Scw10p, a cell-wall glucanase/transglucosidase important for cell-wall stability in *Saccharomyces cerevisiae*

Sergej Sestak,[†] Ilja Hagen,[‡] Widmar Tanner and Sabine Strahl[§]

Correspondence

Sabine Strahl

sstrahl@hip.uni-heidelberg.de

Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, Universitätsstrasse 31, D-93053 Regensburg, Germany

Glycosyl hydrolases and transferases are crucial for the formation of a rigid but at the same time plastic cell wall in yeasts and fungi. The *Saccharomyces cerevisiae* glucan hydrolase family 17 (GH17) contains the soluble cell-wall proteins Scw4p, Scw10p, Scw11p and Bgl2p. For Bgl2p, endoglucanase/glucanotransferase activity has been demonstrated, and Scw11p has been shown to be involved in cell separation. Here, Scw4p and Scw10p, which show 63% amino acid identity, were characterized. *scw4* and *scw10* single mutants were sensitive towards cell-wall destabilizing agents, suggesting a role in cell-wall assembly or maintenance. Simultaneous deletion of *SCW4* and *SCW10* showed a synergistic effect, and activated the cell-wall compensatory mechanism in a PKC1-dependent manner. Both the amount of cell-wall chitin and the amount of mannoproteins attached to chitin were increased in mutant *scw4scw10*. Deletion of *CHS3* proved the critical role of chitin in *scw4scw10*. However, the mannoprotein Sed1p and the glucan synthase Fks2p were also crucial for cell-wall stability in mutant *scw4scw10*. The exchange of two conserved glutamate residues localized in the putative catalytic domain of GH17 family members strongly suggests that Scw10p acts as a 1,3- β -glucanase or as a 1,3- β -glucanotransferase. In addition, the synthetic interactions between Bgl2p and Scw10p which support a functional cooperation in cell-wall assembly were analysed. The data suggest that Scw4p and Scw10p act as glucanases or transglucosidases in concert with other cell-wall proteins to assure cell-wall integrity.

Received 30 April 2004

Revised 29 June 2004

Accepted 1 July 2004

INTRODUCTION

The yeast cell wall is composed of three major components: glucan, mannoproteins and chitin (Kollar *et al.*, 1997; reviewed by Klis *et al.*, 2002). While a 1,3- β -glucan–chitin complex constitutes the inner rigid structure, mannoproteins form the outer layer of the cell wall, and 1,6- β -glucan interconnects all cell-wall components. The main group of cell-wall mannoproteins are covalently attached to 1,3- β -glucan, either via a remnant of a GPI-anchor (GPI-proteins) or directly through an unidentified alkali-sensitive linkage (PIR-proteins). A second group of cell-wall proteins can

be released from intact cells using reducing agents and/or from isolated cell walls using SDS. The majority of the latter are considered as S–S-linked or non-covalently bound cell-wall proteins (Cappellaro *et al.* 1998; reviewed by Klis *et al.*, 2002).

Cell-wall compensatory mechanisms are known to be responsible for remodelling the cell wall to prevent cell lysis whenever its integrity is compromised by environmental stresses, by cell-wall perturbing compounds or in cell-wall mutants (Popolo *et al.*, 2001; Lagorce *et al.*, 2003; Garcia *et al.*, 2004; Hagen *et al.*, 2004). The PKC1-MAPK pathway is essential for cell-wall integrity (Heinisch *et al.*, 1999). It is activated during vegetative growth in G1- to S-phase transition, during mating, and in response to cell-wall damage. Persistent activation of the PKC1 pathway was found in cells with cell walls altered due to treatment with Calcofluor White or zymolyase and in several cell-wall mutants (De Nobel *et al.*, 2000; Lagorce *et al.*, 2003; Garcia *et al.*, 2004). However, the cell-wall compensation seems also to be activated via other signal-transduction pathways, including protein kinase A, HOG- and Ca²⁺/calciurein-dependent pathways (Lagorce *et al.*, 2003; Garcia *et al.*,

[†]Present address: Institute of Chemistry, Slovak Academy of Sciences, Dubravska cesta 9, 84238 Bratislava, Slovak Republic.

[‡]Present address: Center of Excellence for Fluorescent Bioanalysis, Josef-Engert-Str 9, D-93053 Regensburg, Germany.

[§]Present address: University of Heidelberg Institute for Plant Science, Cell Chemistry, Im Neuenheimer Feld 360, D-69120 Heidelberg, Germany.

Abbreviations: CFW, Calcofluor White; CR, Congo Red; HPAEC PAD, high performance anion-exchange chromatography with pulsed amperometric detection; TFA, trifluoroacetic acid.

2004). The known PKC1-activated transcription factors are SBF (Swi4p/Swi6p; Madden *et al.*, 1997) and Rlm1p (Watanabe *et al.*, 1995; Dodou & Treisman, 1997). The expression of several cell-wall related genes was shown to be regulated by the PKC1 pathway through these transcription factors (Igual *et al.*, 1996; Jung & Levin, 1999).

The yeast cell wall is a dynamic structure which has to be remodelled during growth and other morphogenetic processes by a continuous process of synthesis and degradation of cell-wall material (Klis *et al.*, 2002). 1,3- β -Glucan is the main component of the yeast cell wall, and mainly responsible for its rigidity. Endogenous 1,3- β -glucanases and transglucosidases play an important role in the modification of cell-wall glucan (Cid *et al.*, 1995; Smits *et al.*, 2001). Glucosyl hydrolases (GH) and related enzymes have been classified on the basis of amino-acid-sequence similarities within the CAZy (Carbohydrate-Active enZYmes) database (Henrissat & Bairoch, 1996). Several of these families have been implicated to be important for the architecture of the cell wall in yeast.

The GH72 family of 1,3- β -glucanosyltransglycosylases comprises Gas1p and its homologues Gas2p, Gas3p, Gas4p and Gas5p (reviewed by Popolo & Vai, 1999). Glucanosyltransferase activity of Gas1p was demonstrated *in vitro* (Mouyna *et al.*, 2000). Gas1p is a GPI-protein: it acts *in vivo* as an assembly enzyme that transfers segments of 1,3- β -glucan on branching points of other glucans. The lack of Gas1p cross-linking activity causes aberrant cell morphology and activates the PKC1 pathway (reviewed by Popolo *et al.*, 2001). Compared to wild-type cells, *gas1 Δ* mutants show a series of phenotypes such as increased levels of cell-wall chitin, more mannoproteins linked to chitin, and resistance towards zymolyase, all of which indicate that cell-wall compensatory mechanisms are crucial for survival of this mutant.

The exoglucanases Exg1p, Exg2p and Ssg1p/Spr1p are members of the GH5 family (reviewed by Cid *et al.*, 1995). Exg1p has a possible function in cell-wall expansion during vegetative growth. The sporulation-specific exoglucanase Ssg1p/Spr1p is probably involved in the process of spore formation and in the morphogenesis of ascospore walls.

Crh1p, Crh2p/Utr2p and Crr1p show homology with bacterial β -glucanases and eukaryotic endotransglycosidases, and belong to the GH16 family. Crh1p and Crh2p are localized at the cell surface in chitin-rich areas (Rodriguez-Peña *et al.*, 2000). They play a putative role in cell-wall assembly at the sites of polarized cell growth, whereas Crr1p seems to be involved in sporulation. Deletion of *CRH1* in combination with *CRH2* shows additive effects in hypersensitivity to Congo Red and an increased amount of alkali-soluble glucan.

The endoglucanases Eng1p and Acf2p/Eng2p belong to the GH81 family. Eng1p, an extracellular endo-1,3- β -glucanase, is cell-cycle regulated and localizes asymmetrically to the daughter site of the septum (Baladron *et al.*, 2002). This

indicates that Eng1p might be involved in the dissolution of the septum together with chitinase. In *Schizosaccharomyces pombe*, Eng1p is required for the dissolution of the primary septum during cell separation (Martin-Cuadrado *et al.*, 2003).

Biotinylation of yeast cell walls has identified the soluble cell-wall proteins Scw3p/Sun4p, Scw4p, Scw10p and Scw11p (Cappellaro *et al.*, 1998). Scw3p shows sequence homology to β -glucosidase of *Candida wickerhamii* (Skory & Freer, 1995). It is a member of the *Saccharomyces cerevisiae* SUN gene family, and plays a role in the final steps of cell-wall septation (Mouassite *et al.*, 2000).

Scw4p, Scw10p and Scw11p, together with Bgl2p, belong to the GH17 family. Scw11p has been characterized as a daughter-specific G1-regulated protein that is important for cell separation (Colman-Lerner *et al.*, 2001). Bgl2p is one of the most abundant cell-wall proteins (Mrša *et al.*, 1993). It was characterized as an endo-1,3- β -glucanase and glucanosyltransferase that introduces β -1,6-linkages in 1,3- β -glucan chains (Mrša *et al.*, 1993; Goldman *et al.*, 1995). Deletion of *BGL2* does not obviously affect cell morphology, whereas overexpression is harmful for cell viability (Mrša *et al.*, 1993). Scw4p and Scw10p share 63 % amino acid identity and an apparent molecular mass of 66 kDa (Cappellaro *et al.*, 1998). Both proteins contain an ER-translocation sequence and a Kex2p processing site. Expression of the *SCW10* gene is cell-cycle regulated, with a maximum of expression in the G1-phase (Spellman *et al.*, 1998). Transcriptome analyses suggest an important role for Scw10p in cell-wall assembly and/or stability, since *SCW10* expression is highly induced in several cell-wall mutants (Lagorce *et al.*, 2003; Hagen *et al.*, 2004). A homologue of Scw10p in *Candida albicans* CaMP65 was characterized as a major immunodominant antigen. This 65 kDa mannoprotein is particularly observed in extra-cellular fractions of hyphae (La Valle *et al.*, 2000).

Here, we analyse the role of Scw10p in cell-wall stability in combination with other members of the GH17 family. Mutagenesis of two conserved glutamate residues indicated that Scw10p functions as a glucosyl hydrolase/transglycosidase. Analyses of *scw* mutants showed that the PKC1 pathway is activated and cell-wall structure altered in the absence of *SCW4* and *SCW10*. Our data suggest that Scw10p acts in concert with Scw4p and Bgl2p to assure cell-wall integrity.

METHODS

Strains, media and growth conditions. The *S. cerevisiae* strains used are listed in Table 1. Yeast strains were grown in YPD or in synthetic minimal medium with auxotrophic supplements and 2 % glucose (SD) (Kaiser *et al.*, 1994). Deletion mutants were selected on either SD or YPD medium containing 200 μ g geneticin ml⁻¹ (G418; GIBCO).

Deletion constructs. Standard procedures were used for all DNA manipulations (Sambrook *et al.*, 1989). All cloning and transformations were made in *Escherichia coli* strain DH5 α . Oligonucleotide

Table 1. Yeast strains used in this study

Strain	Genotype	Reference
SEY6210	MAT α <i>ura3-52 leu2-3,112 his3-Δ100 trp1-Δ901 lys2-801 suc2-Δ9</i>	Robinson <i>et al.</i> , 1988
<i>scw4</i>	SEY6210 except <i>scw4::LEU2</i>	Cappellaro <i>et al.</i> , 1998
<i>scw10</i>	SEY6210 except <i>scw10::HIS3</i>	Cappellaro <i>et al.</i> , 1998
<i>bgl2</i>	SEY6210 except <i>bgl2::URA3</i>	Cappellaro <i>et al.</i> , 1998
<i>scw4scw10</i>	SEY6210 except <i>scw4::LEU2 scw10::HIS3</i>	Cappellaro <i>et al.</i> , 1998
<i>scw4scw10bgl2</i>	SEY6210 except <i>scw4::LEU2 scw10::HIS3 bgl2::URA3</i>	Cappellaro <i>et al.</i> , 1998
SES101	SEY6210 except <i>sed1::kanMX4</i>	This study
SES102	SEY6210 except <i>scw4::LEU2 scw10::HIS3 sed1::kanMX4</i>	This study
SES103	SEY6210 except <i>chs3::kanMX4</i>	This study
SES104	SEY6210 except <i>scw4::LEU2 scw10::HIS3 chs3::kanMX4</i>	This study
SES105	SEY6210 except <i>fks2::kanMX4</i>	This study
SES106	SEY6210 except <i>scw4::LEU2 scw10::HIS3 fks2::kanMX4</i>	This study

sequences are available upon request. Yeast transformations were carried out according to the method of Gietz *et al.* (1995). Deletions of *SCW4*, *SCW10* and *BGL2* were performed as described by Cappellaro *et al.* (1998). For deletion of *CHS3*, the plasmid P43160 (*chs3::KanMX4*; EUROSCARF) was used. For disruption of *SED1*, the *sed1::KanMX4* cassette was amplified from genomic DNA of strain Y04012 (EUROSCARF) using oligonucleotides SED1A and SED1B. For disruption of *FKS2*, *fks2::KanMX4* was amplified from genomic DNA of strain Y06979 (EUROSCARF) using oligonucleotides FKS2A and FKS2B. Purified PCR products were transformed directly into yeast. Correct integrations were tested by PCR analysis.

Plasmid construction

Plasmid pSE10GAL (*GAL-SCW10^{HIS}*). A *Bam*HI site was introduced immediately upstream of the *SCW10* start codon, and an enterokinase cleavage site followed by a *Xba*I site were introduced immediately upstream of the *SCW10* stop codon by PCR using oligonucleotides BAM-F, XBA and genomic DNA of strain SEY6210 as a template. The PCR product was subcloned into vector pYES2/CT (Invitrogen) digested with *Bam*HI and *Xba*I. In the resulting construct, *SCW10^{HIS}* is under the control of the inducible *GAL1* promoter.

Plasmid pSE10 (*SCW10^{HIS}*). A 1292 bp *Hind*III–*Pme*I fragment of pSE10GAL was subcloned into pRS416HA (Lommel *et al.*, 2004) digested with *Hind*III and *Sma*I, resulting in pRS416SCW. An 880 bp fragment of the 5′-untranslated region of *SCW10* was amplified using oligonucleotides HIND and BAM-R and yeast genomic DNA as a template. The PCR product was subcloned as a *Hind*III–*Bam*HI fragment into pRS416SCW, resulting in pSE10.

Plasmids pSE10-1 (*SCW10E326Q*) and pSE10-2 (*SCW10E380Q*). Glu-326 and Glu-380 respectively, were exchanged for glutamine by PCR. The primer pairs E326-F/E326-R and E380-F/E380-R, respectively, were used. pSE10 served as template. DNA sequence analysis confirmed the basepair exchanges.

Plasmids pRS424SCW and pRS425SCW (*SCW10^{HIS}*). A 3550 bp *Pvu*II–*Pvu*II fragment from plasmid pSE10 was subcloned into pRS424 and pRS425 (Christianson *et al.*, 1992), respectively.

Plasmid NEV-BGL (*BGL2^{HA}*). A 960 bp DNA fragment was amplified on genomic DNA using oligonucleotides BGL-E and BGL-HAE. An *Eco*RI site was thereby introduced immediately upstream of the *BGL2* start codon, as well as one copy of the haemagglutinin (HA) epitope followed by an *Eco*RI site. The PCR product was subcloned into NEV-E cut with *Eco*RI (gift of Dr J. Stolz).

Phenotypic tests. Yeast cells were grown on YPD or SD medium. Portions of 10⁵ cells were harvested, washed and resuspended in 1 ml sterile water. A series of fivefold dilutions was prepared in sterile water and 3 μ l of each dilution was spotted on YPD plates containing Calcofluor White (CFW), Congo Red (CR) or caffeine at the required concentration.

Zymolyase sensitivity. Yeast cells were grown on YPD to exponential phase. Cells equivalent to 1 OD₆₀₀ unit were harvested, washed and resuspended in 50 mM Tris/HCl, pH 7.5, to OD₆₀₀ 0.7–0.8. Zymolyase 100T was added to a final concentration of 10 μ g ml^{−1}, and OD₆₀₀ was monitored at room temperature.

Cell wall preparation. Yeast cells were grown on YPD to late exponential phase. Cells equivalent to 100 OD₆₀₀ units were harvested, washed with 25 ml ice-cold water, 1 ml 50 mM Tris/HCl, pH 7.5, and resuspended in 200 μ l of the same buffer. An equal volume of glass beads was added and cells were lysed by vortexing four times (with 1 min intervals on ice) for 1 min. After puncturing the bottom of the tube, the cell lysate was collected. Cell walls were collected by centrifugation for 5 min at 3000 r.p.m. at 4 °C. Cell walls were washed five times with 1 ml ice-cold water and freeze-dried overnight.

Cell wall analysis

Enzymic hydrolysis. Freeze-dried cell walls equivalent to 50 OD₆₀₀ units (~4 mg) of cells were resuspended either in 0.5 ml of 50 mM Tris/HCl, pH 7.5, containing 20 U of recombinant 1,3- β -glucanase (Quantazyme; Qbiogene) or in 0.5 ml 50 mM potassium acetate, pH 5.5, containing 1 U of recombinant 1,6- β -glucanase (Glyko) and digested at 37 °C for 20 h. Sodium azide (0.02 %) was used as a preservative. The insoluble material was pelleted (20 000 *g* for 10 min) and the supernatant stored at 4 °C. Pellets were washed two times with 0.5 ml water. Subsequently, the 1,3- β -glucanase-resistant material was digested with 1,6- β -glucanase. The 1,6- β -glucanase-resistant material, on the other hand, was digested with 1,3- β -glucanase using the same conditions as above. Supernatants were collected after centrifugation. Total carbohydrate content of the soluble material was measured using the phenol-sulphuric acid method (Dubois *et al.*, 1956). The soluble material was freeze-dried and hydrolysed in TFA (see TFA-hydrolysis). Glucose and mannose contents were quantified by HPAEC PAD (see below).

Trifluoroacetic acid (TFA) hydrolysis. Hydrolysis of total cell walls was carried out in 0.5 ml 4 M TFA in sealed tubes at 100 °C for 4 h. Tubes were cooled on ice and centrifuged (20 000 *g* for 1 min). TFA from 200 μ l of the supernatants was evaporated under nitrogen.

The dry residue was washed with 0.5 ml methanol two times and resuspended in 200 µl Milli-Q water. Carbohydrates were separated and quantified by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC PAD) using a Dionex Bio-LC system.

Glucan analysis. To remove mannoproteins, cell walls were treated with 1 mg trypsin (Boehringer Mannheim) in 0.5 ml 50 mM Tris/HCl, pH 7.5, at 37 °C for 2 h. After washing three times with 1 ml water, trypsinated cell walls were extracted in 0.5 ml 1 M NaOH at 75 °C for 1 h. Alkali-soluble glucans were estimated from soluble NaOH-extracted material by precipitating carbohydrates with two volumes of ethanol at -20 °C. After NaOH extraction, the insoluble material was washed to neutrality and digested for 15 h with 1 mg of Zymolyase 100T (Seikagaku) in 0.5 ml 50 mM Tris/HCl, pH 7.5, at 37 °C. Alkali-insoluble glucans were estimated from zymolyase supernatants precipitated by ethanol. The total carbohydrate content of each fraction was measured as described above. Aliquots of the alkali-soluble fractions were spotted onto nitrocellulose, and 1,6-β-glucan was quantified by Western blot analysis using an anti-1,6-β-glucan polyclonal antibody (Lussier *et al.*, 1998).

Measurement of chitin. Cell walls were digested for 15 h with 1 mg of Zymolyase 100T (Seikagaku) in 0.5 ml 50 mM Tris/HCl, pH 7.5, at 37 °C. The insoluble material was pelleted, washed with 1 ml water, resuspended in 0.5 ml 4M HCl and hydrolysed at 100 °C for 10 h. After cooling on ice and neutralization with saturated Na₂CO₃, the N-acetylglucosamine (GlcNAc) content was measured according to the spectrophotometric method of Davidson (1966). Total chitin was measured in the HCl-hydrolysates of the mock-treated cell walls. GlcNAc values were expressed in µg per mg dry cell wall.

Western blot analysis. Proteins were fractionated by SDS-PAGE and transferred to nitrocellulose. The monoclonal antibody anti-HA (16B12; Babco) was used at 1:8000 dilution, anti-*c-myc* (9E10; Babco) at 1:5000 dilution, and His-probe (H-3; Santa Cruz Biotechnology) at 1:2000 dilution. The polyclonal antibody anti-Bgl2p (Mrša *et al.*, 1993) was used at 1:2000, and anti-1,6-β-glucan (Lussier *et al.*, 1998) at 1:2000 dilution. Protein-antibody complexes were visualized by enhanced chemiluminescence using the Amersham ECL system.

Indirect assay of MPK1p activity. pHPS100, a *CEN/ARS* vector containing both a *lexA-RLM1* fusion and a *lacZ* reporter gene preceded by *lexA* binding sites (Kirchraht *et al.*, 2000), was used for the indirect assay of Mpk1p activity. Transformants with pHPS100 were grown on SD lacking tryptophan at 30 °C to OD₆₀₀ = 1. Aliquots of the cells were diluted to an OD₆₀₀ of 0.5 in SD containing 5 mM caffeine, or mock treated and incubated at 30 °C for 2 h. Cells were collected, washed and lysed, and β-galactosidase activity was determined with ONPG as substrate (Stagljar *et al.*, 1998). β-Galactosidase units were calculated as 1000 × OD₄₂₀/OD₆₀₀ × *t* (where *t* is in min). Five independent transformants from each strain were measured.

RESULTS

Cell-wall architecture is altered in mutant *scw4scw10*

To analyse the function of Scw4p and Scw10p in cell-wall assembly, *scw4* and *scw10* mutants were analysed. Growth rates and general cell morphology of both mutants were similar to wild-type (data not shown; Cappellaro *et al.*, 1998). However, both mutants showed sensitivity to CFW and CR (Fig. 1a), and cell-wall chitin levels were slightly increased compared to wild-type (data not shown). These phenotypes were more pronounced in a *scw4scw10* double mutant. When compared to wild-type yeast, *scw4scw10* mutant cells displayed slower growth rates, morphological abnormalities and hypersensitivity towards CFW, CR and caffeine at concentrations which do not affect single mutants (Fig. 1a, b; Cappellaro *et al.*, 1998).

To study the cell-wall defects in more detail, we analysed the cell-wall composition of mutant *scw4scw10*. The chemical cell-wall analysis showed that levels of chitin (Table 2) and alkali-soluble glucan (Table 3) were significantly increased in mutant *scw4scw10*. Moreover, *scw4scw10* mutant cells

Fig. 1. Sensitivity of (a) *scw4*, *scw10* and *bgl2* mutants, and (b) *scw4scw10* and *scw4scw10bgl2* mutants to Calcofluor White (CFW), Congo Red (CR) and caffeine. A sample containing 5×10^4 cells and a series of fivefold dilutions from the sample were spotted onto YPD plates containing CFW, CR or caffeine, as indicated. Plates were incubated for 2–4 days at 30 °C.

Table 2. Chitin levels in *Saccharomyces cerevisiae* *scwΔ* mutants

Chitin content is expressed as μg *N*-acetylglucosamine per mg cell-wall dry weight (μg GlcNAc mg^{-1}). Values are mean $\pm$ SD of at least three independent determinations. WT, Wild-type.

Strain	Chitin content
SEY6210 (WT)	14.6 ± 0.8
<i>scw4scw10</i>	27.3 ± 2.9
<i>scw4scw10chs3</i>	1.5 ± 0.4
<i>scw4scw10bgl2</i>	19.8 ± 2.1
<i>scw4scw10sed1</i>	38.0 ± 2.7
<i>scw4scw10fks2</i>	40.3 ± 3.5

showed higher resistance to zymolyase (Fig. 2a) compared to wild-type. Increased chitin levels and changes in zymolyase sensitivity indicated that the cell-wall composition was changed, probably due to cell-wall compensatory mechanisms. Similar observations have been made for *gas1Δ* and *fks1Δ* mutants (Popolo *et al.*, 2001). To analyse whether the PKC1 pathway is activated in mutant *scw4scw10*, a PKC1 pathway-dependent β -galactosidase reporter *Rlm1-lacZ* (Kirchrath *et al.*, 2000) was used. Under normal growth conditions, β -galactosidase activity was at least three times higher in mutant *scw4scw10* relative to wild-type (Table 4). Further activation of the PKC1 pathway by 5 mM caffeine was possible (Table 4). These data suggested that the PKC1 pathway is important for cell-wall stability in the absence of Scw4p and Scw10p.

Since chitin synthase 3 (*CHS3*) is a major target gene of the PKC1-pathway, *CHS3* was disrupted in mutant *scw4scw10*. A 90% reduction of cell-wall chitin in mutant strain *scw4scw10chs3* compared to wild-type yeast was observed (Table 2). Mutant *scw4scw10chs3* cells grew in big clumps, showed irregular cell shapes and were hypersensitive towards caffeine and SDS (data not shown),

Table 3. Recovery of alkali-soluble and insoluble glucan from deproteinized cell walls of *S. cerevisiae* mutants

Values shown represent total hexoses (in μg per mg cell-wall dry weight) determined in ethanol precipitates of alkali extracts of trypsinized cell walls (1 M NaOH at 75 °C for 1 h) and in zymolyase hydrolysates of alkali-resistant material (1 mg ml^{-1} Zymolyase 100T). Values are mean $\pm$ SD of at least three independent determinations. WT, Wild-type.

Strain	Glucan recovery ($\mu\text{g mg}^{-1}$)	
	Soluble	Insoluble
SEY6210 (WT)	225 ± 10	115 ± 7
<i>scw4scw10</i>	270 ± 14	109 ± 7
<i>bgl2</i>	295 ± 14	99 ± 5
<i>scw4scw10bgl2</i>	310 ± 17	86 ± 4

Fig. 2. Sensitivity of mutants towards zymolyase. Symbols in (a): ▲, wild-type; ●, *scw4scw10*; ▼, *scw4scw10chs3*; ■, *scw4scw10bgl2*. Symbols in (b): ▲, wild-type; ●, *scw4scw10*; ■, *scw4scw10sed1*; ▼, *scw4scw10fks2*. Cells were suspended in 50 mM Tris/HCl, pH 7.5, and Zymolyase 100T at a final concentration of 10 $\mu\text{g ml}^{-1}$ was added. The decrease of OD₆₀₀ was monitored.**Table 4.** Indirect assessment of Mpk1p activity

Specific β -galactosidase activities were determined from cell lysates prepared from five independent transformants each. The activity is expressed in β -galactosidase units (U), where 1 U = $1000 \times A_{420} / \text{OD}_{600} \times t$, where t is in min. WT, Wild-type; -Caffeine, caffeine absent; +Caffeine, caffeine present.

Strain	β -Galactosidase activity	
	-Caffeine	+Caffeine
SEY6210 (WT)	14 ± 2	48 ± 2
<i>scw4scw10</i>	48 ± 4	98 ± 7
<i>scw4scw10bgl2</i>	29 ± 4	63 ± 5

Table 5. Sequential enzymic hydrolysis of cell walls isolated from the wild-type and from *scw4scw10*

Total hexoses are expressed in µg per mg cell-wall dry weight; values are mean±SD of three independent determinations. Levels of glucose and mannose are shown as a percentage of total hexoses. WT, Wild-type (SEY6210).

Strain		Glucanase			
		1,6-β-	1,3-β-	1,3-β-	1,6-β-
		Step 1	Step 2	Step 1	Step 2
WT	Total hexoses	620±60	350±30	830±60	170±35
	Glucose, mannose	28·7, 71·3	94·7, 5·3	57·9, 42·0	50·8, 49·1
<i>scw4scw10</i>	Total hexoses	630±45	325±20	610±50	370±40
	Glucose, mannose	25·6, 74·4	92·5, 7·5	54·4, 45·5	47·7, 52·3

pointing to an important role for *CHS3* in the cell-wall integrity of mutant *scw4scw10*. However, deletion of *CHS3* in mutant *scw4scw10* did not change the zymolyase-resistant phenotype of the parental strain (Fig. 2a). Thus, zymolyase resistance is not caused by elevated cell-wall chitin levels in the mutant *scw4scw10*.

To further analyse cell-wall composition, the amounts of mannose, glucose and *N*-acetylglucosamine (GlcNAc) were determined in cell walls of wild-type and mutant *scw4scw10* after total wall hydrolysis with TFA. Despite an approximately twofold increase in GlcNAc, due to increased chitin levels (Table 2), no significant change in mannose and glucose content could be detected. The mannose/glucose/GlcNAc ratio in the wild-type was 52/47/1·4 and in mutant *scw4scw10* 54/44/2·3. However, the amount of alkali-soluble glucan was increased in mutant *scw4scw10* (Table 3), suggesting that cross-linking between cell-wall components, rather than total levels of glucan and mannan, might be affected in mutant *scw4scw10*.

The glucan structure of mutant *scw4scw10* was further analysed. Cell walls were sequentially digested with recombinant 1,3-β-glucanase (Quantazyme) and 1,6-β-glucanase, and vice versa. The total hexoses released were determined in all hydrolytic fractions and, in addition, glucose and mannose were determined by HPAEC on Dionex in TFA hydrolysates of freeze-dried enzymic fractions (described in Methods). First, after digestion with 1,6-β-glucanase, the residual insoluble material was treated with 1,3-β-glucanase (Table 5). The 1,6-β-glucanase should release all mannoproteins attached to either 1,3-β-glucan or to chitin via 1,6-β-glucan. Subsequently, 1,3-β-glucanase degrades 1,3-β-glucan. Second, cell walls were first treated with 1,3-β-glucanase followed by 1,6-β-glucanase digestion (Table 5). In this case, the 1,3-β-glucanase fraction contains degraded 1,3-β-glucan and mannoproteins attached to 1,3-β-glucan. Subsequently, with 1,6-β-glucanase only mannoproteins attached to chitin should be released. Similar amounts of cell-wall sugars were released with 1,6-β-glucanase from wild-type and mutant *scw4scw10*, which indicates that the

same amount of mannoproteins was attached to the cell wall via 1,6-β-glucan. In contrast, the amount of total hexoses released by 1,3-β-glucanase from cell walls of mutant *scw4scw10* was reduced by approximately 25% compared to wild-type (Table 5). Subsequent hydrolysis of the 1,3-β-glucanase-resistant material with 1,6-β-glucanase released more than twice the amount of hexoses (Table 5). The increase of both glucose and mannose indicated an alteration in the cross-linking of mannoproteins to the cell wall via 1,6-β-glucan. Our data suggest that in mutant *scw4scw10* mannoproteins are preferentially attached to chitin rather than to 1,3-β-glucan. This is further substantiated by the observation that the amount of 1,6-β-glucan (detected with 1,6-β-glucan antibodies) was reduced in the alkali-soluble fraction of mutant *scw4scw10* (data not shown).

In summary, these results suggest that defects in the 1,3-β-glucan structure of the *scw4scw10* double mutant might be the reason for an altered cross-linking of cell-wall polymers, specifically GPI-mannoproteins and chitin. Similar results have been reported for the *gas1Δ* and the *fks1Δ* mutants (Kapteyn *et al.*, 1997).

The fact that in mutant *scw4scw10* more mannoproteins were linked to chitin but zymolyase resistance was not affected when *CHS3* was deleted suggested that further factors are involved in the compensation of cell-wall defects in mutant *scw4scw10*. To study this, we focused on two proteins which play an important role in cell-wall stability during stress and in stationary phase, but also when the amount of GPI-anchored cell-wall mannoproteins is decreased: Sed1p, a mannoprotein identified to be responsible for zymolyase resistance in stationary phase; and Fks2p, the alternative subunit of glucan synthase, which is upregulated in stationary phase and stress conditions (Shimoi *et al.*, 1998; Mazur *et al.*, 1995; Jung & Levin, 1999; De Nobel *et al.*, 2000; Hagen *et al.*, 2004). The single disruption of *SED1* or *FKS2* genes had no effect on growth or CFW and zymolyase sensitivity of exponential-phase cells (data not shown). However, the same deletions in

Fig. 3. Sed1p and Fks2p are important for cell-wall stability of mutant *scw4scw10*. Deletion of *SED1* or *FKS2* in *scw4scw10* causes hypersensitivity to CFW and CR. For details, see Fig. 1. WT, Wild-type.

mutant *scw4scw10* decreased growth rates and enhanced cell-morphology aberrations (data not shown). In addition, in mutants *scw4scw10sed1* and *scw4scw10fks2*, sensitivity towards CFW (Fig. 3) and chitin levels (Table 2) were increased, whereas cells were less resistant to zymolyase (Fig. 2b). These results indicated that, in addition to Chs3p, Sed1p and Fks2p are also crucial for cell-wall integrity in the absence of Scw4p and Scw10p.

Taken together, our results suggest that Scw4p and Scw10p play a crucial role in the assembly of a stable cell wall, and that the concerted action of several compensatory effects is necessary to mitigate cell-wall defects due to the loss of Scw4p and Scw10p function.

Glu-326 and Glu-380 are essential for Scw10p activity

Our cell-wall analyses suggested that the glucan/chitin/mannan meshwork is altered in the absence of *SCW4* and/or *SCW10*. Scw4p and Scw10p belong to the GH17 family of endo-1,3- β -glucanases and 1,3- β -glucan transglucosidases (CAZy database; <http://afmb.cnrs-mrs.fr/CAZY/>), suggesting that Scw10p and/or Scw4p might act as glucan hydrolases or transglucosidases. To support this assumption, we focused our further analyses on Scw10p since it is strongly upregulated in several cell-wall mutants, such as *gas1* Δ , *kre6* Δ and *mn9* Δ . To purify Scw10p, a HIS-tag was fused to the C-terminus of *SCW10* and the tagged protein (Scw10p^{HIS}) was expressed from a 2 μ -plasmid under the control of the *GAL1* promoter. The tagged protein was functional *in vivo*, since it fully complemented the growth defect of mutant *scw4scw10* in the presence of cell-wall-perturbing agents (data not shown). Scw10p^{HIS} was purified to homogeneity and tested for hydrolytic as well as transglycosylase activity using laminarin, pustulan, chitin, yeast glucan, *p*-nitrophenylglucoside and isolated intact cell walls as substrates. However, no enzymic activity could be detected (data not shown).

To obtain evidence for Scw10p being a hydrolase/transglucosidase, a mutational analysis was performed. The three-dimensional structures of two plant enzymes of the

GH17 family, endo-1,3- β -glucanase and lichenase of barley, have been resolved, and two glutamate residues identified as being essential for enzyme activity (Varghese *et al.*, 1994). Both glutamate residues are conserved in all members of the GH17 family (Fig. 4a). To analyse whether these residues are also essential for Scw10p function, Glu-326 and Glu-380 were replaced with glutamine. In contrast to wild-type Scw10p, neither of the mutant proteins Scw10pE326Q and Scw10pE380Q complemented the sensitivity of *scw4scw10* to cell-wall-perturbing compounds (Fig. 4b). Protein amounts were similar to wild-type Scw10p (data not shown). These data strongly support the assumption that Scw10p acts as a glucan hydrolase or transglucosidase on yeast cell-wall glucan.

Interconnection of Bgl2p with Scw4p and Scw10p

We wished to analyse whether or not there is an interconnection between Scw4p/Scw10p and Bgl2p, a further member of the GH17 family. Deletion of *BGL2* does not result in a dramatic change of the growth phenotype, although Bgl2p is one of the most abundant cell-wall proteins (Mrša *et al.*, 1993). In a similar manner to the *scw4* and *scw10* single mutants, the *bgl2* mutant showed increased sensitivity towards CFW and CR (Fig. 1a). In addition, the amount of alkali-soluble glucan was increased in mutant *bgl2* (Table 3). However, in contrast to the *scw4scw10* mutant, *bgl2* cells showed increased zymolyase sensitivity (data not shown). To analyse the genetic interaction between *BGL2* and *SCW4/SCW10*, the *BGL2* gene was deleted in mutant *scw4scw10*. The triple *scw4scw10bgl2* mutant showed a minor increase in sensitivity towards CFW compared to mutant *scw4scw10* (Fig. 1b). However, all other phenotypes, such as growth rate (data not shown) and sensitivity towards caffeine and zymolyase (Figs 1b and 2a), were much less pronounced compared to those of *scw4scw10* and resembled wild-type features. Also, total cell-wall chitin content was similar to that of wild-type (Table 2). On the other hand, activation of the PKC1 pathway was less pronounced in *scw4scw10bgl2* compared to *scw4scw10* (Table 4). These data suggested that Scw4p/Scw10p and Bgl2p might have antagonistic functions

Fig. 4. Glu-326 and Glu-380 are crucial for Scw10p activity. (a) Amino acid alignment of Bgl2p, Scw4p, Scw10p and barley 1,3- β -glucanase. Identical amino acids are shown in black, conserved amino acids in grey. Arrows indicate catalytic glutamate residues of barley 1,3- β -glucanase. (b) Sensitivity towards CFW, CR and caffeine of mutant *scw4scw10* transformed with plasmid pRS416 (*scw4scw10*) or the same plasmid harbouring either *SCW10* (pSE10) or mutated *SCW10E326Q* (pSE10-1) and *SCW10E380Q* (pSE10-2) (two independent transformants).

in vivo. Therefore, the mutual influence of Scw10p and Bgl2p was further analysed. When *SCW10* was over-expressed in a *bgl2* mutant, a dramatic increase in sensitivity towards CFW was observed, whereas overexpression in mutant *scw4* had no effect (Fig. 5a). When Scw10p protein levels were checked in wild-type and *bgl2* cells by Western blot analysis, the amount of Scw10p was reduced in mutant *bgl2* (Fig. 5b) and Scw10p levels were found to be restored by introducing *BGL2* on a plasmid. In addition, moderately enhanced expression of Bgl2p increased Scw10p levels in wild-type cells (Fig. 5c). Summarizing, our data indicate that levels of Bgl2p and Scw10p have to be balanced for the formation of an intact cell wall.

DISCUSSION

Cell-wall analyses and the phenotypes of *scw4* and *scw10* deletion mutants have shown that Scw10p and Scw4p are crucial for yeast cell-wall stability. Scw10p in particular seems to play a specific role in cell-wall integrity, as concluded from the mRNA expression pattern (Spellman *et al.*, 1998) and genome-wide transcriptome analysis (Lagorce *et al.*, 2003; Hagen *et al.*, 2004).

Scw10p is a member of GH17, a family of mainly plant 1,3- β -glucanases (EC 3.2.1.39) and (1,3-1,4)- β -glucanases (EC 3.2.1.73). Catalytic amino-acid residues of 1,3- β -glucanase isoenzyme GII and the (1,3-1,4)- β -glucanase isoenzyme EII from barley have been investigated (Chen *et al.*, 1993), and amino acids Glu-231 and Glu-232 have been identified as the likely catalytic nucleophiles in GII and EII, respectively. Amino acid Glu-288 represents the putative catalytic acid at the active site of both enzymes. The three-dimensional structures of both enzymes show potential catalytic glutamate residues located in a deep substrate-binding cleft at a distance which enables their function in the catalytic reaction (Varghese *et al.*, 1994). Finally, site-directed mutagenesis of 1,3- β -glucanase demonstrates a 20 000-fold and 350-fold decrease in activity, respectively, when Glu-231 or Glu-288 is changed for glutamine (Chen *et al.*, 1995). In all yeast members of the GH17 family, the catalytic glutamate residues of both barley enzymes are conserved (Fig. 4a). In the case of Scw10p, these are Glu-326 and Glu-380 at the C-terminus of the protein. The exchange of either of them for glutamine caused the loss of Scw10p function. Thus, Scw10p most likely acts as a glucanase. The reason that our biochemical analyses did not reveal any glucanase or

Fig. 5. (a) Overexpression of *SCW10* negatively affects cell-wall stability of mutant *bgl2*. Mutants *scw4* and *bgl2* transformed with pRS424SCW and pRS425SCW, respectively, were grown on YPD containing CFW, as indicated. (b) and (c): Western blot analysis of cell-wall extracts probed with anti-HIS (b) and anti-MYC (c) antibody. The reduced expression level of *SCW10* in mutant *bgl2* (b) can be restored to the level of the wild-type (WT) by simultaneous expression of *BGL2*. Mutant *bgl2* and the wild-type strain expressing *SCW10^{HIS}* (pRS424SCW) and *BGL2* (NEV-BGL) are indicated. Overexpression of *BGL2^{HA}* increases the expression level of *SCW10* (c). The wild-type strain with *SCW10^{MYC}* integrated at the genomic *SCW10* locus transformed with *BGL2^{HA}* is shown.

glucosyltransferase activity could be due to very specific substrate requirements.

Synergistic effects of the double deletion of *SCW4* and *SCW10* concerning the sensitivity to cell-wall-perturbing agents indicated a similar function for both proteins. On the one hand, this was supported by the finding that the amount of Scw10p was increased in mutant *scw4* (data not shown). On the other hand, however, we observed a clear functional difference of both proteins. The phenotypes of the single deletion mutants were slightly different, and overproduction of Scw10p did not complement the sensitivity of mutant *scw4* towards CFW. Moreover, *SCW4* and *SCW10* are differently regulated. The expression of *SCW4* does not change during the cell cycle, whereas *SCW10* is cell-cycle regulated, reaching a maximum of expression during G1-phase (Spellman *et al.*, 1998). It seems that Scw10p has a similar, but still distinct, function compared to Scw4p. Neither protein can fully complement the other, but the presence of one of them is sufficient for normal cell growth. Similar observations have been made with Crh1p and Crh2p (Rodriguez-Peña *et al.*, 2000), which also show significant homology. Deletion of the corresponding genes causes additive sensitivity towards CFW and CR. Both genes show a different pattern of transcriptional regulation: *CRH1* is cell-cycle regulated, whereas *CRH2* expression does not vary during the cell cycle. Consistent with the observed expression pattern, Crh1p-GFP is found to localize at the incipient bud site and in later stages around the

septum area, whereas Crh2p is found mainly at a bud neck throughout the whole cell cycle.

Mutants defective in the synthesis of cell-wall components show altered cell-wall composition and architecture. This is due to mechanisms that compensate for the loss in strength of the cell wall (Popolo *et al.*, 2001; Smits *et al.*, 2001). An increased level of chitin is one of the typical compensatory mechanisms of cell-wall mutants (Popolo *et al.*, 1997; Garcia-Rodriguez *et al.*, 2000). Chitin is glycosidically linked to non-reducing branches of both 1,3- β -glucan and 1,6- β -glucan (Kollar *et al.*, 1995, 1997). It is essential to the insolubility of the wall material, and chitin incorporation results in transfer of the wall material from the alkali-soluble to the alkali-insoluble fraction (Hartland *et al.*, 1994). Activation of the PKC1 pathway and increased cell-wall chitin indicate a compensatory mechanism in mutant *scw4scw10*. The chitin participating in linkages to both 1,3- β -glucan and 1,6- β -glucan is synthesized by Chs3p (Kollar *et al.*, 1995). Chs3p has been shown to be responsible for chitin accumulation in *gas1 Δ* and *fks1 Δ* mutants (Valdivieso *et al.*, 2000; Garcia-Rodriguez *et al.*, 2000). Deletion of *CHS3* in these mutants causes severe growth defects. Similarly, in mutant *scw4scw10*, deletion of *CHS3* stressed its role for cell-wall integrity. Interestingly, in spite of the fact that there is more chitin in the cell wall of mutant *scw4scw10*, the alkali-soluble fraction increased as well. Thus it seems that cross-linking of glucan and chitin is impaired in this mutant. These results, and the absence of significant

differences between wild-type and mutant *scw4scw10* in the amounts of glucose and mannose, indicate that cross-linking of cell-wall polymers, rather than overall composition, is changed in the mutant. Alternative cross-linking of cell-wall mannoproteins to chitin has also been shown for 1,3- β -glucan mutants *gas1 Δ* and *fks1 Δ* (Kapteyn *et al.*, 1997).

Another feature in common between the *scw4scw10* and *fks1 Δ* and *gas1 Δ* mutants is resistance to zymolyase (Popolo *et al.*, 2001). The resistance has been attributed to changes in the mannoprotein layer (Vink *et al.*, 2002), with a specific function for some of the cell-wall proteins (Van Der Vaart *et al.*, 1995). In mutant *scw4scw10*, zymolyase resistance is not lost after deletion of *CHS3*. Increased chitin levels and altered cell-wall structure in mutants *scw4scw10sed1* and *scw4scw10fks2* suggest an important role for both Sed1p and Fks2p in the cell-wall compensation of the *scw4scw10* mutant. The reduced compensation is evident also from reduced zymolyase resistance. This indicates that an additional survival mechanism can be activated for yeast to cope with specific cell-wall stresses. In the case of multiple GPI-mutants, the overexpression of specific cell-wall-related genes has been shown to be responsible for specific compensation (Hagen *et al.*, 2004).

Deletion mutant *bgl2* presented as a mutant without any specific phenotype. On the other hand, the overexpression of *BGL2* seems to be lethal (Mrša *et al.*, 1993). We could demonstrate in this study that cells carrying the *BGL2* deletion behave as cells with a slightly impaired glucan structure. However, this defect does not affect normal cell growth and does not activate compensatory mechanisms. An unexpected phenotype was found when *BGL2* was deleted in mutant *scw4scw10*. Most of the growth and phenotype parameters were much less pronounced compared to *scw4scw10* and resembled wild-type features, including reduced activation of the PKC1 pathway. The deletion of *BGL2* in mutant *scw4scw10* does not result in a wild-type phenotype. The sensitivity of *scw4scw10bgl2* towards CFW, as well as an increased alkali-soluble fraction, indicates a weakened cell wall. On the other hand, deletion of *BGL2* stabilizes the cell-wall structure of the *scw4scw10* mutant and reduces compensatory mechanisms. The effect of the *BGL2* deletion in *scw4scw10* was shown to be specific. The deletion of any of the other cell-wall-related genes tested, namely *SCW11*, *CRH1*, *CRH2* and *EXG1*, in mutant *scw4scw10* could not restore the phenotype in a similar manner to the *scw4scw10bgl2* mutant (data not shown). Thus, we conclude that the Scw4/10 proteins and Bgl2p might have antagonistic functions, and that levels of Bgl2p and Scw10p need to be in balance for stable cell-wall formation.

In summary, the resemblance of the *scw4scw10* mutant to the *gas1 Δ* and *fks1 Δ* mutants indicates that Scw4p and Scw10p play an important role in the correct assembly of glucan structure and the cross-linking of glucan with other cell-wall polymers. The evidence that the glutamate residues

essential for 1,3- β -glucanase activity in the GH17 family are essential for the function of Scw10p strongly supports this indication.

ACKNOWLEDGEMENTS

We wish to thank I. Fuchs for excellent technical assistance. J. P. Latgé and L. Lehle are gratefully acknowledged for assistance in measuring transglucosidase activity and HPAEC measurement, respectively. We thank Drs H. Bussey for providing 1,6- β -glucan antibodies, J. J. Heinisch for plasmids, V. Farkas for radioactive oligosaccharides and J. Stolz for many helpful discussions. This work has been supported by European Union Grant QLK3-CT-2000-01537, by the Deutsche Forschungsgemeinschaft (SFB 521) and by the Fonds der Chemischen Industrie.

REFERENCES

- Baladron, V., Ufano, S., Dueñas, S., Martín-Cuadrado, A. B., del Rey, F. & Vazquez de Aldana, C. R. (2002). Eng1p, an endo-1,3- β -glucanase localized at the daughter side of the septum, is involved in cell separation in *Saccharomyces cerevisiae*. *Eukaryot Cell* **1**, 774–786.
- Cappellaro, C., Mersa, V. & Tanner, W. (1998). New potential cell wall glucanases of *Saccharomyces cerevisiae* and their involvement in mating. *J Bacteriol* **180**, 5030–5037.
- Chen, L., Fincher, G. B. & Hoj, P. B. (1993). Evolution of polysaccharide hydrolase substrate specificity. *J Biol Chem* **268**, 13318–13326.
- Chen, L., Garrett, T. P. J., Fincher, G. B. & Hoj, P. B. (1995). A tetrad of ionizable amino acids is important for catalysis in barley β -glucanases. *J Biol Chem* **270**, 8093–8101.
- Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H. & Hieter, P. (1992). Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**, 119–122.
- Cid, V. J., Duran, D., del Rey, F., Snyder, M. P., Nombela, C. & Sanchez, M. (1995). Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. *Microbiol Rev* **59**, 345–386.
- Colman-Lerner, A., Chin, T. E. & Brent, R. (2001). Yeast Cbk1 and Mob2 activate daughter-specific genetics programs to induce asymmetric cell fates. *Cell* **107**, 739–750.
- Davidson, E. A. (1966). Analysis of sugars in mucopolysaccharides. *Methods Enzymol* **8**, 52–60.
- De Nobel, H., Ruiz, C., Martin, H., Morris, W., Brul, S., Molina, M. & Klis, F. M. (2000). Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2-mediated increase in *FKS2-lacZ* expression, glucanase resistance and thermotolerance. *Microbiology* **146**, 2121–2132.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**, 350–356.
- Garcia, R., Bermejo, C., Grau, C., Perez, R., Rodriguez-Pena, J. M., Francois, J., Nombela, C. & Arroyo, J. (2004). The global transcriptional response to transient cell wall damage in *Saccharomyces cerevisiae* and its regulation by the cell integrity signaling pathway. *J Biol Chem* **279**, 15183–15195.
- Garcia-Rodriguez, L. J., Trilla, J. A., Castro, C., Valdivieso, M. H., Duran, A. & Roncero, C. (2000). Characterization of the chitin biosynthesis process as a compensatory mechanism in the *fks1* mutant of *Saccharomyces cerevisiae*. *FEBS Lett* **478**, 84–88.

- Gietz, R. D., Schiestl, R. H., Willems, A. R. & Woods, R. A. (1995). Studies of the transformations of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* **11**, 355–360.
- Goldman, R. C., Sullivan, P. A., Zakula, D. & Capobianco, J. O. (1995). Kinetics of β -1,3 glucan interaction at the donor and acceptor sites of the fungal glucosyltransferase encoded by the *BGL2* gene. *Eur J Biochem* **227**, 372–378.
- Hagen, I., Ecker, M., Lagorce, A. & 8 other authors (2004). Sed1p and Srl1p are required to compensate for cell wall instability in *Saccharomyces cerevisiae* mutants defective in multiple GPI-anchored mannoproteins. *Mol Microbiol* **52**, 1413–1425.
- Hartland, R. P., Vermeulen, C. A., Klis, F. M., Sietsma, J. H. & Wessels, J. G. (1994). The linkage of (1-3)- β -glucan to chitin during cell wall assembly in *Saccharomyces cerevisiae*. *Yeast* **10**, 1591–1599.
- Heinisch, J. J., Lorberg, A., Schmitz, H. P. & Jacoby, J. J. (1999). The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in *Saccharomyces cerevisiae*. *Mol Microbiol* **32**, 671–680.
- Henrissat, B. & Bairoch, A. (1996). Updating the sequence-based classification of glycosyl hydrolases. *Biochem J* **316**, 695–696.
- Igal, J. C., Johnson, A. L. & Johnston, L. H. (1996). Coordinated regulation of gene expression by the cell cycle transcription factor SWI4 and the protein kinase C MAP kinase pathway for yeast cell integrity. *EMBO J* **15**, 5001–5013.
- Jung, U. S. & Levin, D. E. (1999). Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signaling pathway. *Mol Microbiol* **34**, 1049–1057.
- Kaiser, C., Michaelis, S. & Mitchel, A. (1994). *Methods in Yeast Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Kapteyn, J. C., Ram, A. F. J., Groos, E. M., Kollar, R., Montijn, R. C., Van Den Ende, H., Llobell, A., Cabib, E. & Klis, F. M. (1997). Altered extent of cross-linking of β -1,6-glucosylated mannoproteins to chitin in *Saccharomyces cerevisiae* mutants with reduced cell wall β -1,3-glucan content. *J Bacteriol* **179**, 6279–6284.
- Kirchrath, L., Lorberg, A., Schmitz, H.-P., Gengenbacher, U. & Heinisch, J. J. (2000). Comparative genetic and physiological studies of the MAP kinase Mpk1p from *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. *J Mol Biol* **300**, 743–758.
- Klis, F. M., Mol, P., Hellingwerf, K. & Brul, S. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* **26**, 239–256.
- Kollar, R., Petrakova, E., Ashwell, G., Robbins, P. W. & Cabib, E. (1995). Architecture of the yeast cell wall: the linkage between chitin and β (1-3)-glucan. *J Biol Chem* **270**, 1170–1178.
- Kollar, R., Reinhold, B. B., Petrakova, E., Yeh, H. J. C., Ashwell, G., Drgonova, J., Kapteyn, J. C., Klis, F. M. & Cabib, E. (1997). Architecture of the yeast cell wall: β (1 $\rightarrow$ 6)-glucan interconnects mannoprotein, β (1 $\rightarrow$ 3)-glucan, and chitin. *J Biol Chem* **272**, 17762–17775.
- Lagorce, A., Hauser, N. C., Labourdette, D., Rodriguez, C., Martin-Yken, H., Arroyo, J., Hoheisel, J. D. & Francois, J. (2003). Genome-wide analysis of the response to cell wall mutations in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* **278**, 20345–20357.
- La Valle, R., Sandini, S., Gomez, M. J., Mondello, F., Romagnoli, G., Nisini, R. & Cassone, A. (2000). Generation of recombinant 65-kilodalton mannoprotein, a major antigen target of cell-mediated immune response to *Candida albicans*. *Infect Immun* **68**, 6777–6784.
- Lommel, M., Bagnat, M. & Strahl, S. (2004). Aberrant processing of the WSC family and Mid2p cell surface sensors results in cell death of *Saccharomyces cerevisiae* O-mannosylation mutants. *Mol Cell Biol* **24**, 46–57.
- Lussier, M., Sdicu, A.-M., Shahinian, S. & Bussey, H. (1998). The *Candida albicans* *KRE9* gene is required for cell wall β -1,6-glucan synthesis and is essential for growth on glucose. *Proc Natl Acad Sci U S A* **95**, 9825–9830.
- Madden, K., Sheu, Y. J., Baetz, K., Andrews, B. & Snyder, M. (1997). SBF cell cycle regulator as a target of the yeast PKC-MAP kinase pathway. *Science* **275**, 1781–1784.
- Martin-Cuadrado, A. B., Duenas, E., Sipiczki, M., Vazquez de Aldana, C. R. & del Rey, F. (2003). The endo- β -1,3-glucanase eng1p is required for dissolution of the primary septum during cell separation in *Schizosaccharomyces pombe*. *J Cell Sci* **116**, 1689–1698.
- Mazur, P., Morin, N., Baginsky, W., El-Sherbeini, M., Clemas, J. A., Nielsen, J. B. & Foor, F. (1995). Differential expression and function of two homologous subunits of yeast 1,3- β -D-glucan synthase. *Mol Cell Biol* **15**, 5671–5681.
- Mouassite, M., Camougrand, N., Schwob, E., Demaison, G., Laclau, M. & Guerin, M. (2000). The “SUN” family: yeast *SUN4/SCW3* is involved in cell septation. *Yeast* **16**, 905–919.
- Mouyna, I., Fontaine, T., Vai, M., Monod, M., Fonzi, W. A., Diaquin, M., Popolo, L., Hartland, R. P. & Latge, J.-P. (2000). Glycosylphosphatidylinositol-anchored glucanoyltransferases play an active role in the biosynthesis of the fungal cell wall. *J Biol Chem* **275**, 14882–14889.
- Mrša, V., Klebl, F. & Tanner, W. (1993). Purification and characterization of the *Saccharomyces cerevisiae* *BGL2* gene product, a cell wall endo- β -1,3-glucanase. *J Bacteriol* **175**, 2102–2106.
- Popolo, L. & Vai, M. (1999). The Gas1 glycoprotein, a putative wall polymer cross-linker. *Biochim Biophys Acta* **1426**, 385–400.
- Popolo, L., Gilardelli, D., Bonfante, P. & Vai, M. (1997). Increase in chitin as an essential response to defects in assembly of cell wall polymers in the *gsp1Δ* mutant of *Saccharomyces cerevisiae*. *J Bacteriol* **179**, 463–469.
- Popolo, L., Gualtieri, T. & Ragni, E. (2001). The yeast cell-wall salvage pathway. *Med Mycol* **39**, 111–121.
- Rodriguez-Peña, J. M., Cid, V. J., Arroyo, J. & Nombela, C. (2000). A novel family of cell-wall related proteins regulated differently during the yeast life cycle. *Mol Cell Biol* **20**, 3245–3255.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- 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.
- Skory, C. D. & Freer, S. N. (1995). Cloning and characterization of a gene encoding a cell-bound, extracellular β -glucosidase in the yeast *Candida wickerhamii*. *Appl Environ Microbiol* **61**, 518–525.
- Smits, G. J., van den Ende, H. & Klis, F. M. (2001). Differential regulation of cell wall biogenesis during growth and development in yeast. *Microbiology* **147**, 781–794.
- Spellman, P. T., Sherlock, G., Zhang, M. Q., Iyer, V. R., Anders, K., Eisen, M. B., Brown, P. O., Botstein, D. & Futcher, B. (1998). Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Mol Biol Cell* **9**, 3273–3297.
- Stagljär, I., Korostensky, C., Johnsson, N. & Te Heeden, S. (1998). A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins *in vivo*. *Proc Natl Acad Sci U S A* **95**, 5187–5192.
- Valdivieso, H. N., Ferrario, L., Vai, M., Duran, V. & Popolo, L. (2000). Chitin synthesis in *gas1* mutant of *Saccharomyces cerevisiae*. *J Bacteriol* **182**, 4752–4757.

Van Der Vaart, J. M., Caro, L. H. P., Chapman, J. W., Klis, F. M. & Verrips, C. T. (1995). Identification of three manno-proteins in the cell wall of *Saccharomyces cerevisiae*. *J Bacteriol* **177**, 3104–3110.

Varghese, J. N., Garrett, T. P. J., Colman, P. M., Chen, L., Hoj, P. B. & Fincher, G. B. (1994). Three-dimensional structures of two plant

β -glucan endohydrolases with distinct substrate specificities. *Proc Natl Acad Sci U S A* **91**, 2785–2789.

Vink, E., Vossen, J. H., Ram, A. F., van den Ende, H., Brekelmans, S., de Nobel, H. & Klis, F. M. (2002). The protein kinase Kic1 affects 1,6- β -glucan levels in the cell wall of *Saccharomyces cerevisiae*. *Microbiology* **148**, 4035–4048.