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Sed1p and Srl1p are required to compensate for cell wall instability in *Saccharomyces cerevisiae* mutants defective in multiple GPI-anchored mannoproteins

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Summary

The covalently linked cell wall protein Ccw12p of *Saccharomyces cerevisiae* is a GPI-anchored protein (V. Mrsa *et al.*, 1999, *J Bacteriol* 181: 3076–3086). Although only 121 amino acids long, the haemagglutinin-tagged protein released by laminarinase from the cell wall possesses an apparent molecular mass of >300 kDa. A membrane-bound form with an apparent molecular mass of 58 kDa is highly O- and N-glycosylated and contains the GPI anchor. With a half-life of 2 min, the membrane form is transformed to the >300 kDa form. The deletion mutant *ccw12*Δ grows slower than the wild type, is highly sensitive to Calcofluor white and contains 2.5 times more chitin. Further, compared with wild-type yeast, significantly more proteins are released from intact cells when treated with dithiothreitol. Interestingly, these defects become less pronounced when further GPI-anchored cell wall proteins are deleted. Mutant ΔGPI (simultaneous deletion of *CCW12*, *CCW13/DAN1*, *CCW14*, *TIP1* and *CWP1*) is similar in many respects to wild-type yeast. To find out how the cell wall is stabilized in mutant ΔGPI, a genome-wide transcription analysis was performed. Of 159 significantly regulated genes, 14 encode either known or suspected cell wall-associated proteins. Analysis of genes affected in transcription revealed that *SED1* and *SRL1* in particular are required to reconstruct cell wall stability in the absence of multiple GPI-anchored mannoproteins.

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Introduction

The cell wall of *Saccharomyces cerevisiae* consists of β-1,3-glucan, β-1,6-glucan, chitin and various kinds of mannoproteins, which are interconnected to form a macromolecular complex (Klis *et al.*, 2002). Cell wall proteins play an important role, both as structural components and as enzymes involved in cell wall assembly. Depending on their linkage to the cell wall, they are divided into two classes. One class is composed of proteins that are extracted with SDS and SH reagents. These proteins are considered to be either non-covalently entrapped in or S-S-linked to the cell wall and are named soluble cell wall proteins (Scwp) (Cappellaro *et al.*, 1998). The other class consists of proteins that are covalently linked to the glucan framework. These covalently linked cell wall proteins (Ccwp) can be grouped into two subclasses depending on their linkage to glucan. PIR (proteins with internal repeats)-Ccwps are bound directly to β-1,3-glucan through an unidentified linkage and can be released from the cell wall by β-1,3-glucanase or by mild alkali extraction (Mrsa *et al.*, 1997; Kapteyn *et al.*, 1999; Klis *et al.*, 2002). The second subclass, the glycosylphosphatidylinositol (GPI)-Ccwps are bound to β-1,6-glucan through a processed form of the GPI anchor (Lu *et al.*, 1994; 1995; Montijn *et al.*, 1994; Kollar *et al.*, 1997). In the transfer step from the plasma membrane to the cell wall, the GPI anchor is split, the glucosaminyl and phosphatidylinositol moiety is lost, and the protein is postulated to be linked to β-1,6-glucan via a residue containing phosphoethanolamine and several mannosyl residues (Kollar *et al.*, 1997; Fujii *et al.*, 1999). As β-1,6-glucan is linked in turn to β-1,3-glucan, GPI-Ccwps can be extracted from the cell wall with both β-1,6- and β-1,3-glucanases (Kapteyn *et al.*, 1996).

In the genome of *S. cerevisiae*, about 60–70 proteins have been identified as containing a GPI-anchoring

sequence (Caro *et al*., 1997; Klis *et al*., 2002). Forty of them are expected to remain attached to the plasma membrane, and the rest are thought to be transferred to the cell wall. Biochemical approaches have identified 22 of these latter proteins directly in different cell wall extracts (Roy *et al*., 1991; Van der Vaart *et al*., 1995; Bony *et al*., 1997; Kitagaki *et al*., 1997; Moukadiri *et al*., 1997; Mrsa *et al*., 1997; 1999; Hamada *et al*., 1998; Shimoi *et al*., 1998; Rodriguez-Pena *et al*., 2000). GPI-Ccwps are involved in a number of phenomena including sexual agglutination (Lipke and Kurjan, 1992; Cappellaro *et al*., 1994), cell surface hydrophobicity (Alexandre *et al*., 2000), flocculation (Teunissen *et al*., 1993) and resistance towards lytic enzymes in stationary phase cells (Shimoi *et al*., 1998). With the exception of the agglutinins, the functions or enzymatic activities of covalently linked cell wall proteins remain mostly obscure.

Although mutations in many GPI proteins cause reduced cell wall stability, none of these proteins is essential for viability. In order to gain insights into the contribution of this protein class to cell wall integrity, we analysed mutants with multiple deletions of GPI protein-encoding genes. Altogether, the genes *CCW12*, *CCW13*/*DAN1*, *CCW14*, *CWP1* and *TIP1* were deleted to create the mutant ΔGPI. Cwp1p und Tip1p have been described as major cell wall components (Shimoi *et al*., 1995; Van der Vaart *et al*., 1995). Ccw12p, Ccw13p and Ccw14p are released from the wall by β-1,3-glucanase and, after SDS–PAGE chromatography, they remain in the stacking gel (Mrsa *et al*., 1999), indicating that they are part of the complex cell wall subunits described by Kollar *et al*. (1997). Deletion of *CCW12* results in hypersensitivity towards Calcofluor white. This property is lost when additional disruptions of *CCW13* and *CCW14* are introduced into cells. As repair and compensatory mechanisms guaranteeing cell wall integrity are known to exist (Heinisch *et al*., 1999; Popolo *et al*., 2001; Klis *et al*., 2002; Lagorce *et al*., 2003), the question arose as to what extent these may be involved in compensation for the loss of a significant amount of the mannan layer. To obtain information concerning this question, transcriptional analysis of mutant ΔGPI was performed using DNA arrays. One hundred and fifty-nine significantly regulated genes were identified. Among those, we identified *SED1* and *SRL1* as being required to stabilize the cell wall in the absence of multiple GPI-anchored mannoproteins.

Results and discussion

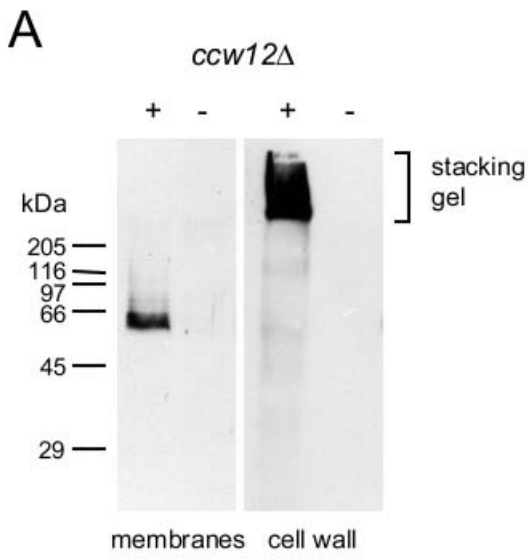
Characterization of the post-translational modifications of Ccw12p

To analyse functions of GPI-anchored mannoproteins, Ccw12p was used as a model protein. Ccw12p is homologous to the N-terminal half of the Sed1p, and its expression decreases in the presence of α-factor (Seidel and Tanner, 1997; Mrsa *et al*., 1999). The protein is released from the cell wall by digestion with laminarinase (Mrsa *et al*., 1999). The processed and unmodified Ccw12p has a predicted molecular mass of ≈10 kDa. However, when released from the cell wall by laminarinase, the protein stays in the stacking gel during SDS–PAGE, corresponding to an apparent molecular mass of >300 kDa (Mrsa *et al*., 1999). This is thought to be caused by a covalent association of the protein via a GPI anchor remnant with part of the glucan–chitin network of the cell wall (Kollar *et al*., 1997).

To study the maturation of Ccw12p in more detail, a haemagglutinin (HA) tag was fused in triplicate directly after the signal peptidase cleavage site, resulting in a 121-amino-acid version of Ccw12p. Ccw12p^{HA} was expressed on a 2μ plasmid in *ccw12*Δ and fully complements the growth defects of this mutant (data not shown). Isolated cell walls of the transformed strain were extracted with SDS and subsequently incubated with laminarinase. By Western blot analysis of Ccw12p^{HA}, a polydisperse band with an apparent molecular mass of >300 kDa could be detected in the stacking gel (Fig. 1A). In crude membrane preparations, a 58 kDa form of Ccw12p^{HA} was detected by SDS–PAGE, and the same form was observed in a *sec18* mutant at the non-permissive temperature (data not shown), indicating that the biggest increase in molecular weight of Ccw12p^{HA} most probably occurs outside the cell. The membrane form corresponds to the protein after cleavage of the signal peptide, glycosylation and addition of the GPI anchor. The increase in size caused by addition of the GPI anchor was determined in a *gpi1*Δ mutant, defective in GPI anchor synthesis at the restrictive temperature (Leidich and Orlean, 1996). This mutant shows a defect in the synthesis of *N*-acetylglucosaminyl-phosphatidylinositol, which is the first intermediate in GPI synthesis (Leidich *et al*., 1994). Ccw12p^{HA} was expressed in mutant *gpi1*Δ, and the apparent molecular mass of the membrane form of the protein was compared at permissive and restrictive temperature. A decrease of about 5 kDa was observed (Fig. 1B), proving that the 58 kDa version of Ccw12p^{HA} is GPI anchored.

Ccw12p has three predicted attachment sites for N-linked carbohydrate chains and contains nearly 40% serine and threonine residues. To determine the increase in size caused by N-linked sugars, the membrane preparation was treated with endoglycosidase H to remove all N-chains. A reduction in molecular weight of 5 kDa was observed, corresponding to a core glycosylation at the three N-glycosylation sites (Fig. 1B).

The addition of N-linked sugar chains and the GPI anchor contribute only a comparatively small amount to the molecular weight of the membrane form of Ccw12p^{HA}.



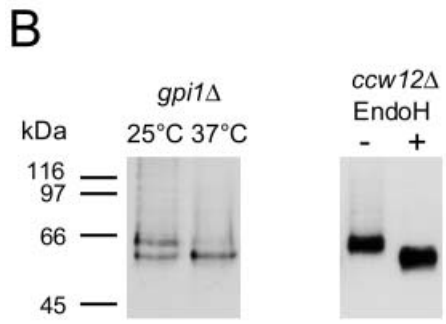


Fig. 1. A. Localization of Ccw12p. Western blot of crude membranes (corresponding to 1 OD cells) and laminarinase extracts of isolated cell walls (corresponding to 20 OD cells). *ccw12*Δ mutant cells were transformed with pME11 carrying *CCW12*^{HA} (+) and empty vector as a control (–). Blots were probed with anti-HA monoclonal antibody. B. Analysis of Ccw12p modifications. Right. Ccw12p^{HA} was expressed in the *gpi1*Δ mutant. Crude membranes were prepared from cells growing at permissive (25°C) and restrictive (37°C) temperatures. Left. Crude membranes were prepared from *ccw12*Δ mutant cells transformed with pME11 and treated with endoglycosidase H (+) or mock treated (–) as described in *Experimental procedures*. Ccw12p was detected with anti-HA monoclonal antibody.

By removing all sugar chains with fluoric acid, the size of the protein decreased to ≈16 kDa, which correlates well with the expected size predicted from the amino acid sequence of the HA-tagged Ccw12p (data not shown). It was therefore likely that O-mannosylation is mainly responsible for the increase in size of the protein from 16 kDa to 58 kDa. Although further modifications of Ccw12p in the Golgi apparatus, especially the addition of outer chain carbohydrates, cannot be ruled out, the absence of detectable intermediate forms in membrane preparations favours the interpretation of a highly O-mannosylated, solely core N-glycosylated modification of Ccw12p. Most probably, part of the glucan–chitin meshwork is extracted together with Ccw12p by β-glucanases from the cell wall, which leads to the high apparent molecular mass of the protein as observed during SDS–PAGE in the stacking gel.

In order to determine the transfer rate of Ccw12p^{HA} from the membrane-bound form to the cell wall, a time course was performed in the presence of cycloheximide. Cells were harvested at different time points after the addition of cycloheximide (100 μg ml^{-1}). Cell wall and membrane proteins were extracted as described above. As shown in Fig. 2, the inhibition of protein synthesis leads to a rapid decline in the 58 kDa form of Ccw12p^{HA} with an estimated half-life of about 2 min. This observation indicates a fast transfer reaction of Ccw12p to the glucan polymer. Similar time intervals are assumed to be required for GPI proteins in general to be transferred to the cell wall.

Phenotypic characterization of ccw12Δ *and multiple deletions of GPI-anchored proteins*

Deletions of many GPI protein encoding genes including *CCW12* result in hypersensitivity to cell wall-perturbing agents such as Calcofluor white (CFW) and Congo red (CR) (Ram *et al*., 1994; Mrsa *et al*., 1999; see Fig. 3). The deletion mutants barely display significant morphological phenotypes (Van der Vaart *et al*., 1995; Moukadiri *et al*., 1997; Mrsa *et al*., 1999). Therefore, hypersensitivity reflects an increased amount of corresponding cell wall polymers, chitin for example, which is brought about as a consequence of the activation of the cell wall integrity pathway in response to cell wall defects (Heinisch *et al*., 1999; Popolo *et al*., 2001; Klis *et al*., 2002; Lagorce *et al*., 2002).

To elucidate the role of GPI proteins in maintaining the structural integrity of the cell wall, concomitant deletion

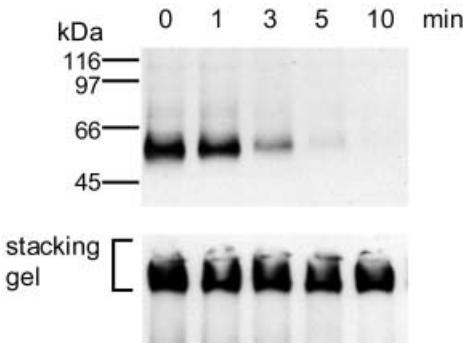


Fig. 2. The membrane-associated form of Ccw12p represents an intermediate. Ccw12p^{HA} was expressed in the *ccw12*Δ mutant. Cells were grown overnight to early logarithmic phase and harvested after the addition of cycloheximide (100 μg ml^{-1}) at the time points indicated. Crude membranes (top) and laminarinase extracts of cell walls (bottom) were resolved on SDS–polyacrylamide gels and analysed by Western blotting using anti-HA monoclonal antibody.

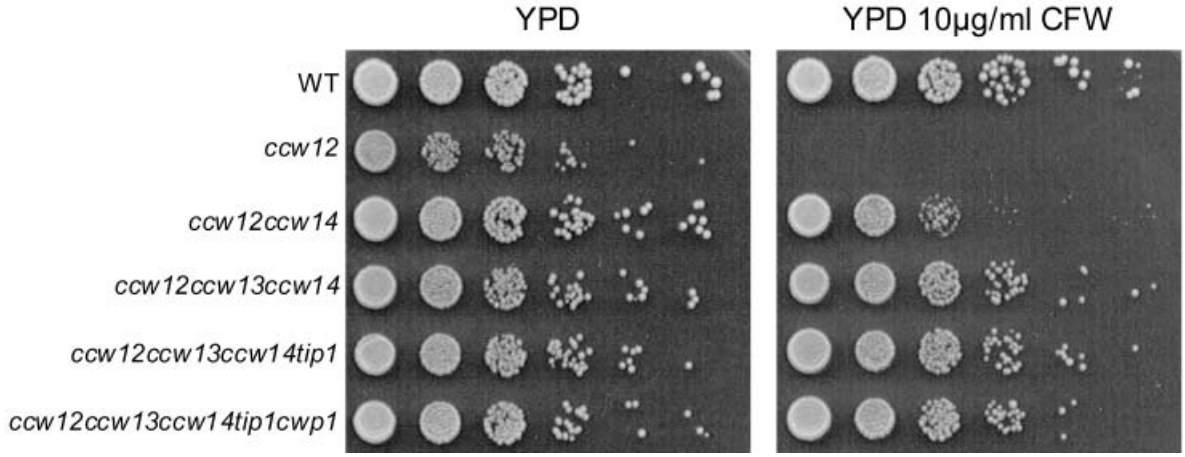


Fig. 3. Additional deletions of GPI-anchored proteins reduce the Calcofluor white hypersensitivity of a *ccw12Δ* mutant. Approximately 10^8 cells ml^{-1} of the various strains as well as serial 10-fold dilutions were spotted (5 µl each) on YPD and YPD plus 10 µg ml^{-1} Calcofluor white plates.

mutants of up to five GPI protein-encoding genes were constructed as described in *Experimental procedures*. Together with *CCW12*, *CCW13*, *CCW14*, in addition *TIP1* and *CWP1*, two major GPI-anchored cell wall proteins were chosen for deletion (ΔGPI mutant). Surprisingly, none of the performed knock-outs exacerbated the phenotype of the single *ccw12Δ* mutant. On the contrary, as shown in Fig. 3, the strong CFW hypersensitivity of mutant *ccw12Δ* was decreased in the multiple mutants. An analogous phenomenon was observed when the amount of proteins released from intact cells by dithiothreitol (DTT) was determined (Fig. 7, compare lanes 2, 3 and 6). Similarly, growth rates recovered in the multiple mutants. Whereas the *ccw12Δ* single mutant displayed an increased generation time of about 40% (Mrsa *et al*., 1999), the doubling time of the multiple mutants was the same as for wild type (data not shown).

To understand these effects on a molecular level, the overall cell wall composition of the various mutants was analysed (see *Experimental procedures*). Whereas only minor changes in the glucan–mannan ratio were observed (data not shown), the amount of chitin increased 2.5-fold in *ccw12Δ* and returned almost to wild-type amounts in the multiple deletion mutants (Table 1). Although the overall cell wall composition of the ΔGPI mutant seemed to be fairly similar to that of wild-type cells, electron microscopy revealed pronounced morphological differences. The inner glucan layer was considerably thicker and most probably less compact in the mutant (180–250 nm compared with 100–140 nm for the wild type), whereas the length of the mannan brush-like fibres was reduced and less regular (Fig. 4). This indicates a major change in the cell wall morphology of the ΔGPI mutant. The deleted proteins possibly contribute to the fibrillar structure at the external surface of the wall in wild-type cells.

The global transcriptional response of the ΔGPI mutant

The antagonistic behaviour and the changes in cell wall morphology may be caused by an induction of other, possibly unknown, cell wall-related genes, which compensate for the deleted GPI proteins and maintain (although with a different overall structure) the integrity of the cell wall. Also, an increased activity of the enzyme(s) responsible for transferring the membrane form of GPI-anchored proteins to the cell wall glucan–chitin meshwork was expected to act in a compensatory manner. Therefore, a genome-wide expression analysis was performed comparing the ΔGPI mutant with the corresponding wild-type strain. Yeast strains were grown to an OD_{600} of 1 overnight. Pelleted cells were instantly shock frozen in liquid nitrogen and mechanically disrupted in a microdismembrator as described in *Experimental procedures*. Total RNA was

Table 1. Comparison of the chitin content of wild type and *ccw* mutants.

Strain	% Glc-NAc
WT	100
ccw12Δ	245
ccw12ccw13ccw14Δ	130
ΔGPI	105
ccw12ccw13ccw14sed1Δ	200

Cell walls from log-phase cells (corresponding to OD 100 ml^{-1}) were washed three times with water, extracted twice with 0.5 ml of SDS/ME buffer for 3 min at 95°C, washed again three to five times with 1 ml of water, lyophylized, and the dry weight was determined. Dry cell walls were suspended in 0.5 ml of 50 mM Tris-Cl, pH 7.5, plus 0.5 mg of zymolyase 100T and incubated at 37°C overnight. Supernatant was removed, and the cell walls were hydrolysed in 0.5 ml of 4 M HCl for 10 h at 100°C, then neutralized with 0.5 ml of saturated Na_2CO_3. Chitin was determined as N-acetyl-glucosamine with Ehrlich reagent (Davidson, 1966). ΔGPI refers to mutant *ccw12ccw13ccw14tip1cwp1Δ*.

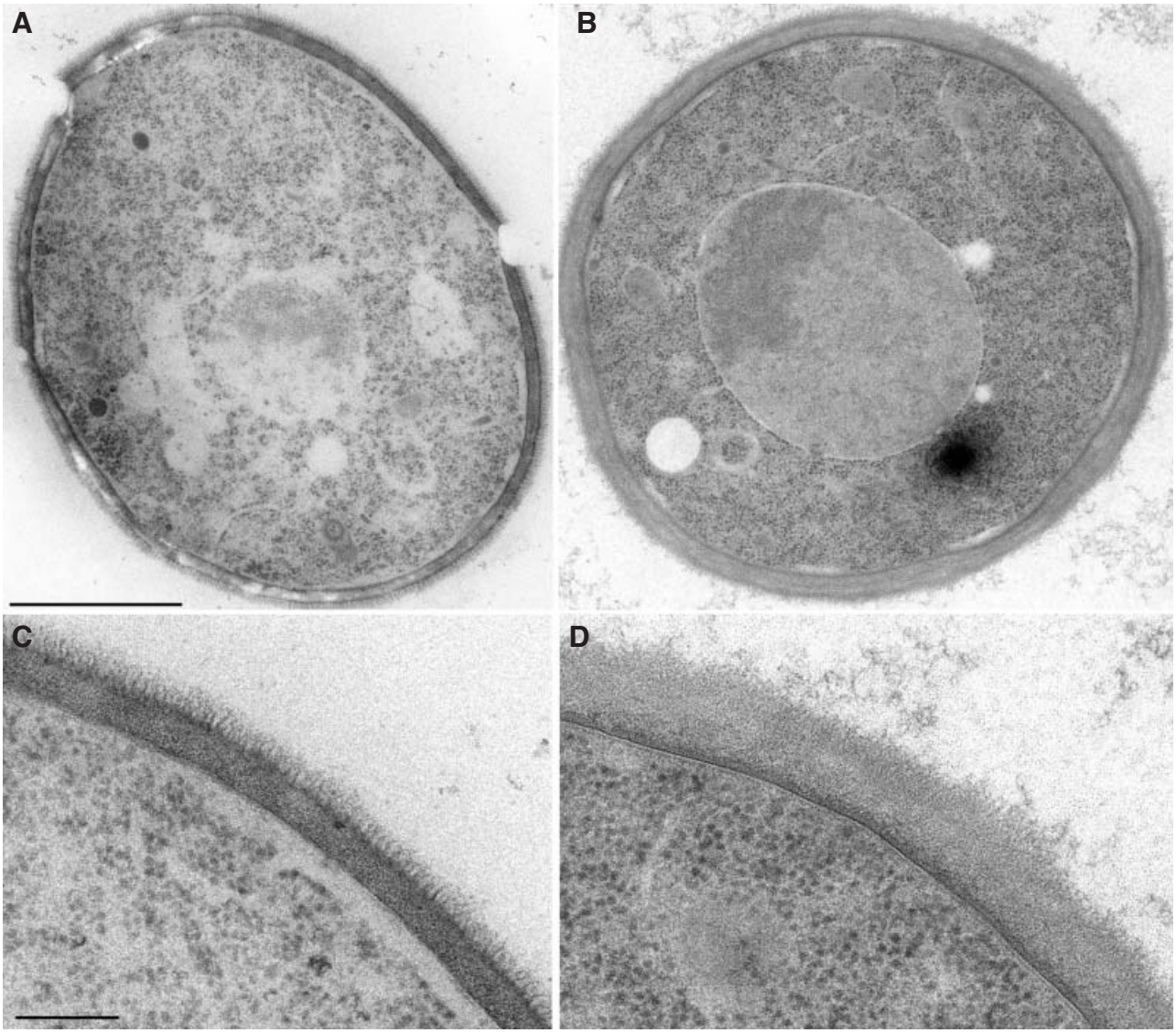


Fig. 4. Ultrathin sections of high-pressure frozen, freeze-substituted yeast cells.
A and C. SEY6210 (wild type).
B and D. ΔGPI.
Bar for A and B: 1 μm. Bar for C and D: 0.2 μm. For details, see *Experimental procedures*.

isolated and labelled with ^{33}P during first-strand cDNA synthesis. Probes were hybridized to Eurofan yeast arrays containing 6103 *S. cerevisiae* open reading frames (ORFs) (Hauser *et al*., 1998). Reproducibility was assured by repeating hybridization at least five times using the same filter arrays with the respective converse probe. After quantification, the data obtained were analysed using the M-CHIPS software (Fellenberg *et al*., 2002; 2003; http://www.mchips.org/).

Comparing wild-type and ΔGPI mutant, 159 genes were identified that displayed at least a 1.5-fold, and 34 genes with at least a twofold difference in signal intensity. Among these, 60 genes were activated in response to the deletion of the five GPI-anchored proteins, and 99 genes displayed diminished signals (Table 2; see also http://www.biologie.uni-Regensburg.de/Botanik/Strahl/data/GPIprofile.xls). Among the strongest regulated genes are *SCW10* (putative β-1,3-glucanase), *PRB1* (vacuolar endopeptidase) and YOR389w (putative membrane protein); all these were also upregulated in a Δ*gas1* and a Δ*mnn9* mutant, and the first two in a Δ*kre6* mutant (Lagorce *et al*., 2003). To verify the DNA array data, mRNA levels of six representative genes (*SCW10*, *TOS1*, *SED1*, *CRH2*, *YOR389w*, *YPL088w*) were analysed further by Northern blot (Fig. 5). All expression factors obtained by quantifying the mRNA levels in Northern blot

Table 2. Summary of genes differentially expressed in ΔGPI mutant.

	No. of genes
Expression ≥ 1.5-fold	159
Induced	60
Repressed	99
Expression ≥ 2.0-fold	34
Induced	14
Repressed	20

analyses were largely in accordance with those measured in the array.

In analysing the data, integral cell wall proteins or enzymes involved in cell wall biogenesis were our main focus. In addition to known cell wall proteins, we screened for potential cell wall-related proteins by *in silico* sequence analysis. All genes regulated by more than a factor of 1.5 were checked for whether they possess a signal sequence for secretion using IPSORT prediction (Bannai *et al*., 2002). The strongest differentially expressed genes encoding known cell surface-localized proteins (Table 3) can basically be divided into two groups: on the one hand, *SCW10*, *BGL2* and *CRH2*, which encode known or putative β-glucanases or transglycosidases, and on the other hand, *PRY2*, *SED1*, *TOS1* and *SRL1*, which encode putative structural mannoproteins.

SCW10 displayed the highest induction of all genes (Table 3). Scw10p and Bgl2p are soluble cell wall proteins that belong to the CAZy (carbohydrate active enzymes) family 17 of glycoside hydrolyases (GH) (Cappellaro *et al*., 1998). For Bgl2p, endo-β-1,3-glucanase and glucosyltransferase activity has been demonstrated (Mrsa *et al*., 1993; Goldman *et al*., 1995). The gene encoding Scw4p, another member of the CAZy GH 17 family that is 74% identical to Scw10p, is also significantly upregulated (E-factor +1.4) in mutant ΔGPI. In contrast, *CRH2*, which encodes a putative β-1,3-1,4-glucanase of the CAZy GH 16 family (Rodriguez-Pena *et al*., 2000), is significantly repressed in the ΔGPI mutant. Crh2p was found to localize in the cell wall mainly at the bud neck throughout the

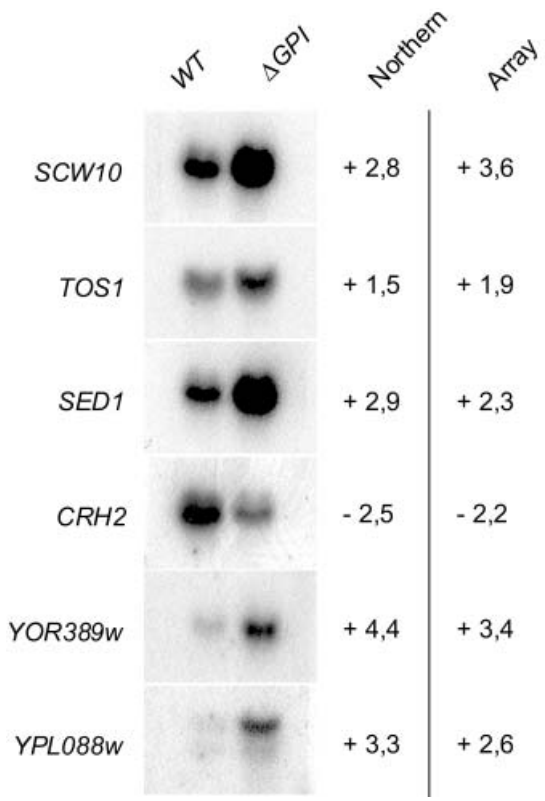


Fig. 5. Comparison of gene expression by Northern blot analysis with that determined by the DNA array. Northern blot analyses were performed using 20 μg of total RNA, isolated from wild type (WT; SEY6210) and ΔGPI mutant. Signals were quantified, and the calculated expression changes in transcripts were compared with the data obtained in the DNA array. For details, see *Experimental procedures*.

Table 3. Genes regulated in mutant DGPI.

Gene	ORF	E-factor[a]	Gene annotation
Genes coding for known cell surface-associated proteins			
SCW10	YMR305c	+3.6	Putative glucanase
PRY2	YKR013w	+2.3	Cell wall protein
SED1	YDR077w	+2.3	GPI protein
TOS1	YBR162c	+1.9	Cell wall protein, Aga1p homologue
DFG5	YMR238w	+1.8	GPI-anchored, putative mannosidase
SRL1	YOR247w	+1.7	Cell wall protein
BGL2	YGR282c	+1.6	Endo-1,3-glucanase
CRH2	YEL040w	–2.2	Putative glucanase
ORFs encoding putative cell surface-associated proteins			
PST1	YOR389w	+3.4	Signal peptide, putative membrane protein
	YPL277c	+3.3	Membrane protein, YOR389w homologue
	YDR055w	+1.5	Secreted by regenerating protoplasts
	YLR414c	+1.5	Signal peptide, putative membrane protein
	YLR413w	–1.6	Signal peptide, putative membrane protein
	YDR134c	–4.3	Putative GPI protein, pseudogene

a. The E-factor indicates the *n*-fold higher and lower expression, respectively, of the corresponding gene in the ΔGPI mutant compared with the wild type.

whole budding cycle (Rodriguez-Pena *et al*., 2002). To date, it is well established that all these enzymes affect cell wall stability; however, their precise functions are not known. In the cell wall mutants Δ*gas1*, Δ*kre6* and Δ*mnn9*, the *SCW10* and *BGL2* genes are also significantly upregulated, whereas *CHR2* is downregulated (Lagorce *et al*., 2003), suggesting that they play a general role in assembly and/or remodelling of the glucan–chitin meshwork and, therefore, in maintaining yeast cell wall integrity.

SED1 encodes a GPI protein that is 31% identical in amino acid composition to Ccw12p in its N-terminal half (Hardwick *et al*., 1992). Initially described as a major cell surface protein in the stationary phase (Shimoi *et al*., 1998), it was also characterized as a stress-induced gene, regulated by the PKC1 pathway (Jung and Levin, 1999). The observation that overexpression of *SED1* resulted in resistance to zymolyase, an enzyme preparation that contains β-1,3-glucanase as its main activity (Shimoi *et al*., 1998), implicates a task for the Sed1 protein in affecting cell wall porosity. *PRY2*, *TOS1* and *SRL1* were recently identified in a sequence-based approach for identification of cell wall proteins (Terashima *et al*., 2002). Little is known about their function, but the high serine and threonine content of these proteins (Tos1p 26%, Pry2p 34% and Srl1p 43%) is typical of integral cell wall proteins and indicates an intense O-mannosylation. Tos1p is highly homologous to the GPI proteins Aga1p and Tir2p, but it lacks a GPI attachment signal as also do Srl1p and Pry2p, indicating that these three proteins are non-covalently bound to cell wall polymers.

In addition, in mutant ΔGPI, *DFG5* is upregulated, which encodes a GPI-anchored membrane protein, required for filamentous growth, cell polarity and cellular elongation (Mosch and Fink, 1997). *DFG5* is also upregulated in the mutants Δ*fks1*, Δ*knr4*, Δ*kre6*, Δ*gas1* and Δ*mnn9*, indicating a general role in cell wall integrity (Lagorce *et al*., 2003). Dfg5p together with its homologue Dcw1p might be involved in the transfer reaction of GPI proteins to the cell wall (Kitagaki *et al*., 2002). The deletion of both these genes is lethal, indicating the necessity of GPI proteins for cell growth.

Using the IPSORT program, we identified six regulated ORFs encoding as yet unidentified potential extracellular proteins, which may be involved in maintaining cell wall integrity in the ΔGPI mutant (Table 3). Four of the uncharacterized ORFs were positively and two negatively regulated. YOR389w and the closely related YPL277c are both upregulated to a very similar degree, 3.4- and 3.3-fold respectively. Hydropathy plot analysis revealed at least one potential transmembrane span for both gene products, suggesting that these proteins are localized in membranes. Although Pst1p (YDR055w) has not been demonstrated to be attached to the cell wall, evidence exists for a cell surface localization. *In silico* analysis revealed a GPI attachment signal, and Pst1p has been found to be secreted from protoplasts that regenerate their cell wall (Caro *et al*., 1997; Pardo *et al*., 1999). The two neighbouring ORFs, YLR414c and YLR413c, both encode potential membrane proteins, each with at least four transmembrane domains according to their hydropathy profile. YDR134c is one of the most repressed ORFs in the array and displays a high homology to *CCW12*. Both gene products are 70% identical, but YDR134c lacks a GPI-anchoring sequence because a stop codon truncates the polypeptide and reduces the chain length from 94 to 48 amino acids, which we confirmed by DNA sequence analysis. Among the known and putative cell surface-associated mannoproteins identified in this DNA array, Sed1p, Pst1p, Pry2p, Tos1p and Srl1p represent a set of mannoproteins that is also upregulated in several cell wall mutants, especially in Δ*mnn9* (Lagorce *et al*., 2003). *PST1* expression is also induced upon treatment with antifungal agents, such as amphotericin B or caspofungin (Agarwal *et al*., 2003). Although the exact function of these proteins is not yet known, they are induced as a result of different cell wall stress situations and, therefore, seem to be major players in the maintenance of cell wall structure.

Interestingly, the DNA array data revealed that most of the characteristic target genes of the PKC1 and HOG pathways (Heinisch *et al*., 1999; Jung and Levin, 1999; Rep *et al*., 2000) are not or only little affected in the ΔGPI mutant. This is in contrast to other cell wall mutants such as Δ*kre6*, Δ*gas1* or Δ*mnn9* where, e.g. the MAP kinase *SLT2* is significantly upregulated (Lagorce *et al*., 2003). Upon induction of the PKC1 pathway, cell wall chitin levels are increased. This is reflected by the fact that the flux in the chitin pathway is increased mainly because of upregulation of both *GFA1* and *CHS3* in such mutants (Lagorce *et al*., 2002). However, in the ΔGPI mutant, expression of *CHS3* is not affected, and cell wall chitin levels are similar to wild type (Table 1), further supporting the view that the PKC1 pathway is not strongly induced upon deletion of five GPI-anchored cell wall mannoproteins. *SCW10* and *DFG5* expression is also regulated by the Ca^{2+}/calcineurin-dependent pathway (Yoshimoto *et al*., 2002). Further, the Ca^{2+}/calcineurin-dependent pathway and the 'global stress' response mediated by Msn2/4p were suggested to act in combination with the PKC1 pathway in the cell wall compensatory mechanism (Lagorce *et al*., 2003). However, in mutant ΔGPI, none of the components of those signal transduction pathways is significantly increased (data not shown).

Promoter analysis of the known highly upregulated genes was performed to identify common motifs that might contribute to the regulation of those genes in mutant ΔGPI. Therefore, 1000 bp non-coding upstream sequence of *SCW10*, *PRY2*, *SED1*, *TOS1*, *DFG5*, *SRL1* and *BGL2* was analysed using MATINSPECTOR (Quandt *et al*., 1995). This

algorithm localizes regulatory motifs that emerge more frequently in the whole genome than would be expected on a random basis. Among others, we found consensus sequences corresponding to binding sites of the transcription factors encoded by *RLM1*, *CRZ1*, *MSN2/4* and *HSF1*; however, none of these was common to all upstream sequences analysed (data not shown). The transcription factor Rlm1p is controlled by the PKC1 pathway and activates several cell wall-related genes (Dodou and Treisman, 1997; Jung and Levin, 1999). *CRZ1* is the major effector of calcineurin-regulated gene expression (Yoshimoto *et al*., 2002). *MSN2/4* and *HSF1* code for transcription factors that bind to STRE and HSE motifs respectively (Sorger, 1991; Martinez-Pastor *et al*., 1996). We found Rlm1p binding sites only in the promoter regions of *SED1* and *DFG5*, Msn2p/Msn4p binding sites in the promoter regions of *SED1* and Crz1p sites in the promoter of *PRY2*. Only Hsf1p binding sites are present in the promoter regions of all genes analysed, except *DFG5*. The *in silico* analyses support our finding that none of the major pathways involved in cell wall compensation is activated in mutant ΔGPI.

We also analysed the promoter regions of *SCW10*, *PRY2*, *SED1*, *TOS1*, *DFG5*, *SRL1* and *BGL2* using the Regulatory Sequence Analysis Tools (Van Helden *et al*., 2000; http://rsat.ulb.ac.be/rsat/). We performed a matrix-based pattern discovery using the tool CONSENSUS (matrix length 10), which extracts shared motifs from a set of unaligned sequences, followed by PASTER, which scans DNA sequences with a profile matrix. Interestingly, we could identify the consensus motif 5′CCT(T/G/C)(T/C)TT(C/G)(G/c)(T/G)3′, which is present once in the promoter regions of *PRY2*, *DFG5*, *BGL2* and *SRL1* and twice in *SCW10*, *SED1* and *TOS1*. Using the same matrix, we performed a genome-scale pattern matching using genome-scale PASTER. The same consensus motif was localized in the promoter regions of the ORFs YOR389w and YPL277c, which are also highly upregulated in mutant ΔGPI, confirming the significance of our finding. This motif is also present in the upstream sequence of *FKS3*, which is slightly upregulated in mutant ΔGPI (data not shown). Fks3p shows homology to the β-1-3-glucan synthase Fks1p; however, its function in cell wall integrity is not known. The *FKS3* gene is upregulated in the cell wall mutant Δ*gas1* (Lagorce *et al*., 2003), supporting an important role for the cell wall compensatory mechanism. Our *in silico* data revealed a DNA motif that is present in the promoter region of several genes upregulated in mutant ΔGPI. However, whether this consensus sequence represents a new regulatory DNA motif and whether it is important for gene regulation in mutant ΔGPI remains to be shown in the future.

Deletion analysis

We also analysed whether the mutant ΔGPI upregulated genes are in fact important for cell wall integrity in the absence of multiple GPI-anchored proteins. Therefore, *SCW10*, *SED1*, *SRL1*, *TOS1*, YOR389w and YPL277c were disrupted in mutant *ccw12ccw13ccw14*Δ, which shows the same growth rate and phenotypes as mutant ΔGPI (Figs 3 and 7; and data not shown). For comparison, single knock-outs were constructed in a wild-type background to reveal additive or synergistic effects. Growth rates and cell wall-related phenotypes such as sensitivity to CFW and proteins released from cells by DTT were analysed in all resulting single and multiple deletion strains.

All multiple deletion mutants constructed were viable. Additional deletion of *SCW10*, YOR389w, YPL277c and *TOS1* in mutant *ccw12ccw13ccw14*Δ did not alter the phenotypes of the mutant (data not shown).

These data suggest that none of these proteins is solely necessary to maintain cell wall integrity in the absence of multiple GPI-anchored proteins. Interestingly, homologues are present for all these genes in the *S. cerevisiae* genome. Sequence similarity query showed that the N-terminal halves of YOR389w and YPL277c are 98% identical. Scw10p and its homologue Scw4p are 74% identical. Further, Tos1p and the uncharacterized ORF YJL171c align to 90.5%. The observed paired homologues suggest that these genes might act as functional homologues and potentially compensate for each other in the deletion strains. Functional redundancy is supported by the finding that the double deletion mutant *scw4scw10*Δ displays additive effects when compared with the individual single mutants (Cappellaro *et al*., 1998; S. Sestak, unpublished results). However, we cannot completely rule out the possibility that the deletion of additional genes in mutant *ccw12ccw13ccw14*Δ might activate other compensatory mechanisms such as the PKC1 pathway.

In contrast, deletion of *SRL1* and *SED1* dramatically affects all cell wall-related phenotypes of mutant *ccw12ccw13ccw14*Δ. Interestingly, growth rates and the phenotypes of *ccw12ccw13ccw14sed1*Δ and *ccw12ccw13ccw14srl1*Δ resemble those of a *ccw12*Δ single mutant (Figs 6 and 7; data not shown). As shown in Fig. 6, the quadruple mutants are highly sensitive to CFW, comparable to mutant *ccw12*Δ, which agrees with the chitin content being almost equal to that of the single *ccw12*Δ mutant (Table 1). Also, the amount of protein released by DTT from intact *ccw12ccw13ccw14*Δ cells increases in the absence of *SED1* and *SRL1* (Fig. 7, lanes 3–5).

Taken together, our results suggest that the upregulation of *SED1* and *SRL1* is required for maintenance of cell wall integrity in the absence of multiple GPI-anchored proteins and that other genes cannot replace this function. A concomitant deletion of *CCW12* and *SED1* did not change the CFW hypersensitivity or the growth defect of the single *ccw12*Δ deletion (data not shown), which excludes a co-operative function for these proteins.

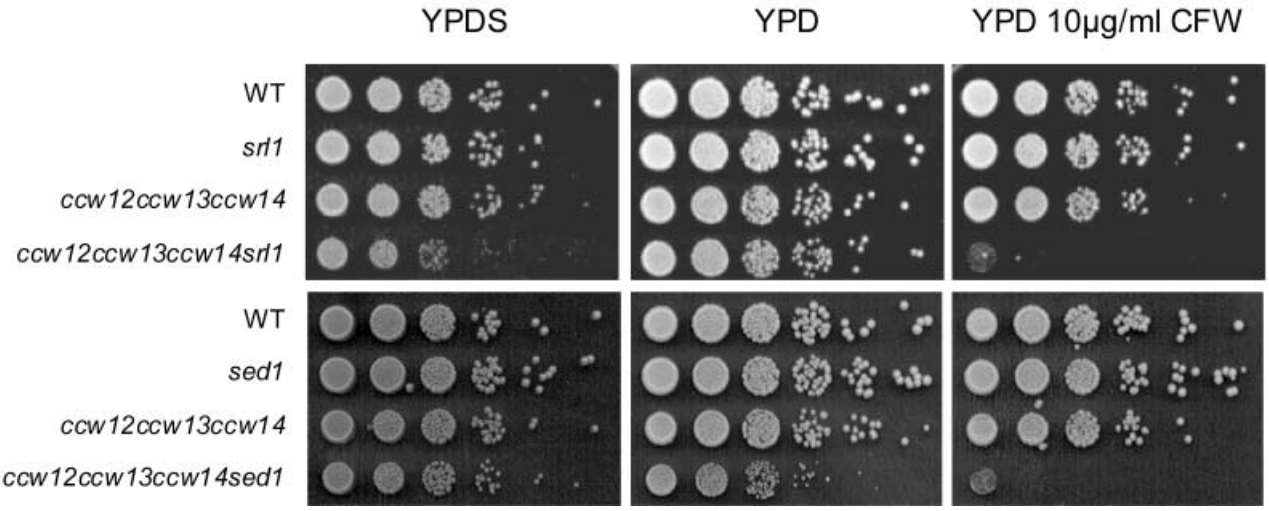


Fig. 6. Functional *SRL1* and *SED1* are important for Calcofluor white resistance of *ccw12ccw13ccw14*Δ. Approximately 10^8 cells ml^{-1} of the various strains as well as serial 10-fold dilutions were spotted (5 µl each) on YPDS, YPD and YPD plus 10 µg ml^{-1} Calcofluor white plates.

Conclusions

A set of cell wall-related genes has been uncovered that is important for the compensation of cell wall defects caused by the simultaneous deletion of several GPI protein-encoding genes. The single deletion of *CCW12* results in an increase in cell wall chitin very probably caused by the activation of known cell wall compensation mechanisms such as the PKC1 pathway. This compensatory mechanism seems to be less important when an additional two to four GPI-anchored cell wall proteins are deleted and the mannan layer of the cell wall is severely affected (Fig. 4). The chitin content in these multiple mutants is as low as in wild-type cells and so is the CFW sensitivity. In this situation, the cell wall proteins Sed1p and Srl1p are important for cell wall stabilization. It is not known which cell wall components are affected by this new compensation, but an increased amount of Sed1p, itself a GPI-anchored cell wall protein, might contribute to a functional mannan layer. Electron microscopy pictures indicate that the cell wall layer internal to the mannoprotein fimbriae is considerably thickened, although less compact (Fig. 4). A differently structured glucan layer, possibly caused by different levels of the glucanases/transglycosidases coded by *SCW10*, *BGL2* and *CRH2*, seems to be the best guess concerning further cell wall changes under these conditions.

Experimental procedures

Yeast strains and culture conditions

Saccharomyces cerevisiae strains used in this work are shown in Table 4. Yeast cells were grown in synthetic complete medium or YPD medium containing 1% yeast extract, 2% bactopeptone and 2% glucose (Kaiser *et al*., 1994).

Deletion constructs

Standard procedures were used for all DNA manipulations (Sambrook *et al*., 1989). All cloning and transformations were carried out in *E. coli* host DH5α.

Deletions of *ccw13::TRP1* and *ccw14::HIS3* were performed as described previously (Mrsa *et al*., 1999); for *sed1::kanMX*, a disrupted *SED1* ORF was amplified from −438 to +1244 by polymerase chain reaction (PCR) using genomic DNA from Euroscarf strain Y14012 as a template and oligonucleotides SED1A and B. The purified PCR product was transformed directly. For *srl1::kanMX*, the deletion of

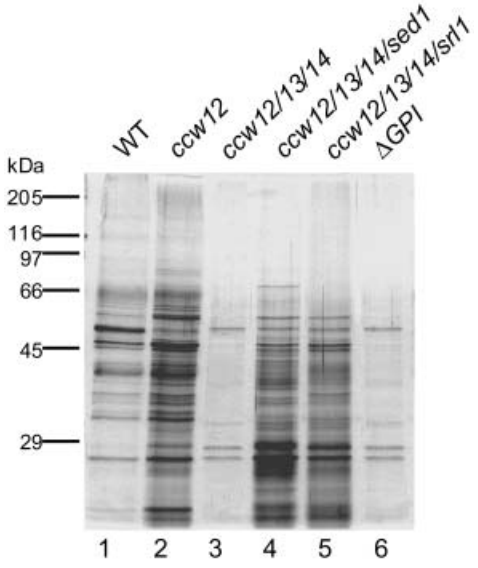


Fig. 7. Release of proteins from intact cells of mutants deleted in GPI-anchored proteins. Log-phase cells after washing were incubated for 90 min at 4°C while vigorously shaking in 25 mM Tris-HCl buffer, pH 8.0, containing 5 mM DTT. Supernatant corresponding to 20 OD ml^{-1} was separated on a 10% SDS gel, and proteins were detected by silver staining.

Table 4. Yeast strains used in this work.

Strain	Genotype	Reference
SEY6210	*MATα, ura3-52, leu2-3, 112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9*	Robinson *et al*. (1988)
HAI101	SEY6210 except *sed1::KanMX*	This work
HAI103	SEY6210 except *srl1::KanMX*	This work
HAI2341	SEY6210 except *ccw12::URA3*, *ccw13::TRP1*, *ccw14::HIS3*, *sed1::KanMX*	This work
HAI2342	SEY6210 except *ccw12::URA3*, *ccw13::TRP1*, *ccw14::HIS3*, *srl1::KanMX*	This work
MEY12A	SEY6210 except *ccw12::URA3*	Mrsa *et al*. (1999)
MEY1214	SEY6210 except *ccw12::URA3*, *ccw14::TRP1*	This work
MEY234	SEY6210 except *ccw12::URA3*, *ccw13::TRP1*, *ccw14::HIS3*	Mrsa *et al*. (1999)
MEY2341	SEY6210 except *ccw12::URA3*, *ccw13::TRP1*, *ccw14::HIS3*, *tip1::KanMX*	This work
MEY2342 ΔGPI	SEY6210 except *ccw12::URA3*, *ccw13::TRP1*, *ccw14::HIS3*, *tip1::KanMX cwp1::LEU2*	This work
gpi1Δ	*MAT*a, *ura3-52 hisG::hisG gpi1::URA3*	Leidich and Orlean (1996)

SRL1, the plasmid P42503 from Euroscarf was used; for *tip1::kanMX*, primer pairs TIP1A and B and TIP1C and D were used to amplify N- and C-terminal fragments of the *TIP1* ORF by PCR, using genomic DNA as a template. Both were used together with TIP1A and D in a megaprimer PCR using the *kanMX* cassette as a template. The purified PCR product was used directly for transformation. The deletion construct *cwp1::LEU2* was obtained as an analogue to the PCR strategy described above for *tip1::kanMX*, except using primer pairs CWP1A and B and CWP1C and D and a *LEU2* marker as a template.

Vectors

PME11. Two DNA fragments containing *CCW12* and untranslated regions from −282 to +58 and +59 to +607 were amplified by PCR using primer pairs CCW12-1 and -2 and CCW12-3 and -4 respectively. Genomic DNA was used as template. Both were used in a megaprimer PCR to amplify the HA tag using a 111 bp *Not*I fragment of pAxl2 (Roemer *et al*., 1996) as template. Flanking *Eco*RI restriction sites were used to clone the fragment into pRS425.

Phenotypic tests

For growth tests in the presence of different cell wall-perturbing agents, yeast cells were grown in YPD medium to exponential phase. Cells were harvested, washed once with sterile water and resuspended in sterile water at a concentration OD_{600} of 1. Serial 10-fold dilutions were spotted on solid medium containing the agents at different concentrations using a metal replicating tool. Growth on plates was scored after 2 days of incubation at 30°C.

Preparation of cell walls and crude membranes

Yeast cells were grown in synthetic complete medium (Kaiser *et al*., 1994). At a concentration of 1 OD_{600}, 50 ml of cells was harvested, washed with 20 ml of 50 mM Tris-HCl, pH 7.5, 0.3 mM $MgCl_2$ and resuspended in 200 μl of the same buffer plus 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine, 0.25 mM TLCK, 50 μg ml^{-1} TPCK, 10 μg ml^{-1} antipain, 1 μg ml^{-1} leupeptin and 1 μg ml^{-1} pepstatin. An equal volume of glass beads was added, and the cells were homogenized by vortexing, for 1 min, four times (with 1 min intervals on ice). The bottom of the tube was punctured, and the homogenate was collected. Cell debris was removed by centrifugation for 5 min at 3000 r.p.m. at 4°C. The pellet (cell walls) was stored at −20°C. Crude membranes were collected from the supernatant by centrifugation for 60 min at 20 000 r.p.m. at 4°C (Sorvall SS34 rotor).

Deglycosylation by endoglycosidase H digestion

Ten microlitres of the respective protein fraction was suspended in 10 μl of EndoH buffer (100 mM KPO_i, pH 5.5, 0.2 M 2-mercaptoethanol, 0.04% SDS, 1 mM PMSF) and digested with 0.5 μl of endoglycosidase H (Calbiochem) for 1–2 h at 37°C.

Cell lysis and RNA isolation

Yeast cells were grown to a OD_{600} of 1 in a volume of 50 ml and harvested. For microarray analysis, yeast cells were mechanically disrupted as described by Hauser *et al*. (1998). RNA was prepared using the RNeasy midi system (Qiagen). For Northern blot analysis, RNA was prepared using a hot phenol method. Ten OD_{600} of a logarithmic growing cell culture was pelleted and resuspended in 400 μl of 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% SDS. After the addition of 400 μl of acid phenol, samples were incubated for at least 30 min at 65°C, cooled on ice for 5 min and subsequently centrifuged for 5 min at 14 000 r.p.m. at 4°C. To the upper phase, 400 μl of acid phenol was added and centrifuged once more. The water phase was extracted with about one volume of chloroform and subsequently ethanol precipitated. After an additional washing step with 70% (v/v) ethanol, the RNA was pelleted, dried and resuspended in 50 μl of H_2O.

Probe generation and hybridization

Single-strand cDNA synthesis was performed as described previously (Hauser *et al*., 1998). Arrays were prehybridized for 2–3 h in Church buffer. After denaturing for 5 min at 100°C, the probe was hybridized in 5 ml of the same buffer for at least 20 h at 65°C. Filters were subsequently washed briefly at room temperature in 40 mM $NaPO_i$, pH 7.2, 0.1% SDS and again for 30 min at 65°C.

Filters were regenerated with boiling buffer (5 mM $NaPO_i$, pH 7.2, 0.1% SDS), first briefly rinsed with 100 ml and then, within the same volume, they were left to cool down to room temperature.

Image and data analysis

Signal detection was performed with a phosphofluoroimager Storm 860 (Molecular Dynamics) and quantified using the AIS (analytical imaging station) software from Imaging Research. M-CHIPS software was used for further analysis of the data obtained (Fellenberg *et al*., 2002; 2003; available at http://www.mchips.org/). A complete list of numerical and graphical images of the raw data can be found online at http://www.dkfz.de/funct_genome/yeast-data.html.

Northern analysis

RNA was separated on formaldehyde–agarose gels (20–30 μg per lane) according to the method of Ausubel *et al*. (1994) and transferred to Porablot NCL membranes (Macherey and Nagel). Probes for each ORF of interest were amplified by PCR using gene-specific oligonucleotides and genomic DNA as template. The PCR products were gel purified and labelled with [γ-^{32}P]-dCTP (50 μCi 200 ng^{-1} DNA) using the Decalabel™ DNA labelling system (MBI-Fermentas). Blots were prehybridized in 40 mM $NaPO_i$, pH 7.2, 0.1% SDS for at least 3 h, and hybridization was performed in the same buffer at 65°C overnight. Filters were washed once briefly in 2× SSC, 0.1% SDS at room temperature and subsequently twice at 68°C for 15 min each in 0.1× SSC, 0.1% SDS. Signals were quantified with a phosphor imager using the OPTIQUANT software from Packard BioScience.

Western blot analysis

Proteins were fractionated by disc SDS–PAGE (8–12% PA gels) under reducing conditions and transferred to nitrocellulose. The anti-HA monoclonal antibody (16B12; Babco) was used at a 1:8000 dilution. Protein–antibody complexes were visualized by enhanced chemiluminescence using the Amersham ECL system.

Electron microscopy

Yeast cells were sedimented in their culture tubes (Falcon, 50 ml) at 1 *g*. The pellet was taken up in cellulose capillaries (200 μm diameter) and high-pressure frozen as described previously (Cappellaro *et al*., 1994; Hohenberg *et al*., 1994). Freeze-substitution was done in either pure acetone–1% OsO_4, or methanol–0.5% glutaraldehyde–0.5% uranyl acetate for 42 h at –90°C, 6 h at –60°C, 4 h at –40°C. After washing twice in pure acetone at –40°C, the capillaries with the cells were embedded in Epon. Polymerization was done at 60°C. Ultrathin sections were stained with uranyl acetate and lead citrate. Recording of the electron micrographs was done using a slow-scan CCD camera (Tietz-TVIPS), mounted to a Philips transmission electron microscope CM12 (FEI), at an effective magnification of 16 000–34 000.

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