
11/13†	14	523677–531475	VAC7, <i>MSG5</i>
23/44†	4	791592–798528	<i>STB3</i>
24	11	487450–497450‡	<i>GCN3</i> , YKR027w, <i>SAP190</i>
43	2	243957–253522	YBR004c, YBR005w, <i>UGA2</i> , YBR007c

* Bold type is used in cases where the ORF responsible for the suppression has been determined by subcloning.

† Two clones with identical inserts were identified.

‡ This clone was only sequenced from the left flank. The right flank was estimated based on mean insert size and restriction analysis.

the *Bam*HI site which was used for the insertion of the genomic fragments) was cloned into YEplac181 resulting in plasmid pEV017, which contained the *MSG5* ORF and could suppress the *kic1* mutant. Clone 23 and 44 also showed an identical insert, in which the *STB3* gene was the only complete ORF. In addition, 2.3 kb of the 3' end of the *SEC7* coding sequence was present in this insert. This 2.3 kb fragment was removed by an *Xho*I/*Sal*I digest of clone 44, using the *Xho*I site in the insert and the *Sal*I site in the pMA3a vector, 0.3 kb from the insert. The resulting plasmid (pEV020) retained the ability to suppress the *kic1* mutant.

Phenotypic screens. Calcofluor white sensitivity was analysed as described previously (Ram *et al.* 1998). Precultures were concentrated or diluted to OD₅₃₀ 10. Subsequently, ten-fold dilution series were made of which 4 μ l of each dilution was spotted onto YPD or SD plates containing 0, 10 and 50 μ g calcofluor white ml⁻¹. Plates were incubated for 3 days at 28 °C.

K1 killer toxin sensitivity was measured using the halo assay (Brown *et al.* 1994), with some modifications. Precultures were concentrated or diluted to OD₅₃₀ 10 and 45 μ l cells were seeded in 13 ml killer agar medium. On the surface, 5 μ l of a dilution series of 10⁰, 5 $\times$ 10⁻¹, 10⁻¹, 5 $\times$ 10⁻² and 10⁻² of isolated killer toxin was spotted. Killer toxin was isolated according to Brown *et al.* (1994). In short, the medium of the K1 killer toxin-producing strain was concentrated 1000-fold by ultrafiltration using a 10 kDa Amicon filter and was used as such in the halo assay. In all experiments, samples and controls were treated with toxin from the same isolation. Plates were incubated for 4–6 days at 20 °C. The diameters of the haloes were measured for each toxin dilution. Relative apparent sensitivities were calculated according to Reneke *et al.* (1988). In short, the diameter of the growth inhibition zone is proportional to the logarithm of the K1 killer toxin dose applied in the centre of the zone. Plotting these parameters against each other allows estimation of the dose required to produce an inhibition zone of a given diameter. The ratio of this dose estimated for wild-type cells divided by the dose found for mutant cells is termed the 'relative apparent sensitivity'.

Zymolyase sensitivities were measured essentially as described by De Nobel *et al.* (1990). Yeast strains were grown to equivalent optical densities, and 1 OD₅₃₀ unit was taken for analysis. Cells were washed once and resuspended in 900 μ l 10 mM Tris/HCl pH 7.5. The OD₅₃₀ was followed for 1 h after the addition of 100 μ l Zymolyase 20T (10 mg ml⁻¹ in

10 mM Tris/HCl pH 7.5; Zymolyase 20T was from Kirin Brewery).

Isolation of cell walls. Cell walls from cells grown to early exponential phase were isolated according to Van Rinsum *et al.* (1991). Walls were extracted twice in 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 2% (w/v) SDS, 0.3% (v/v) β -mercaptoethanol for 5 min at 100 °C. Walls were extensively washed in distilled water and subsequently freeze-dried.

Determination of 1,6- β -glucan levels. The levels of 1,6- β -glucan in the alkali-insoluble cell wall fraction were basically determined in accordance with Brown *et al.* (1994). Essentially, cells were grown for 24 h at 28 °C to stationary phase, harvested and washed twice in distilled water. Samples were split up into four aliquots and three fractions were each three times extracted in 3% (w/v) NaOH at 75 °C for 1 h. The remaining fraction was freeze-dried and used for the determination of the cell dry weight. The alkali-insoluble material was washed twice in 100 mM Tris/HCl, pH 7.5 and then washed in distilled water. The pellet was resuspended in 10 mM Tris/HCl, pH 7.5, 10% (v/v) glycerol, 1 mg Zymolyase 100T ml⁻¹ (Kirin Brewery) and incubated at 37 °C overnight. Following incubation, samples were centrifuged at 14000 g for 5 min, and the supernatant was dialysed against distilled water using a Spectra/POR 3 (6000–8000 molecular mass cut-off) dialysis membrane. The glucose content of the residue – 1,6- β -glucan and chitin – was determined by the phenol/sulfuric acid method (Dubois *et al.*, 1956). In later experiments, the procedure was as follows. Cells were grown for 24 h in YPD to early stationary phase, washed twice in 30 mM Tris/HCl, 1 mM EDTA, pH 7.4, and collected in five aliquots. Two were freeze-dried to quantify the cell dry weight, whereas the three other samples were resuspended in 600 μ l 50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, pH 7.4 and broken with glass beads. Walls were collected and extracted twice in 50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, 2% SDS, 40 mM β -mercaptoethanol, pH 7.4, at 100 °C for 5 min. Walls were extensively washed in distilled water and suspended in 10 mM Tris/HCl, pH 7.5, with 1 mg Zymolyase 100T ml⁻¹ (w/v) (Kirin Brewery) and incubated at 37 °C for 16 h. Following incubation, solutions were centrifuged for 5 min at 15000 g and the supernatant was dialysed against distilled water using a Spectra/POR 3 (3500 molecular mass cut-off) dialysis membrane. The residue was hydrolysed in 2 M trifluoroacetic acid at 100 °C for 4 h, freeze-dried, and subsequently glucose levels were determined using the D-glucose oxidase assay.

Northern analysis. RNA was isolated from early exponential phase cells with hot acidic phenol (Ausubel *et al.*, 1998). Fifteen microgrammes of RNA was loaded on a 1% agarose gel containing 2.4% formaldehyde. Following electrophoresis, the RNA was blotted onto Hybond-N⁺ (Amersham) through capillary paper transfer and UV cross-linked to the membrane.

RESULTS

CWH30 is allelic to KIC1

The *cwh30-1* mutant was isolated in a general screen for cell wall mutants (Ram *et al.*, 1994), based on their hypersensitivity to the cell wall perturbing agent calcofluor white. The gene mutated in *cwh30-1* was identified by functional complementation of the hypersensitivity to calcofluor white, using a YCp50-based genomic library. Out of 40000 transformants, only 15 were able to grow in the presence of 50 µg calcofluor white ml⁻¹. The plasmids from these transformants were recovered and upon retransformation only five were able to complement the calcofluor white hypersensitivity of the *cwh30-1* mutant. All five complementing plasmids contained the same genomic insert (data not shown). The complementing activity of this insert resided in a 4.4 kb fragment that remained after *Hind*III truncation of the fragment, but was lost after further truncation to 4.2 kb with *Xba*I. The 4.4 kb fragment contained the *YHR102w* ORF, which previously has been named *KIC1* for kinase interacting with Cdc31 (Sullivan *et al.*, 1998). The *Xba*I truncation removed part of the 5' upstream region of the *KIC1* gene, which seemed essential for complementation of the mutant. Additional evidence that *CWH30* was allelic to *KIC1* came from the resulting diploid of the *cwh30-1* mutant and the *kic1* disruption strain JV143. The JV143 strain has been disrupted for *kic1* but is supported by a plasmid-borne *P_{GAL1}:KIC1* fusion. When the diploid was cultured on glucose-containing medium, the calcofluor white hypersensitivity caused by the recessive *cwh30-1* mutation was not complemented (not shown), indicating that *CWH30* is allelic to *KIC1*.

Disruption of KIC1

To generate a *KIC1* knock-out mutant, the *KIC1* gene was replaced by the *TRP1* marker in the diploid AR835 wild-type strain, and by the *HIS3* marker in the diploid HAB251-15B wild-type strain. For both heterozygous diploids (JV68 and JV67), tetrad analysis resulted in two wild-type colonies and two very poorly growing mutant colonies (data not shown). The poor growth of the *kic1Δ* strain could not be suppressed by osmotic support of the medium (data not shown). In addition our results show that mutant spores failed to germinate on medium with galactose as the sole carbon source (data not shown). Microscopic analysis showed that *kic1Δ* cells were enlarged and round, forming large clumps, indicating a cell separation defect which is in accordance with previously described phenotypes of *kic1* mutants (data not shown). In addition, the cells were very sensitive to pipetting and centrifugation. Fig. 1 shows that the *kic1Δ* mutant displayed calcofluor white hypersensitivity. This

Fig. 1. Both *kic1Δ* and *P_{GAL1}:KIC1* strains are calcofluor white hypersensitive. Tenfold serial dilutions of precultures of wild-type (FY834), *kic1Δ* (JV80) and *P_{GAL1}:KIC1* (JV144) were spotted onto YEPD and YEPD containing 50 µg calcofluor white ml⁻¹. Cells were grown for 2 days at 28 °C. JV80 grows faster than the original *kic1Δ* strain, probably because it has acquired a second-site suppressor mutation.

was similar to that of the *cwh30-1* mutant (Ram *et al.*, 1994; data not shown). The morphological defects, the hypersensitivity to calcofluor white and the fragility of the cells are all in agreement with a role in cell wall integrity for *KIC1*.

Construction of *P_{GAL1}:KIC1*

The *kic1Δ* cells were not only very fragile, but they also had low transformation efficiencies, and even heterozygous diploids sporulated very poorly. In addition, *kic1Δ* cells occasionally developed second-site suppressor mutations. To circumvent the technical problems of working with a *kic1Δ* strain, a repressible *KIC1* allele was constructed by placing it under the control of the *GAL1* promoter in the pYEura3 plasmid. The activity of the *P_{GAL1}:KIC1* construct was confirmed by its ability to complement the *kic1Δ* growth defect (data not shown). This plasmid was transformed into a diploid heterozygous for *kic1* and this strain was sporulated. When germinated on YPGal medium, spores lacking the endogenous *KIC1* gene grew indistinguishably from spores with the endogenous *KIC1* gene (data not shown). However, when germinated on YEPD medium, the spores lacking the endogenous *KIC1* gene had a noticeable growth defect, albeit not as severe as a *kic1Δ* strain without the *P_{GAL1}:KIC1* plasmid (data not shown). This suggests that under glucose repression conditions there still is some expression of the *KIC1* gene. Under these conditions however, cells still display calcofluor white hypersensitivity and other defects in cell wall integrity (see Figs 1, 2 and 4). The *kic1Δ* mutant carrying the *P_{GAL1}:KIC1* plasmid will be referred to as the *P_{GAL1}:KIC1* mutant. Interestingly, when grown on nonselective media (e.g. YEPD), the *kic1Δ* strain was not cured of the *P_{GAL1}:KIC1* plasmid. In addition, no viable colonies were found when *P_{GAL1}:KIC1* cells were put on media containing 5'-FOA (data not shown).

P_{GAL1}:KIC1 mutant cells are resistant to Zymolyase

Zymolyase is a commercial enzyme preparation with both 1,3-β-glucanase and protease activities, which can

(d)

Fig. 2. *KIC1* gene dosage affects cell wall composition. (a) Cells from wild-type (□), *kic1*Δ (◆), *P_{GAL1}:KIC1* (●), and wild-type with 2μ *KIC1* (△) were precultured, washed and incubated in the presence of Zymolyase 20T. (b) Isolated cell walls of wild-type (□), *kic1*Δ (◆), *P_{GAL1}:KIC1* (●), and wild-type with 2μ *KIC1* (△) were incubated in the presence of Zymolyase 20T. (c) *KIC1* and *PBS2* have opposing effects on Zymolyase sensitivity. Wild-type (□), *P_{GAL1}:KIC1* (◆), *pbs2*Δ (●) and *P_{GAL1}:KIC1 pbs2*Δ (△) cells were precultured, washed and incubated in the presence of Zymolyase 20T. (d) mRNA levels of cell wall protein encoding genes *SED1*, *CWP2* and *CWP1* in wild-type and the *P_{GAL1}:KIC1* mutant. Actin mRNA levels are shown as a loading reference.

be used to assay differences in cell wall structure and composition (De Nobel *et al.*, 1990; Ram *et al.*, 1994; De Groot *et al.*, 2001). Wild-type cells were sensitive to

treatment with Zymolyase 20T, whereas *Kic1*-deficient cells were resistant (Fig. 2a). Conversely, *KIC1* expressed from a high-copy plasmid resulted in hypersensitivity to

Fig. 3. *KIC1* expression levels affect K1 killer toxin sensitivity. Relative apparent sensitivity to the K1 killer toxin was determined as described in Methods. (a) Some examples of the halo plate assay. Tenfold dilution series of isolated K1 killer toxin were spotted onto seeded plates. After 4 days at 20 °C, halo diameters were measured and relative apparent sensitivities were calculated. (b) Relative apparent sensitivities of several strains. Strains displayed in the upper panel were grown on SD-based medium with selective amino acid mix. Strains displayed in the lower panel were grown in YEPD-based medium.

Zymolyase (Fig. 2a). The major substrate for Zymolyase, i.e. 1,3- β -glucan, forms the inner layer of the cell wall. The outer layer consists of mannoproteins, which in intact cells limits the permeability to macromolecules (Zlotnik *et al.*, 1984; De Nobel *et al.*, 1990). It is conceivable that (at least part) of the Zymolyase sensitivity of intact cells can be attributed to changes in the protein outer layer and thus in cell wall permeability. One way to investigate this is to compare the Zymolyase sensitivity of intact cells and isolated walls, in which the inner layer is now exposed to Zymolyase. The increase in Zymolyase resistance of cell walls from the P_{GAL1}::*KIC1* mutant was much less dramatic compared to intact cells (Fig. 2b), but was still significant. Cell walls derived from a strain with high copy numbers of *KIC1* now were more resistant to Zymolyase than wild-type (Fig. 2b). Taken together, these data suggest that *KIC1* affects cell wall permeability. This might be caused by altered mannoprotein levels (see below) and possibly to some extent altered glucan levels.

Expression of some cell wall proteins is altered in the *kic1* mutant

The mRNA expression levels of some known cell wall proteins in the P_{GAL1}::*KIC1* mutant showed increased levels of both *SED1* and *CWP2* in comparison to wild-

type levels (Fig. 2d). *CWP1* mRNA levels, however, remained unaffected (Fig. 2d). Interestingly, both *sed1Δ* and *cwp2Δ* mutants are more sensitive to Zymolyase than wild-type (Van der Vaart *et al.*, 1995; Shimoi *et al.*, 1998). Overexpression of *SED1* resulted in Zymolyase resistance (Shimoi *et al.*, 1998). This strongly suggests that at least part of the Zymolyase resistance of the P_{GAL1}::*KIC1* mutant is caused by increased levels of *SED1* and *CWP2*.

The P_{GAL1}::*KIC1* mutant is more resistant to K1 killer toxin

K1 killer toxin has been a powerful tool for identifying genes involved in 1,6- β -glucan synthesis. Lower sensitivity to this toxin is generally associated with decreased levels of 1,6- β -glucan in the cell wall, which is a receptor for the toxin (Boone *et al.*, 1990; Brown *et al.*, 1993). K1 killer toxin sensitivities were compared using the halo assay. Fig. 3(a) shows the halo assays of some strains tested, to exemplify the difference in sensitivity. The K1 killer toxin sensitivity of various strains compared to their corresponding wild-type, is depicted graphically in Fig. 3(b). Note that the overexpression studies were performed on supplemented SD-based media, as opposed to the other strains which were tested on YEPD media. The halo assays performed on SD-

Table 4. *KIC1* gene dosage affects 1,6- β -glucan levels

Strain	Alkali-insoluble 1,6- β -glucan (%)*
Wild-type	100
<i>kic1</i> Δ ‡	72 $\pm$ 7
Wild-type†	100
<i>kic1</i> Δ †‡	71 $\pm$ 8
Wild-type + 2 μ <i>KIC1</i> †	112 $\pm$ 1

* Measured as μ g alkali-insoluble glucan (mg dry weight cell wall)⁻¹ and expressed as percentages relative to wild-type as described in Methods. Values represent mean $\pm$ SEM ($n=3$).

† These strains were grown on SD medium.

‡ Strain with improved growth rate, probably resulting from a second-site suppressor mutation.

based media commonly showed larger haloes than on YEPD media, but the sensitivities compared to wild-type remained consistent.

The sensitivity of the *kic1* Δ mutant (JV83) to the K1 killer toxin was very low (not shown). The *P_{GAL1}:KIC1* mutant (JV142) retained a low sensitivity to the toxin, although not to the same extent as the deletion mutant. Conversely, overexpression of *KIC1* in wild-type (JV39 with plasmid p62) resulted in an increased sensitivity to the K1 killer toxin (see Fig. 3b). The *KIC1* gene dosage effect on K1 killer toxin sensitivity might reflect altered 1,6- β -glucan levels.

KIC1 affects cell wall 1,6- β -glucan levels

To determine if the changes in K1 killer toxin sensitivity can be attributed to changes in 1,6- β -glucan levels, the alkali-insoluble cell wall fraction was analysed. As expected, the *kic1* Δ strain showed a marked decrease in 1,6- β -glucan levels (Table 4). Consistently, overexpression of *KIC1* resulted in a slight increase in 1,6- β -glucan levels (Table 4). These data are in agreement with the data from the K1 killer toxin assay. The gene dosage effect of *KIC1* on 1,6- β -glucan levels suggests a role for *KIC1* in 1,6- β -glucan deposition.

The *P_{GAL1}:KIC1* mutant displays synthetic growth defects with *kre* mutants

The proposed role of *KIC1* in 1,6- β -glucan deposition in the cell wall is further supported by the strong genetic interaction occurring between a *P_{GAL1}:KIC1* mutant and *kre* mutants, which have lowered 1,6- β -glucan levels in the cell wall. The *P_{GAL1}:KIC1* mutant was crossed with *kre6* Δ , *kre9* Δ and *kre1* Δ mutants, and the resulting diploids were sporulated. Double mutants were selected on galactose-containing media and the growth phenotypes were analysed on glucose-containing media. *kic1* showed a strongly enhanced growth defect with both *kre6* Δ and *kre9* Δ . Interestingly, when the *P_{GAL1}:KIC1* mutant was crossed with *kre1* Δ , enhancement of the

Fig. 4. *KIC1* displays synthetic growth phenotypes with several *kre* mutants. The *P_{GAL1}:KIC1* mutant strain was crossed with *kre6* Δ , *kre9* Δ , *kre1* Δ and *fks1* Δ . Tetrads were dissected on YPGal, and the double and single mutants were tested for growth on YEPD in tenfold serial dilutions.

growth defect was minor (Fig. 4). In contrast, *KIC1* did not show an enhanced growth defect with *FKS1*, a mutant impaired in 1,3- β -glucan synthesis (Fig. 4). This suggests that the growth defects of *Kic1*-deficient cells are to a large extent related to the biogenesis of 1,6- β -glucan, and offers further support for the notion that *KIC1* is involved in the regulation of 1,6- β -glucan biogenesis.

Multicopy suppressor screen of the calcofluor white sensitivity of *P_{GAL1}:KIC1* mutant

To further elucidate the regulatory function of *KIC1* in cell wall biosynthesis, we introduced a high-copy pMA3a-based genomic library into the *P_{GAL1}:KIC1* mutant strain JV141. Three thousand five hundred transformants were replica-plated on selective SD medium containing 100 μ g calcofluor white ml⁻¹. Plasmids isolated from the surviving colonies were retransformed into JV141 and the original *cwh30-1* point mutant to ensure plasmid-dependent suppression of the calcofluor white phenotype. Serial dilutions of cells were spotted

Fig. 5. Multicopy suppressors of the calcofluor white hypersensitivity of the *P_{GAL1}::KIC1* mutant. (a) Tenfold serial dilutions of wild-type (FY69)+ YEplac181, *P_{GAL1}::KIC1* (JV141)+YEplac181, *P_{GAL1}::KIC1* (JV141)+2µ *RHO3* (pEV021), *P_{GAL1}::KIC1* (JV141)+2µ *MSG5* (pEV017), *P_{GAL1}::KIC1* (JV141)+pMA3a and *P_{GAL1}::KIC1* (JV141)+pMA3a-*STB3* (pEV020) were spotted onto selective SD media with or without 50 µg calcofluor white ml⁻¹. (b) Tenfold dilution series of wild-type (FY834), *rho3Δ* (EV116), *rho4Δ* (EV077), *kic1Δ*+*P_{GAL1}::KIC1* (JV144) and *kic1Δ* (JV80) were spotted onto YEPD with or without 50 µg calcofluor white ml⁻¹. Cells were grown for 2–3 days at 30 °C.

onto selective SD containing 50 µg calcofluor white ml⁻¹. Partial sequence analysis of the inserts of the seven strongest suppressors revealed five separate genomic regions (Table 3). Several clones were subjected to subcloning and deletion experiments, identifying the genes responsible for the (partial) suppression of the calcofluor white hypersensitivity of the *P_{GAL1}::KIC1* mutant (Fig. 5a). These consisted of (1) *STB3*, a gene encoding a two-hybrid interactor with the Sin3p protein (Kasten & Stillman, 1997), (2) *MSG5*, encoding a dual-specificity protein phosphatase involved in the phero-

mone adaptation response (Doi *et al.*, 1994; Zhan *et al.*, 1997) and, in addition, capable of influencing the phosphorylation state of Slt2p (Watanabe *et al.*, 1995; Martín *et al.*, 2000) and (3) *RHO3*, encoding a small G-protein, which is known to be involved in bud formation and growth, and organization of the actin cytoskeleton and exocytosis (Matsui & Toh-e, 1992b; Imai *et al.*, 1996; Robinson *et al.*, 1999; Adamo *et al.*, 1999). JV141 cells were transformed with either of the before-mentioned plasmids and grown on selective medium containing 5'-FOA to determine if the presence of these

Fig. 6. *RHO3* gene dosage affects K1 killer toxin sensitivity. Relative apparent sensitivity to the K1 killer toxin was determined as described in Methods. (a) *kic1Δ* + P_{GAL1} :*KIC1* (JV144) + YEplac181, *kic1Δ* + P_{GAL1} :*KIC1* (JV144) + 2 μ *RHO3* and *kic1Δ* + P_{GAL1} :*KIC1* (JV144) + 2 μ *RHO4* are compared. (b) Wild-type (FY834), *rho3Δ* (EV116), *rho4Δ* (EV077) and *kic1Δ* + P_{GAL1} :*KIC1* (JV144) was compared. (c) Wild-type (FY834) + YEplac181, wild-type (FY834) + 2 μ *RHO3* and wild-type (FY834) + 2 μ *RHO4* were compared.

multicopy suppressor plasmids allowed the loss of the P_{GAL1} :*KIC1* plasmid. No viable colonies were found, indicating that none of these plasmids could restore (all of) the essential function(s) of *KIC1* (data not shown). *RHO3* was chosen for further analysis.

The *rho3* deletant is impaired in cell wall biogenesis

RHO3 was disrupted and the cells were tested for their sensitivity to calcofluor white. Although not to the same extent as *Kic1*-deficient cells, *rho3Δ* cells showed increased sensitivity to calcofluor white, indicating a defect in cell wall biogenesis (Fig. 5b). In the absence of *RHO3*, the functionally related *RHO4* can suppress the growth defect when overexpressed (Matsui & Toh-e, 1992a), whereas the *rho3 rho4* double mutant is inviable. The double mutant also proved inviable in our genetic

background (data not shown). Cells disrupted for *RHO4* did not show an increased sensitivity to calcofluor white (Fig. 5b). In addition, overexpression of *RHO4* did not restore the calcofluor white hypersensitivity of the P_{GAL1} :*KIC1* mutant (data not shown). This suggests that the function that is suppressed by overexpression of *RHO3* in the P_{GAL1} :*KIC1* mutant, is not shared by *RHO4*.

Overexpression of the *RHO3* gene in the P_{GAL1} :*KIC1* mutant produced an increase in sensitivity to the K1 killer toxin, in contrast to overexpression of the *RHO4* gene (Fig. 6a). Deletion of *RHO3* resulted in a decrease in K1 killer toxin sensitivity (Fig. 6b), and a decrease of about 40 % in cell wall 1,6- β -glucan (3.3 % of cell dry weight in wild-type cells compared to 1.9 %). In contrast, deletion of *RHO4* had no effect on K1 killer toxin sensitivity (Fig. 6b). Overexpression of the *RHO3* gene in wild-type also resulted in an increase in K1 killer toxin sensitivity (Fig. 6c). However, when *RHO4* was overexpressed in wild-type, cells displayed a decrease in killer sensitivity (Fig. 6c). A possible explanation for this is that high levels of Rho4 might compete with Rho3. Taken together, the observations suggest that the levels of *RHO3* influence the level of cell wall 1,6- β -glucan and thus the sensitivity of the cells to the K1 killer toxin. This further implicates *KIC1* in 1,6- β -glucan biogenesis, evidently for a part through *RHO3*.

KIC1 antagonizes the *PBS2*–*HOG1* pathway in cell wall biogenesis

Cell wall phenotypes caused by overexpression of the MAP kinase kinase *PBS2* from the HOG pathway show a remarkable resemblance to some of the phenotypes shown by P_{GAL1} :*KIC1* mutant cells. Similar to cells that overexpress *PBS2* (Jiang *et al.*, 1995; Lai *et al.*, 1997), P_{GAL1} :*KIC1* mutant cells were less sensitive to K1 killer toxin, and showed a modest decrease in 1,6- β -glucan levels (Fig. 3b; Table 4). The reverse phenotypes were found in the *pbs2Δ* mutant and cells overexpressing *KIC1*, i.e., hypersensitivity to the K1 killer toxin (Jiang *et al.*, 1995; Lai *et al.*, 1997; Fig. 3b) and an increase in 1,6- β -glucan levels (Jiang *et al.*, 1995; Table 4). A P_{GAL1} :*KIC1 pbs2Δ* double mutant was generated and this strain displayed an intermediate sensitivity to K1 killer toxin (Fig. 3b). *KIC1* deficiency and deletion of *PBS2* also reversely affected the sensitivity of the cells to cell wall degrading enzymes (Fig. 2c), whereas the double mutant displayed an intermediate phenotype. In summary, our observations in combination with data from the literature indicate that *KIC1* and *PBS2* have opposing roles in cell wall biogenesis.

DISCUSSION

The *KIC1* gene was originally identified in a two-hybrid screen interacting with *CDC31*, which encodes yeast centrin. *KIC1*, member of the PAK1/Ste20 kinase family, encodes a 116 kDa protein which interacts *in vivo* with *CDC31* and has *in vitro* kinase activity dependent on *CDC31*. However, it was shown that *KIC1* did not play

a role in the SPB duplication function of *CDC31*. *KIC1* rather contributes to its function in cell integrity and morphogenesis, since several *kic1* mutants showed aberrant cell wall morphology, wide bud necks, failure in cell separation and cell lysis (Sullivan *et al.*, 1998). Previously, cells deleted for *KIC1* were found to be inviable (Sullivan *et al.*, 1998). However, differences in genetic backgrounds might explain the (albeit very poor) viability of the *kic1Δ* strain in our backgrounds.

The *cwb30/kic1* mutant was originally discovered because it was hypersensitive to the cell wall perturbing agent calcofluor white (Ram *et al.*, 1994). Interestingly, mutant cells were also resistant to the K1 killer toxin, indicating that their walls contained less 1,6- β -glucan. As *KIC1* encodes a protein kinase, this marked it as a potential regulator of 1,6- β -glucan biogenesis. The following evidence supports this. *KIC1* deficiency resulted in decreased sensitivity to the K1 killer toxin and lower levels of 1,6- β -glucan in its walls. Conversely, overexpression of *KIC1* resulted in increased sensitivity to K1 killer toxin and elevated levels of 1,6- β -glucan. In addition, the *P_{GAL1}:KIC1* mutant crossed with various *kre* mutants resulted in double mutants with a synthetic growth defect, whereas a combination of the *P_{GAL1}:KIC1* mutant with the 1,3- β -glucan impaired mutant *fks1Δ* did not result in a synthetic growth defect. Taken together, these results imply a role for *KIC1* in the regulation of 1,6- β -glucan biogenesis. Finally, the expression levels of *RHO3* correlated with the sensitivity to K1 killer toxin and thus probably with 1,6- β -glucan levels. In addition, the mutant phenotypes of *Kic1*-deficient cells were partially suppressed by overexpression of *RHO3*. This is consistent with the postulated role for *KIC1* in regulating 1,6- β -glucan biogenesis.

Besides the defects in 1,6- β -glucan deposition, the *Kic1*-deficient cells also displayed resistance to the cell wall-degrading enzyme mixture Zymolyase, whereas overexpression of *KIC1* resulted in hypersensitivity to Zymolyase. This effect may partly be caused by changes in the cell wall mannoprotein composition, since the external layer of mannoproteins in the cell wall determines the porosity and needs to be removed for efficient cell wall degradation (Zlotnik *et al.*, 1984; De Nobel *et al.*, 1990). There are two lines of evidence that confirm this notion. First, when cell walls were isolated prior to Zymolyase treatment, walls from *Kic1*-deficient cells showed a much less pronounced resistance to Zymolyase compared to wild-type cell walls. Second, two known cell wall protein encoding genes, *SED1* and *CWP2*, were found to be upregulated in the *P_{GAL1}:KIC1* mutant. Interestingly, overexpression of *SED1* leads to Zymolyase resistance (Shimoi *et al.*, 1998), and, reversely, deletion of both *SED1* and *CWP2* results in increased sensitivity to Zymolyase (Van der Vaart *et al.*, 1995; Shimoi *et al.*, 1998). Increased expression of these genes in the *Kic1*-deficient mutants might thus at least in part explain the resistance to Zymolyase. By which mechanism the expression of these cell wall proteins is induced is unknown. It might reflect the induction of a

cell wall repair mechanism as the result of the decrease in 1,6- β -glucan. (Popolo & Vai, 1999; Kapteyn *et al.*, 1999; Klis *et al.*, 2002), but this normally includes induction of *CWP1* expression (Terashima *et al.*, 2000; Kapteyn *et al.*, 2001). However, *CWP1* expression was not induced in the *P_{GAL1}:KIC1* mutant. Alternatively, *Kic1* might have a regulatory role in multiple cell wall biosynthetic steps and not only in 1,6- β -glucan biogenesis.

The identification of *RHO3* as a multicopy suppressor of the *P_{GAL1}:KIC1* mutant suggests that *RHO3* might be a downstream target of *KIC1* in cell wall biogenesis. This is supported by the *RHO3* gene dosage relationship with K1 killer toxin sensitivity and the reduction of 1,6- β -glucan levels in the *rho3Δ* mutant. Whereas *RHO4* contributes to some of the known functions of *RHO3* (Matsui & Toh-e, 1992a; Imai *et al.*, 1996), the effects on cell wall biogenesis are not shared by *RHO4*.

Evidence is accumulating that suggests a role for the *PBS2-HOG1* pathway in cell wall construction. Overexpression of *PBS2* causes resistance to laminarinase, a cell wall degrading enzyme mixture (Lai *et al.*, 1997), and deletion results in hypersensitivity (Fig. 2b; Alonso-Monge *et al.*, 2001). In addition, *PBS2* overexpression results in resistance to the K1 killer toxin and a decrease in cell wall 1,6- β -glucan levels (Jiang *et al.*, 1995). Also, under noninducing conditions the HOG pathway contributes to the maintenance of cell wall architecture (García-Rodríguez *et al.*, 2000). Furthermore, overexpression of some cell wall related genes suppress the hyperosmosensitive phenotype of a *ste11 ssk2 ssk22* mutant. These include *LRE1* and *HLR1*, which can also suppress the osmosensitivity and the glucanase sensitivity of both *pbs2Δ* and *hog1Δ* mutants (Alonso-Monge *et al.*, 2001). Our report further supports a role for *PBS2* in cell wall biogenesis. The *P_{GAL1}:KIC1* mutant and the *pbs2Δ* single mutant cells had reverse phenotypes in both K1 killer toxin and Zymolyase sensitivities. In the *P_{GAL1}:KIC1 pbs2Δ* double mutant an intermediate phenotype was observed (Fig. 3b). These results suggest that *KIC1* and *PBS2* play opposing roles in cell wall biogenesis. The mechanism by which *KIC1* and *PBS2* counteract each other remains obscure. In summary, the protein kinase *Kic1* is involved in regulating cell wall construction in multiple ways and seems to have a specific role in controlling 1,6- β -glucan levels.

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REFERENCES

- Adamo, J. E., Rossi, G. & Brennwald, P. (1999). The Rho GTPase Rho3 has a direct role in exocytosis that is distinct from its role in actin polarity. *Mol Biol Cell* **10**, 4121–4133.
- Al-Aidroos, K. & Bussey, H. (1978). Chromosomal mutants of

- Saccharomyces cerevisiae* affecting the cell wall binding site for killer factor. *Can J Microbiol* **24**, 228–237.
- Alonso-Monge, R., Real, E., Wojda, I., Bebelman, J. P., Mager, W. H. & Siderius, M. (2001). Hyperosmotic stress response and regulation of cell wall integrity in *Saccharomyces cerevisiae* share common functional aspects. *Mol Microbiol* **41**, 717–730.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidmen, J. G., Smith, J. A. & Struhl, K. (editors) (1998). *Current Protocols in Molecular Biology*. New York: Greene Publishing & Wiley Interscience.
- Berben, G., Dumont, J., Gilliquet, V., Bolle, P. A. & Hilger, F. (1991). The YDp plasmids: a uniform set of vectors bearing versatile gene disruption cassettes for *Saccharomyces cerevisiae*. *Yeast* **7**, 475–477.
- Boone, C., Sommer, S. S., Hensel, A. & Bussey, H. (1990). Yeast *KRE* genes provide evidence for a pathway of cell wall beta-glucan assembly. *J Cell Biol* **110**, 1833–1843.
- Breinig, F., Tipper, D. J. & Schmitt, M. J. (2002). Kre1p, the plasma membrane receptor for the yeast K1 viral toxin. *Cell* **108**, 395–405.
- Brown, J. L. & Bussey, H. (1993). The yeast *KRE9* gene encodes an O glycoprotein involved in cell surface beta-glucan assembly. *Mol Cell Biol* **13**, 6346–6356.
- Brown, J. L., Kossaczka, Z., Jiang, B. & Bussey, H. (1993). A mutational analysis of killer toxin resistance in *Saccharomyces cerevisiae* identifies new genes involved in cell wall (1–6)-beta-glucan synthesis. *Genetics* **133**, 837–849.
- Brown, J. L., Roemer, T., Lussier, M., Sdicu, A. M. & Bussey, H. (1994). The K1 killer toxin: molecular and genetic applications to secretion and cell surface assembly. In *Molecular Genetics of Yeast: a Practical Approach*, pp. 217–232. Edited by J. R. Johnston. Oxford: IRL Press at Oxford University Press.
- Buehrer, B. M. & Errede, B. (1997). Coordination of the mating and cell integrity mitogen-activated protein kinase pathways in *Saccharomyces cerevisiae*. *Mol Cell Biol* **17**, 6517–6525.
- Bussey, H. (1991). K1 killer toxin, a pore forming protein from yeast. *Mol Microbiol* **5**, 2339–2343.
- Bussey, H., Saville, D., Hutchins, K. & Palfree, R. G. (1979). Binding of yeast killer toxin to a cell wall receptor on sensitive *Saccharomyces cerevisiae*. *J Bacteriol* **140**, 888–892.
- De Nobel, J. G., Klis, F. M., Priem, J., Munnik, T. & Van Den Ende, H. (1990). The glucanase-soluble mannoproteins limit cell wall porosity in *Saccharomyces cerevisiae*. *Yeast* **6**, 491–499.
- De Groot, P. W. J., Ruiz, C., Vazquez de Aldana, C. R. & 14 other authors (2001). A genomic approach for the identification and classification of genes involved in cell wall formation and its regulation in *Saccharomyces cerevisiae*. *Comp Funct Genomics* **2**, 124–142.
- Doi, K., Gartner, A., Ammerer, G., Errede, B., Shinkawa, H., Sugimoto, K. & Matsumoto, K. (1994). MSG5, a novel protein phosphatase promotes adaptation to pheromone response in *S. cerevisiae*. *EMBO J* **13**, 61–70.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colometric method for determination of sugars and related substances. *Anal Chem* **28**, 350–356.
- García-Rodríguez, L. J., Durán, A. & Roncero, C. (2000). Calcofluor antifungal action depends on chitin and a functional high-osmolarity glycerol response (HOG) pathway: evidence for a physiological role of the *Saccharomyces cerevisiae* HOG pathway under noninducing conditions. *J Bacteriol* **182**, 2428–2437.
- Gietz, R. D. & Sugino, A. (1988). New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74**, 527–534.
- Imai, J., Toh-e, A. & Matsui, Y. (1996). Genetic analysis of the *Saccharomyces cerevisiae* *RHO3* gene, encoding a rho-type small GTPase, provides evidence for a role in bud formation. *Genetics* **142**, 359–369.
- Ivanovska, I. & Rose, M. D. (2001). Fine structure analysis of the yeast centrin, Cdc31p, identifies residues specific for cell morphology and spindle pole body duplication. *Genetics* **157**, 503–518.
- Jiang, B., Ram, A. F. J., Sheraton, J., Klis, F. M. & Bussey, H. (1995). Regulation of cell wall beta-glucan assembly: *PTC1* negatively affects *PBS2* action in a pathway that includes modulation of *EXG1* transcription. *Mol Gen Genet* **248**, 260–269.
- Jiang, B., Sheraton, J., Ram, A. F. J., Dijkgraaf, G. J., Klis, F. M. & Bussey, H. (1996). *CWH41* encodes a novel endoplasmic reticulum membrane N-glycoprotein involved in beta 1,6-glucan assembly. *J Bacteriol* **178**, 1162–1171.
- Kapteyn, J. C., Van Egmond, P., Sievi, E., Van Den Ende, H., Makarow, M. & Klis, F. M. (1999). The contribution of the O-glycosylated protein Pir2p/Hsp150 to the construction of the yeast cell wall in wild-type cells and beta-1,6-glucan-deficient mutants. *Mol Microbiol* **31**, 1835–1844.
- Kapteyn, J. C., ter Riet, B., Vink, E., Blad, S., De Nobel, H., Van Den Ende, H. & Klis, F. M. (2001). Low external pH induces *HOG1*-dependent changes in the organization of the *Saccharomyces cerevisiae* cell wall. *Mol Microbiol* **39**, 469–479.
- Kasten, M. M. & Stillman, D. J. (1997). Identification of the *Saccharomyces cerevisiae* genes *STB1-STB5* encoding Sin3p binding proteins. *Mol Gen Genet* **256**, 376–386.
- Klis, F. M., Mol, P. C., Hellingwerf, K. & Brul, S. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* **26**, 239–256.
- Lai, M. H., Silverman, S. J., Gaughran, J. P. & Kirsch, D. R. (1997). Multiple copies of *PBS2*, *MHP1* or *LRE1* produce glucanase resistance and other cell wall effects in *Saccharomyces cerevisiae*. *Yeast* **13**, 199–213.
- Lussier, M., White, A. M., Sheraton, J. & 17 other authors (1997). Large scale identification of genes involved in cell surface biosynthesis and architecture in *Saccharomyces cerevisiae*. *Genetics* **147**, 435–450.
- Martín, H., Rodríguez-Pachon, J. M., Ruiz, C., Nombela, C. & Molina, M. (2000). Regulatory mechanisms for modulation of signaling through the cell integrity Slt2-mediated pathway in *Saccharomyces cerevisiae*. *J Biol Chem* **275**, 1511–1519.
- Matsui, Y. & Toh-e, A. (1992a). Isolation and characterization of two novel *ras* superfamily genes in *Saccharomyces cerevisiae*. *Gene* **114**, 43–49.
- Matsui, Y. & Toh-e, A. (1992b). Yeast *RHO3* and *RHO4* *ras* superfamily genes are necessary for bud growth, and their defect is suppressed by a high dose of bud formation genes *CDC42* and *BEM1*. *Mol Cell Biol* **12**, 5690–5699.
- Popolo, L. & Vai, M. (1999). The Gas1 glycoprotein, a putative wall polymer cross-linker. *Biochim Biophys Acta* **1426**, 385–400.
- Ram, A. F. J., Wolters, A., Ten Hoopen, R. & Klis, F. M. (1994). A new approach for isolating cell wall mutants in *Saccharomyces cerevisiae* by screening for hypersensitivity to calcofluor white. *Yeast* **10**, 1019–1030.
- Ram, A. F. J., Brekelmans, S. S. C., Oehlen, L. J. W. M. & Klis, F. M. (1995). Identification of two cell cycle regulated genes affecting the beta-1,3-glucan content of cell walls in *Saccharomyces cerevisiae*. *FEBS Lett* **358**, 165–170.
- Ram, A. F. J., Kapteyn, J. C., Montijn, R. C., Caro, L. H. P., Douwes, J. E., Baginsky, W., Mazur, P., Van Den Ende, H. & Klis, F. M.

- (1998). Loss of the plasma membrane-bound protein Gas1p in *Saccharomyces cerevisiae* results in the release of beta-1,3-glucan into the medium and induces a compensation mechanism to ensure cell wall integrity. *J Bacteriol* **180**, 1418–1424.
- Reneke, J. E., Blumer, K. J., Courchesne, W. E. & Thorner, J. (1988). The carboxy-terminal segment of the yeast alpha-factor receptor is a regulatory domain. *Cell* **55**, 221–234.
- Robinson, N. G., Guo, L., Imai, J., Toh-e, A., Matsui, Y. & Tamanoi, F. (1999). Rho3 of *Saccharomyces cerevisiae*, which regulates the actin cytoskeleton and exocytosis, is a GTPase which interacts with Myo2 and Exo70. *Mol Cell Biol* **19**, 3580–3587.
- Roemer, T. & Bussey, H. (1991). Yeast beta-glucan synthesis: *KRE6* encodes a predicted type II membrane protein required for glucan synthesis *in vivo* and for glucan synthase activity *in vitro*. *Proc Natl Acad Sci USA* **88**, 11295–11299.
- Roncero, C., Valdivieso, M. H., Ribas, J. C. & Duran, A. (1988). Isolation and characterization of *Saccharomyces cerevisiae* mutants resistant to calcofluor white. *J Bacteriol* **170**, 1950–1954.
- Rose, M. D., Novick, P., Thomas, J. H., Bothstein, D. & Fink, G. R. (1987). A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromeric-containing shuttle vector. *Gene* **60**, 237–243.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequence with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Shahinian, S. & Bussey, H. (2000). β -1,6-Glucan synthesis in *Saccharomyces cerevisiae*. *Mol Microbiol* **35**, 477–489.
- Sherman, F. & Hicks, J. (1991). Micromanipulation and dissection of asci. *Methods Enzymol* **194**, 21–37.
- Shimoi, H., Kitagaki, H., Ohmori, H., Iimura, Y. & Ito, K. (1998). Sed1p is a major cell wall protein of *Saccharomyces cerevisiae* in the stationary phase and is involved in lytic enzyme resistance. *J Bacteriol* **180**, 3381–3387.
- Sullivan, D. S., Biggins, S. & Rose, M. D. (1998). The yeast centrin, Cdc31p, and the interacting protein kinase, Kic1p, are required for cell integrity. *J Cell Biol* **143**, 751–765.
- Terashima, H., Yabuki, N., Arisawa, M., Hamada, K. & Kitada, K. (2000). Up-regulation of genes encoding glycosylphosphatidylinositol (GPI)-attached proteins in response to cell wall damage caused by disruption of *FKS1* in *Saccharomyces cerevisiae*. *Mol Gen Genet* **264**, 64–74.
- Van Berkel, M. A., Rieger, M., te Heesen, S., Ram, A. F. J., Van Den Ende, H., Aebi, M. & Klis, F. M. (1999). The *Saccharomyces cerevisiae* *CWH8* gene is required for full levels of dolichol-linked oligosaccharides in the endoplasmic reticulum and for efficient N-glycosylation. *Glycobiology* **9**, 243–253.
- Van Der Vaart, J. M., Caro, L. H. P., Chapman, J. W., Klis, F. M. & Verrips, C. T. (1995). Identification of three mannoproteins in the cell wall of *Saccharomyces cerevisiae*. *J Bacteriol* **177**, 3104–3110.
- Van Rinsum, J., Klis, F. M. & Van Den Ende, H. (1991). Cell wall glucomannoproteins of *Saccharomyces cerevisiae* *mn9*. *Yeast* **7**, 717–726.
- Vossen, J. H., Ram, A. F. J. & Klis, F. M. (1995). Identification of *SPT14/CHW6* as the yeast homologue of hPIG-A, a gene involved in the biosynthesis of GPI anchors. *Biochim Biophys Acta* **1243**, 549–551.
- Watanabe, Y., Irie, K. & Matsumoto, K. (1995). Yeast *RLM1* encodes a serum response factor-like protein that may function downstream of the Mpk1 (Slr2) mitogen-activated protein kinase pathway. *Mol Cell Biol* **15**, 5740–5749.
- Winston, F., Dollard, C. & Ricupero-Hovasse, S. L. (1995). Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. *Yeast* **11**, 53–55.
- Zhan, X. L., Deschenes, R. J. & Guan, K. L. (1997). Differential regulation of *FUS3* MAP kinase by tyrosine-specific phosphatases *PTP2/PTP3* and dual-specificity phosphatase *MSG5* in *Saccharomyces cerevisiae*. *Genes Dev* **11**, 1690–1702.
- Zlotnik, H., Fernandez, M. P., Bowers, B. & Cabib, E. (1984). *Saccharomyces cerevisiae* mannoproteins form an external cell wall layer that determines wall porosity. *J Bacteriol* **159**, 1018–1026.

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