A screen for deficiencies in GPI-anchorage of wall glycoproteins in yeast

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Abstract

Many of the genes and enzymes critical for assembly and biogenesis of yeast cell walls remain unidentified or poorly characterized. Therefore, we designed a high throughput genomic screen for defects in anchoring of GPI-cell wall proteins (GPI-CWPs), based on quantification of a secreted GFP-Sag1p fusion protein. *Saccharomyces cerevisiae* diploid deletion strains were transformed with a plasmid expressing the fusion protein under a GPD promoter, then GFP fluorescence was determined in culture supernatants after mid-exponential growth. Variability in the amount of fluorescent marker secreted into the medium was reduced by growth at 18°C in buffered defined medium in the presence of sorbitol. Secondary screens included immunoblotting for GFP, fluorescence emission spectra, cell surface fluorescence, and cell integrity. Of 167 mutants deleted for genes affecting cell wall biogenesis or structure, eight showed consistent hyper-secretion of GFP relative to parental strain BY4743: *tdh3* (glyceraldehyde-3-phosphate dehydrogenase), *gda1* (guanosine diphosphatase), *gpi13* and *mcd4* (both ethanolamine phosphate-GPI-transferases), *kre5* and *kre1* (involved in synthesis of β1,6 glucan), *dcw1*(implicated in GPI-CWP cross-linking to cell wall glucan), and *cwp1* (a major cell wall protein). In addition, deletion of a number of genes caused decreased secretion of GFP. These results elucidate specific roles for specific genes in cell wall biogenesis, including differentiating among paralogous genes.

Keywords

cell wall assembly; gene deletions; GPI; GFP marker

Introduction

In yeasts, glycosylphosphatidylinositol (GPI)-anchorage is essential for viability and for maintenance of normal cellular morphology (Leidich *et al*., 1994; Pittet and Conzelmann, 2007). GPI-anchored glycoproteins serve as surface receptors, hydrolytic enzymes, structural components and adhesion proteins in mating, and in the formation of flocs, mats and biofilms. Fungal biofilms are exploited in bioremediation (Ryan *et al*., 2005), bio-control of insect pests (Aanen *et al*., 2009), food and beverage production (Donalies *et al*., 2008) and to enhance the effectiveness of fertilizers in nutrient-depleted soil (Adesemoye

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and Kloepper, 2009). However, the ability of fungal biofilms to aggressively adhere to and infect a multitude of abiotic and biotic surfaces (Verstrepen and Klis, 2006) signifies a continuous threat to human welfare and human life, due to the inherent resistance of fungal biofilms to currently available antifungals (Jabra-Rizk *et al*., 2004; Nowak and Kurnatowski, 2009).

GPI synthesis and addition have been well characterized in yeast. Briefly, glycoproteins are synthesized on ER-bound ribosomes, and a pre-assembled GPI anchor is substituted for a C-terminal hydrophobic signal by a transamidation reaction in the lumen of the endoplasmic reticulum (ER) membrane prior to their export to the cell surface in specialized vesicles (Castillon *et al*., 2009). GPI anchor biosynthesis involves 20–30 different proteins that work in multiprotein complexes to assemble the anchor in a step-wise fashion (Orlean and Menon, 2007). Proteins with added GPI anchors are further modified with *N*- and *O*-linked sugars in the ER and Golgi prior to their transport to the outer leaflet of the plasma membrane, where they remain tethered through the lipid part of their GPI anchor. In fungi, some GPI anchors are further processed by cleavage at the anchor's glycan part; this cleavage releases the protein with an attached GPI remnant into the cell wall space, where it becomes transglycosylated to cell wall β 1,6-glucan (Lu *et al*., 1995; Van Der Vaart *et al*., 1996). The mechanism and enzymes responsible for cell wall GPI–protein anchorage in fungi remain unresolved and are considered ideal antifungal targets because they are unique to fungi.

A comprehensive genome-wide search for genes required for GPI–cell wall protein (CWP) anchorage in the model eukaryote *Saccharomyces cerevisiae* would contribute to the understanding of known components of this cellular pathway and highlight new ones. Our data would complement existing genome-wide surveys for cell wall genes (De Groot *et al*., 2001, 2003; Lagorce *et al*., 2003; Lussier *et al*., 1997; Murillo *et al*., 2005; Weig *et al*., 2004; Yin *et al*., 2007) in efforts aimed at investigating potential relationships between datasets obtained from different 'omic' approaches (Ge *et al*., 2003). This type of data integration is proving increasingly crucial for comprehensive and reliable understanding of cellular pathways.

We have therefore developed a phenotypic screen for deficiencies in GPI–protein anchorage to the cell wall, using the commercially available *S. cerevisiae* gene deletion library. To our knowledge, no such mechanistically-based screen for wall assembly defects has been done before. Previous attempts for screening for GPI–CWP anchorage genes at a genome-wide level were abandoned because of high variance in the results, low protein yields and the possibility that the critical processes are encoded by redundant genes or by essential genes. All three issues have been successfully addressed, and we report the identification of several genes required for efficient anchorage of GPI-glycoproteins to the cell wall.

Materials and methods

Strains, media and reagents

Homozygous and heterozygote diploid deletion strains in the BY4743 background, isogenic to the sequenced strain S288C, were obtained from the Invitrogen collection. The specific deletions in strains identified in the screen were confirmed by colony PCR (data not shown). Yeast strains were grown in either YPD medium (1% yeast extract, 2% peptone, 2% glucose) or defined medium [0.2% yeast nitrogen base without amino acids, 0.5% ammonium sulphate, 2% glucose, and 0.08% complete synthetic medium (CSM) lacking uracil], containing 1 M sorbitol, buffered to pH 6.5 with 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) and supplemented with 200 μM geneticin (Sigma). *Escherichia coli* strains DH5α and XL10-Gold (Stratagene) were used as hosts for

recombinant DNA manipulation. *E. coli* strains were grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, 0.1% glucose, 0.01% ampicillin).

Construction of the reporter protein GFP–Sag1p

A GFP-labelled GPI–CWP was constructed as follows. An *Eco*RI–*Bgl*II fragment of the yeast-enhanced GFP (yEGFP) gene from *Aequorea victoria* was prepared by PCR, using pMut3-yEGFP (provided by Dr T. Hughes, Yale University, USA) as the PCR template. A *Bgl*II–*Xho*I fragment encoding the last 300 residues at the C-terminus of the cell wall glycoprotein *α*-agglutinin encoded by the *SAG1* gene was similarly prepared, using pH27 as the PCR template (Lipke *et al*., 1989). A *Spe*I–*Eco*RI fragment encoding the invertase secretion signal and cleavage site was synthesized using overlapping oligomers of 51 and 53 base pairs (bp) each (see Supporting information, Table S1), extending the non-overlapping regions with Klenow DNA polymerase (New England BioLabs). Each fragment was subjected to restriction digestion with the indicated enzymes, subjected to DNA gel electrophoresis and recovered from the gel using QIAquick Gel Extraction Kits (Fisher Scientific). A four-fold ligation reaction involving all three restriction fragments described above and vector p416*GPD* (ATCC, Manassas, VA, USA), bearing *Spe*I–*Xho*I sticky ends, was performed using T4 DNA Ligase (New England BioLabs). Constructs were verified by restriction analysis and sequenced to exclude mutations. The resulting plasmids were propagated and purified from *E. coli* using Qiagen plasmid purification kits. The plasmids were transformed into yeast strains using the lithium acetate protocol (Amberg *et al*., 2005).

High-throughput yeast transformations and cell growth

High-throughput transformation of gene deletion strains was accomplished using the Bio101 EZ-Yeast Transformation kit. Transformants were grown at 15–18 °C in 13 × 100 mm borosilicate tubes in CSM — uracil, pH 6.5-buffered selective medium containing 1 M sorbitol. Growth in small test tubes allowed convenient monitoring of cell growth by taking the OD at 2 h intervals. Cultures at $OD_{660} = 0.5$–0.6 (1.6–2.0×10^8 cells/ml) were centrifuged and 500 μl of their cell-free supernatant stored at −80 °C in silicon-coated tubes in the presence of fungi-specific protease inhibitor cocktail (Sigma), a condition that minimized protein loss (data not shown). Stored supernatants were thawed on ice.

Fluorescence microscopy

Yeast strains transformed with pGFP–Sag1p and control samples transformed with plasmid lacking the reporter gene were grown to $OD_{660} = 0.4$–0.6. Cell samples were washed twice with 1 M sorbitol and then incubated in 10 mM Tris–HCl, 1 M sorbitol, pH 7.5, for 2 h to overnight to promote maximal GFP folding. An Olympus fluorescence microscope was used to visualize GFP with a fluorescein filter (excitation at 470 nm, emission at 530 nm) and a ×60 immersion objective with a numerical aperture of 1.2.

High-throughput quantification of GFP levels

GFP amounts in supernatants of 167 cell wall gene deletion mutants and the parental strain were determined by single wavelength fluorescence spectroscopy, using a 96-well FLUOstar Optima microplate-reading fluorimeter from BMG Labtechnologies. Excitation and emission wavelengths were set a 488 and 520 nm, respectively. Complete fluorescence spectra were collected from the supernatants of several deletion mutants. Background fluorescence consisted of a single broad peak centred at 536 nm ($18\ 657\ cm^{-1}$) with a width at half height of 90 nm ($3500\ cm^{-1}$), as determined from wild-type cells transformed with empty vector DNA. This background fluorescence was subtracted from each experimental spectrum according to:

$$\text{Corrected fluorescence spectrum} = \text{experimental spectrum} - C \times \text{background}$$

The multiplier constant C was chosen to produce a corrected spectrum that matched as closely as possible the spectrum of the pure GFP from Clonetech. The only exception was mutant *GPI13/gpi13*. The relative fluorescence for each sample was taken as the height at the maximum point in the corrected spectrum. In the case of the *GPI13/gpi13* this clearly underestimates the fluorescence, since this mutant emits more from the peak at 552 nm than other mutants.

High-throughput immunoblotting

Yeast supernatants were recovered by high-speed centrifugation and 100 µl aliquots of the supernatants were applied to nitrocellulose membranes, using a 96-well BioRad dot-blotter/protein concentrator, as recommended by the manufacturer. This aliquot size was optimal for sensitivity and reproducibility (data not shown). Membranes were allowed to dry overnight and assayed for GFP levels, using monoclonal mouse anti-GFP antibodies and HRP-labelled goat anti-mouse antibodies (Sigma).

Cell wall isolation

For cell wall purifications, 50 ml cell suspension at 10^8 cell/ml was washed three times in Tris–PMSF buffer (10 mM Tris–HCl, 1 mM PMSF, pH 7.8) at 4 °C. After washing, the cells were resuspended in Tris–PMSF containing glass beads (diameter 0.45 mm) at a ratio of 1:1:1 (cell:buffer:glass beads, v/v/v). The cells were then homogenized by vortexing 10 times (each 1 min), with 1 min cooling intervals on iced water. The cell lysate was separated from the beads by repeated washings with 1 M NaCl, pH 7.5. The lysate was centrifuged at $3000 \times g$ for 10 min. The cell wall fraction recovered as insoluble material after centrifugation was washed twice with 1 M NaCl. This fraction was then treated with 1% SDS for 20 min at room temperature. The surfactants were removed by repeated washings with 1 M NaCl, followed by three washings with Tris–PMSF buffer (10 min each). The cell wall fractions were stored in 1 mM PMSF, pH 7.5.

Immunoelectron microscopy

To confirm cross-linking of the GFP–Sag1p to cell wall polysaccharides, immunoelectron micrographs were prepared from wild-type cells. Cells from a mid-exponential culture were washed twice with 1% SDS and twice with 50 mM Tris–chloride, pH 7.5. The cell pellet was incubated overnight in 30 ml 1:500 monoclonal mouse anti-GFP antibody solution. The cells were then washed three times for 10 min each in PBS, 0.5% Tween 20, and incubated overnight in 1:2000 gold-labelled goat anti-mouse antibody. The cells were fixed in 5% formaldehyde. Electron micrographs were prepared in Dr Ronald Gordon's laboratory at Mount Sinai Medical School, New York.

Total protein assay

For each strain tested, triplicate 5 ml cultures were grown to $OD_{660} = 0.6$–0.8, then centrifuged at high speed to remove the cells. The supernatant was frozen at −80 °C, lyophilized, concentrated 12.5-fold by resuspension in 400 µl water, then assayed in triplicate with Coomassie Plus Reagent (Pierce), with BSA as standard.

Results

Localization of GFP–Sag1p to the cell wall

We constructed a reporter plasmid (pGFP–Sag1p) that expressed a secreted and cell wall-anchored form of yGFP fused to the C-terminal 300 residues of the GPI cell wall protein Sag1p. The low copy plasmid p416-GPD was the vector (Figure 1A). Because irreversible damage to the GFP fluorophore occurs at pH levels <3.0 (Yang *et al*., 1996) and *S. cerevisiae* cultures can quickly reach such low pH levels, the cells were grown in medium buffered to pH 6.5 with 50 mM MOPS–Tris. Visualization of GFP fluorescence at the cell surface confirmed that the reporter protein was successfully expressed and processed as a cell surface protein (Figure 1D1). There was significantly less cell surface fluorescence in cells grown in unbuffered medium (data not shown). Cells transformed with vector p414*GPD* alone did not show cell surface fluorescence (Figure 1D3). Isolated cell walls from GFP–Sag1p-expressing cells remained fluorescent after treatment to remove non-covalently bound cell wall soluble protein (Klis *et al*., 2007) (Figure 1C1). Additionally, electron microscopy of GFP–Sag1p-expressing cells showed localization of the marker protein at the cell wall after treatment of the cells with gold-labelled anti-GFP antibodies (Figure 1B). Therefore, the GFP–Sag1p fusion protein was localized in the cell wall.

Optimizing yields and reproducibility

Initially, levels of GFP in the growth supernatants were low and highly variable, due to GFP instability and differences in cell concentration or growth stage. A series of experiments increased both the sensitivity and reproducibility of immunoblot assays. When separate clones of BY4743 were grown at 18 °C and 30 °C, there were similar GFP levels in the supernatants among the different clones at each growth temperature. However, there were significantly greater GFP levels in supernatants from cultures grown at 18 °C (Figure 2A). The amount of secreted GFP–Sag1p also increased after growth in 1 M sorbitol (Figure 2C). In sorbitol-supplemented buffered cultures grown at 18 °C, GFP detected in the culture supernatant was maximal when the cells were at mid-exponential stage, $OD_{660\,nm}$ = 0.5–0.6 (Figure 2C and data not shown).

These studies were the basis for a standardized growth protocol for the pGFP–Sag1-transformed strains. Triplicate cultures of each strain were grown in buffered defined medium with 1 M sorbitol at 18 °C. The cultures were harvested at $OD_{660\,nm}$ = 0.5–0.6 and supernatants were collected and frozen until assay by immunoblotting of 100 μl samples.

Proof of principle: cell wall hypersecreting mutants

The cell wall mutants *kre1/kre1* and *KRE5/kre5* hypersecrete GPI–CWPs (Lu *et al*., 1995). These strains presumably do so because of diminished levels of cell wall *β*-1,6-glucan, which is the cell wall polysaccharide to which the proteins are anchored (Shahinian and Bussey, 2000). As expected for *KRE5/kre5* cells, more GFP was detected in cell-free supernatants from these cells than in those from wild-type (Figure 2B). *KRE5/kre5* cells also showed significantly less cell surface fluorescence than wild-type cells (Figure 1D2 vs. 1D1), a result consistent with reduced cell wall anchorage of the GFP test protein.

eGFP folds slowly and is susceptible to protease digestion in the culture supernatants (Yang *et al*., 1996). Therefore, we assayed secretion of GFP–Sag1p in cell wall mutants *cwp1/cwp1* and *kre1/kre1* after growth in the presence or absence of the aspartyl protease inhibitor pepstatin A (1 μM). In the presence of the inhibitor, both *cwp1/cwp1* and *kre1/kre1* strains hypersecreted relative to wild-type (Figure 2C). Similarly, in the presence of pepstatin A, a *dcw1/dcw1* strain hyper-secreted the marker protein (Figure 2D). Pepstatin A also increased

the marker protein detected from the *dfg5/dfg5* strain, but to levels only slightly greater than the wild-type BY4743. *DFG5* is paralogous to *DCW1*.

High-throughput quantification of GFP–Sag1p

GFP–Sag1p secretion levels were determined for parental strains BY4743 and for 167 strains missing genes implicated in cell wall biogenesis. These genes were selected from three independent groups of genes previously ascribed a cell wall function: (a) 268 genes whose deletions affect sensitivity to K1 killer toxin (Page *et al*., 2003); (b) 103 genes listed in SGD as cell wall genes (http://www.yeastgenome.org); and (C) 171 cell wall-related genes identified in an *in silico* study in *S. cerevisiae* (Coronado *et al*., 2007). All genes common to all three groups and those shared between two groups were selected for screening. Of 45 genes shared between two groups, seven failed to transform and could not be screened.

The GFP fluorescence values from wild-type and mutant culture supernatants were ranked and plotted. The data are shown in Figure 3 (see also Supporting information, Figure S1, Tables S2, S3). Seventeen mutants were hypersecretors, by the criterion that their mean fluorescence was greater than the mean plus standard error (SE) for wild-type. 38% of the 167 gene deletants secreted less GFP–Sag1p than wild-type, on the basis that their mean fluorescence was lower than the mean minus SE for the wild-type. Of the 17 potential hypersecretors, seven were positive in a second immunoblot assay (Figure 4A). The additional hypersecretors confirmed by dot-blot analysis were *YGR192CΔ* (*tdh3/tdh3*), *YLL031CΔ* (*GPI13/gpi13), YKL165CΔ* (*MCD4/mcd4*) and *YEL042WΔ* (*gda1/gda1*). For these four mutants and the hyposecretor *nsr1/nsr1*, complete fluorescence spectra were determined as described in Materials and methods. In accordance with the immunoblot, *nsr1/nsr1* cells showed a fluorescence peak substantially lower than that observed for wild-type cells (Figure 4B, C).

Cell surface fluorescence

To estimate relative cell surface fluorescence intensities for these strains, the samples were photographed with a series of exposure times in a fluorescence microscope and we visually matched the exposure time for each to the wild-type BY4743. For instance, in a comparison of wild-type and *GPI13/gpi13* (Figure 5A, C, respectively) the one-thirtieth of a second exposure for *GPI13/gpi13* cells showed comparable intensity to the one-fifteenth of a second exposure of wild-type cells. Therefore, the fluorescence of *GPI13/gpi13* cells was twice that of the wild-type cells. *MCD4/mcd4* cells were also slightly brighter than wild-type cells (Figure 5A, B). The *tdh3/tdh3* and *gda1/gda1* strains were ~two-fold less bright than wild-type (Figure 5F–H). *GPI13/gpi13* and *gda1/gda1* cells had cell separation defects and increased fluorescence near the septa (Figure 5D, E).

Screens for cell lysis

There was no significant difference between wild-type and mutant cells in the total protein present in growth supernatants (data not shown), suggesting that higher GFP–Sag1p levels in mutant supernatants are not the result of cell lysis.

To confirm the lack of cell lysis or leakage of intracellular protein/components due to cell wall damage, cell-free supernatants were also examined for presence of the cytosolic protein phosphoglycerate kinase (PGK). This enzyme was used as a marker because of its abundance and the commercial availability of an antibody. Immunoblots showed that there was more PGK in supernatants from wild-type BY4743 cells than in those from any of the hyper-secreting mutants (see Supporting information, Figure S2).

Note that cell membrane damage or lysis should release large amounts of PGK into the medium, regardless of whether or not some of the protein is localized in the wall, as has been previously reported (Alloush *et al.*, 1997; Angiolella *et al.*, 1996; de Groot *et al.*, 2007).

Discussion

General approach

The rationale for the phenotypic screen was that strains missing genes required for GPI–CWP processing or cell wall anchorage would retain a GFP-labelled GPI–CWP protein within the cell (hyposecretors), or secrete the fluorescent protein into the growth medium (hypersecretors), relative to wild-type cells. Additionally, defective strains would exhibit altered levels of cell surface fluorescence relative to wild-type cells. Here, we identified strains with each phenotype.

The value of our experimental design lies in its functionality as a high-throughput approach to uncover genes required for fungal adhesin attachment to the fungal wall. Previous studies demonstrate hypersecretion of fluorescently labelled GPI-protein in some strains, although no approach has been taken to survey the genome in its entirety. Genome-wide surveys for genes encoding GPI-mannoproteins have been done *in silico* (De Groot *et al.*, 2003). Mass spectrometry has identified and quantified cell wall GPI-proteins, but this approach has only been extended to a few cell wall mutants (de Groot *et al.*, 2007; Yin *et al.*, 2008). Our genome-wide approach is designed to handle the systematic assay of hundreds of strains at a time. Hyper- and hyposecretion of the marker is reproducibly assayed in a 96-well plate reading fluorimeter. Therefore, the procedure is easily replicable and adaptable to a range of fungi and growth conditions.

Initially, we found that a high-throughput fluorescence assay for secretion of GFP–Sag1p was feasible, but showed unacceptable variability (see Supporting information, Figure S1). This variability resulted from uncontrolled pH, lack of osmotic support and variations in growth. Variability was reduced by growth at reduced temperature in the presence of 1 M sorbitol in defined medium at pH 6.5, to mid-exponential phase (Figure 2). Under these conditions, there was also reproducibility in cell surface fluorescence and minimal cell lysis (Figures 2, 5; see also Supporting information). The assay was validated in cell wall mutant *KRE5/kre5*, known to hypersecrete GPI–CWPs and have reduced GPI–CWP levels at the wall.

Role of cell surface proteases

It is clear that the inclusion of pepstatin A in the culture medium affected the levels of GFP–Sag1p detected in the supernatants (Figure 2C, D). For the *kre1/kre1, cwp1/cwp1, dcw1/dcw1* and *dfg5/dfg5* strains, the amount of marker protein was greater after growth with protease inhibitor. Paradoxically, pepstatin A caused a no change or a very slight reduction in supernatant GFP–Sag1p in wild-type cells and in the *GPI13/gli13* strain (Figure 2D). This unexpected result is explained on the basis that yapsins, GPI-anchored aspartyl proteases, may act as 'shedases' that cleave GPI–CWPs (including the yapsins themselves) from the wall (Gagnon-Arsenault *et al.*, 2008). Based on rough quantification, about 10 ng/ml mannoprotein is shed into the growth medium, or about 10% of the cell surface content in wild-type cells (Figure 4A). We speculate that in wild-type strains, pepstatin A will inhibit shedding of the marker protein into the medium. In the case of the mutants unable to properly anchor GFP–Sag1p to the wall, pepstatin A will inhibit yapsins that have been secreted into the medium (Yao *et al.*, 2009), therefore stabilizing the GFP marker protein. Important to note is that previous studies reporting GPI–CWP hypersecretion in *kre1* and

other cell wall mutants employed antibodies against highly glycosylated GPI–CWPs that are not as susceptible to proteolysis (Lu *et al*., 1995; de Groot *et al*., 2001). These proteins would remain detectable in the absence of protease inhibitors such as pepstatin A (Walsh and Chapman, 1991). The conclusion is that the addition of pepstatin A to growth media does not affect growth, but adds to the sensitivity of the screening assay by enhancing differences in GFP–Sag1p secretion between mutant and wild-type cells. Assays under both conditions provide complementary information by allowing detection of hypo-, mild and hypersecreting phenotypes relative to wild-type. The latter are often detected without inclusion of pepstatin A, as demonstrated in Figure 2B, whereas the addition of pepstatin A brings out weaker phenotypes (Figure 2D). Although at the concentration used in our studies pepstatin A does not affect growth rate (data not shown), we chose to perform the screen without pepstatin A because of important roles assigned to yapsins in cell wall remodelling and maintenance of cell wall integrity (Krysan *et al*., 2005).

Genes required for efficient GPI–CWP anchorage to the cell wall

We screened 167 mutants involved in cell wall assembly and biogenesis (Brown *et al*., 1993; Page *et al*., 2003; Rodriguez-Pena *et al*., 2005; Smits *et al*., 1999). Hyper- or hyposecretion was confirmed by immunoblotting and by collecting complete fluorescence spectra. The strains were also analysed for cell surface fluorescence and levels of cytosolic and total protein in the culture medium. Eight of the selected 167 cell wall mutants exhibited clear deficits in anchoring our GPI–CWP reporter protein to the cell wall. Five of these genes are non-essential (*TDH3, GDA1, KRE1, CWP1* and *DCW1*) and three are essential (*MCD4, GPI13* and *KRE5*).

MCD4 and GPI13

MCD4 and *GPI13* are phosphoethanolamine (EtN-P) transferases involved in GPI-anchor biosynthesis. As in other organisms, these enzymes form part of the GPI anchor biosynthetic complex, where they add ethanolamine phosphate side chains to the mannose residues of the glycan part of the GPI-anchor (Gaynor *et al*., 1999; Taron *et al*., 2000; Toh-e and Oguchi, 2002). To date, three EtN-P transferases have been described in *S. cerevisiae*: Mcd4p, Gpi7p/Las21p and Gpi13p add EtN-P groups to the first, second and third mannose residues, respectively, of the glycan core of the GPI anchor (Gaynor *et al*., 1999). The essentiality of *GPI13* is expected because it adds EtN-P to the C6 hydroxyl group of the third mannose of the GPI anchor, and it is through this EtN-P group that the GPI-anchor is linked to protein via an amide bond (Tiede *et al*., 1999). In *gpi13* disruptants, GPI anchors are not attached to proteins through other EtN-P groups, and reduction of GPI anchoring leads to overall cell wall fragility (Flury *et al*., 2000). Our studies show that cells heterozygous for a *GPI13* deletion hypersecrete GFP–Sag1p and have increased cell surface fluorescence relative to wild-type cells. It is likely that in these cells the synthesis of GPI-proteins is upregulated to compensate for the high failure rate of forming the protein-GPI amide linkage. This would also help to explain the relatively strong fluorescence that we observed in these cells at sites of active cell wall synthesis, such as during daughter cell growth and the formation of new budding cells (Figure 5D). We also observed that *GPI13/gpi13* cells have a septation defect that leads to the formation of multibudded cells (Figure 5E). In this class of mutants, defects in GPI synthesis lead to deficits in anchor addition to precursor GPI-proteins and to subsequent decreases in GPI-protein targeting to the plasma membrane and the cell wall, where they are required for cell wall formation, remodelling and integrity. Similar responses may occur in *GPI13/gpi13* cells that would lead to septation defects and overproduction and hypersecretion of GPI-anchored cell wall proteins that we observed in this strain. Although these defects are predictable in *GPI13/gpi13* cells, this study provides the first experimental evidence that *GPI13* is required for normal GPI-protein anchorage to the fungal wall and that heterozygous deletions of this gene are haplo-insufficient. The value of *GPI13* as an

anti-fungal target deserves closer examination (Hong *et al*., 2000; Almeida, 2005; Grimme *et al*., 2001).

Mcd4p catalyses the addition of EtN-P to the first mannose in the GPI anchor. Mcd4p is required for GPI anchoring, because these mutants exhibit cell wall fragility and are defective in ER-to-Golgi transport of multiple GPI-anchored proteins (Yada *et al*., 2001). This deficit in targeting GPI-proteins to their final destination is believed to cause the aberrant phenotypes observed in *mcd4*[ts] cells, such as cell separation defects and abnormal bud site emergence and cell morphology (Gaynor *et al*., 1999). Since deficits in GPI-anchor biosynthesis inevitably compromise cell wall integrity, it seems logical to assume that *mcd4* mutations can result in activation of the cell wall stress response and the subsequent upregulation of GPI-mannoproteins responsible for repairing and stabilizing cell wall integrity. This would help to explain the increase in cell surface GFP–Sag1p and in the levels of secreted GFP–Sag1p that we observed in *MCD4/mcd4* cells relative to wild-type cells. Mcd4p is essential for fungal growth and dispensable for mammalian cell viability (Yada *et al*., 2001). The value of *MCD4* as a novel antifungal target is corroborated by findings that the terpenoid lactone BE49385A, which inhibits the modification of mannose one of the GPI-anchor with EtN-P, is lethal to yeast cells (Hong *et al*., 1999).

The defects caused by *gpi13* and *mcd4* are similar to that in *gpi7Δ* cells and other mutants deficient in GPI anchor biosynthesis (de Groot *et al*., 2001). However, our screen did not identify *gpi7/gpi7* as either a hyper- or a hyposecretor under the standard growth conditions in the absence of pepstatin A.

GDA1

GDA1 encodes a guanosine diphosphatase (GDPase) which is involved in adding mannosyl residues to proteins and sphingolipids in the Golgi (Lopez-Avalos *et al*., 2001). The donor of the mannosyl residues is the nucleotide sugar GDP-mannose. GDP-mannose is synthesized in the cytosol and translocated into the Golgi lumen by a transporter, Vrg4p (Berninsone *et al*., 1995; Dean *et al*., 1997; Warit *et al*., 2000). Following mannosylation of lumenal proteins and lipids, GDP is converted to GMP by Gda1p. A Golgi membrane antiporter then exchanges the lumenal GMP for cytosolic GDP-mannose (Berninsone *et al*., 1995). Therefore, deletion of Gda1p blocks the transport of GDP-mannose into the lumen of the Golgi and subsequently both *O*- and *N*-glycosylation are impaired (Abeijon *et al*., 1993). Whether the underglycosylated GPI–CWPs can still anchor to the cell wall is currently not known. Our results showed much lower than wild-type levels of GFP–Sag1p at the wall in *gda1/gda1* cells, and hypersecretion of the reporter protein into the growth medium (Figures 4, 5). These observations imply that underglycosylated proteins are excluded from being cross-linked to the wall and are subsequently excreted. These phenotypes are consistent with observations that *C. albicans gda1/gda1* cells are more susceptible to the action of *β*-1,3-glucanases, perhaps due to a thinner outer layer of cell wall proteins which protects the underlying *β*-1,3-glucan network from glucanases (De Nobel *et al*., 1991; Ellerton *et al*., 2008). Notably, we did not see increases in the level of intracellular protein in the growth medium of these *gda1/gda1* cells, so it is likely that increased GFP levels in the medium is not due to leakage of intracellular components. Our data are consistent with the idea that proper glycosylation is required for protein to anchor onto the wall.

TDH3

TDH3 encodes an isozyme of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a highly expressed glycolytic enzyme. The presence of classic cytosolic enzymes at the cell surface has been considered an artifact (Klis *et al*., 2007), but GAPDH can direct intracellular proteins to the cell wall (Alloush *et al*., 1997; Edwards *et al*., 1999; Pardo *et al*.,

1999). For instance, a fusion of intracellular *S. cerevisiae* invertase (*ScSUC2*) to *C. albicans TDH3* expresses invertase activity at the cell surface, presumably from regions in *TDH3* capable of targeting the fusion protein to the cell wall (Delgado *et al*., 2003). Cell wall localization of GAPDH was further established in *S. cerevisiae* by indirect immunofluorescence and flow cytometry analysis with a polyclonal antibody against *S. cerevisiae* GAPDH (Gil-Navarro *et al*., 1997). In *C. albicans* and *S. cerevisiae*, the GAPDH protein is detected at the outer surface of the cell wall and in the cytoplasm of wild-type cells by immunoelectron microscopy (Delgado *et al*., 2001). We note that neither *tdh1/tdh1* nor *tdh2/tdh2* strains hypersecreted GFP–Sag1p in our screen (see Supporting information, Tables S1–S3) and therefore Tdh3p may have a non-redundant role in cell wall biogenesis.

DCW1 and DFG5

DCW1 and *DFG5* encode putative glycosidase/transglycosidases homologous to bacterial family 75 (Cantarel *et al*., 2009). Recently, these genes have been implicated in GPI anchor cleavage and transglycosylation of the GPI-anchor remnant protein to cell wall polysaccharides. However, their exact role in the processes remains to be determined. Dcw1p and Dfg5p are GPI-mannoproteins of the plasma membrane, although in *C. albicans* Dfg5p also localizes to the cell wall (Spreghini *et al*., 2003). Deletion of *DCW1* renders cells hypersensitive to the cell wall-digesting enzyme zymolyase, whereas deletion of *DFG5* does not lead to an observable phenotype (Kitagaki *et al*., 2002). Both single deletants exhibit normal morphology and grow normally in rich and synthetic media. *DCW1* and *DFG5* are functionally redundant and show synthetic lethality, which can be rescued by a plasmid expressing *DFG5* from the *GAL1* promoter. The rescued mutant hypersecretes cell wall GPI-mannoproteins and becomes hypersensitive to cell wall-disrupting agents following glucose repression (Kitagaki *et al*., 2002). In *S. cerevisiae, DFG5* and *DCW1* are required for bud formation (Kitagaki *et al*., 2004), while *DFG5* is required for agar invasion and growth at alkaline pH (Mosch and Fink, 1997). In *C. albicans*, these genes have similar functions: at least one of them is required for growth (Spreghini *et al*., 2003) and *DGF5* is required for hyphal development (Spreghini *et al*., 2003). Clearly, Dcw1p and Dfg5p play an important role in cell wall biogenesis. We have shown that *dcw1/dcw1* null mutants hypersecreted GFP–Sag1p and have reduced levels of the protein at the wall relative to wild-type cells. However, the cells required growth in the presence of pepstatin A for detection of the phenotype.

Conclusion

In conclusion, the fusion protein secretion assay can reliably distinguish between yeast strains with different deletions. The screen has shown that some deletions increase secretion of GPI–CWPs to the medium, whereas others do not. The assay will be useful in screening antifungal drugs and in elucidating the physiological consequences of mutations and potential anti-fungal compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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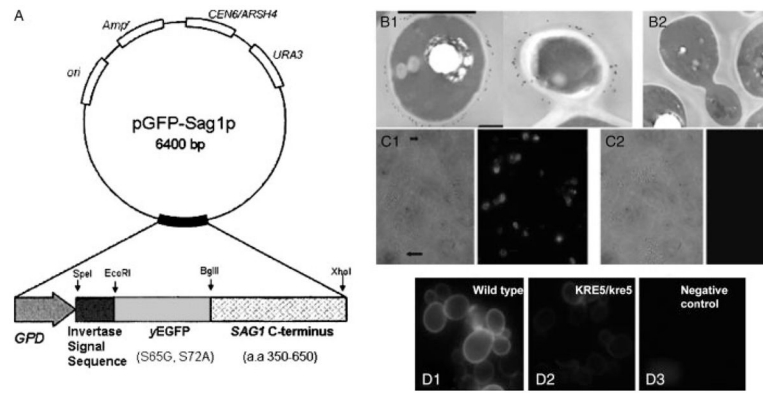
Figure 1.
Cell wall localization of GFP–Sag1p. (A) Schematic of pGFP–Sag1p reporter construct in low copy vector p416-GPD. (B1) Immunoelectron micrographs of wild-type cells expressing GFP–Sag1p labelled with mouse monoclonal anti-GFP as primary antibody and gold-labelled goat anti-mouse as secondary antibody. (B2) Immunoelectron micrograph of non-expressing cells. (C) Phase contrast and GFP fluorescence of isolated washed cell walls from BY4743 cells expressing GFP–Sag1p (C1) and BY4743 cells transformed with empty vector alone (C2). Cell surface fluorescence of: (D1) BY4743–GFP–Sag1p wild-type; (D2) *KRE5/kre5* mutant; and (D3) non-expressing cells
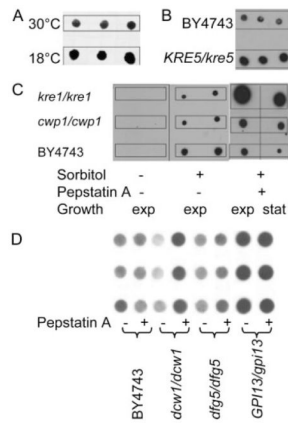
Figure 2.
Immunoblot analyses of cell wall mutants and optimization of assays. (A) Growth at lower temperature increases GFP–Sag1p yields. Triplicate clones of BY4743 were grown at 30 °C (top) and 18 °C before assay. (B) Supernatants from triplicate clones of BY4743 (top) and *KRE5/kre5* mutant grown without pepstatin A. (C) Effect of sorbitol (1 M), pepstatin A (1 μM) and growth phase. Duplicate clones were grown without sorbitol or with sorbitol in the absence (left) or presence (right) of pepstatin A. (D) Effect of growth in pepstatin A (1 μM) on secretion of GFP–Sag1p in wild-type and three deletion strains. Each strain was grown in triplicate under standard conditions
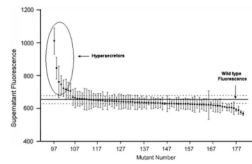
Figure 3.
Supernatant fluorescence values for representative mutants. Mutant strains were grown
without pepstatin A in triplicate at 18 °C to mid-log phase in osmotically stable medium
buffered to pH 6.5, and supernatants were assayed for GFP fluorescence. Mutant names and
genotypes are given in Table S2 (see Supporting information). Mean and SEM values
(dashed lines) are shown for the wild-type BY4743–GFP–Sag1 strain and for mutant strains
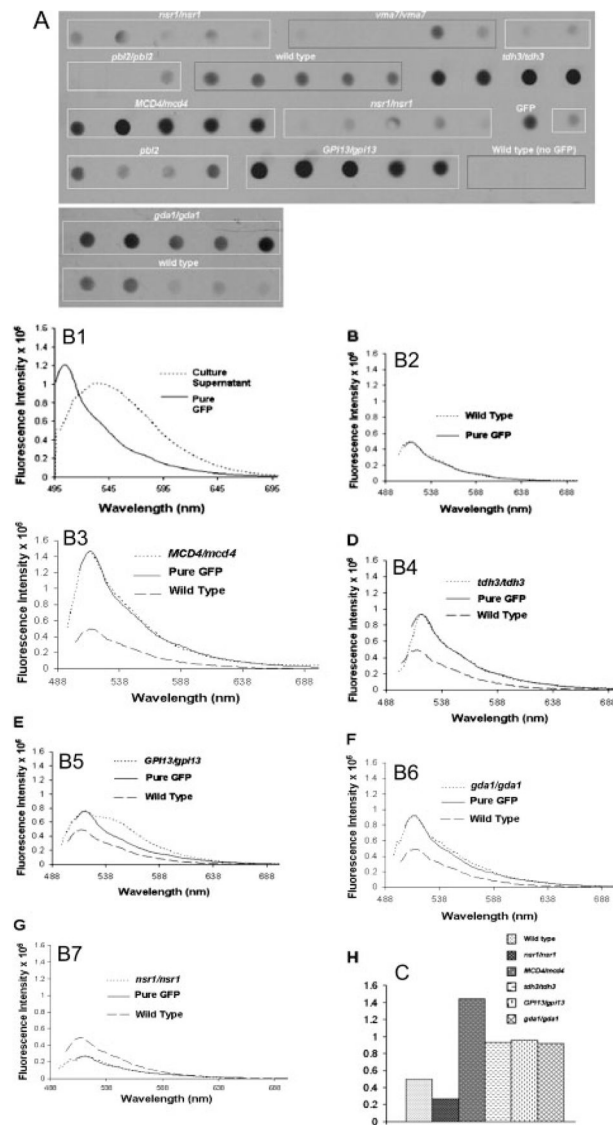(error bars)

Figure 4.
Confirmation of hyper- and hyposecreting mutants. (A) Standard condition immunoblots of growth supernatants from five replicate clones of BY4743 and seven mutants. (B1) Uncorrected spectra for GFP and culture supernatant of BY4743. (B2) corrected spectrum for BY4743. (B3–B7) Background-corrected fluorescence spectra from supernatants from BY4743 (dashed lines), selected mutants (dotted lines) and purified GFP (solid lines) as reference. The relative fluorescence for each sample was taken as the height at the maximum point in the corrected spectrum (C). Cells were grown without pepstatin A
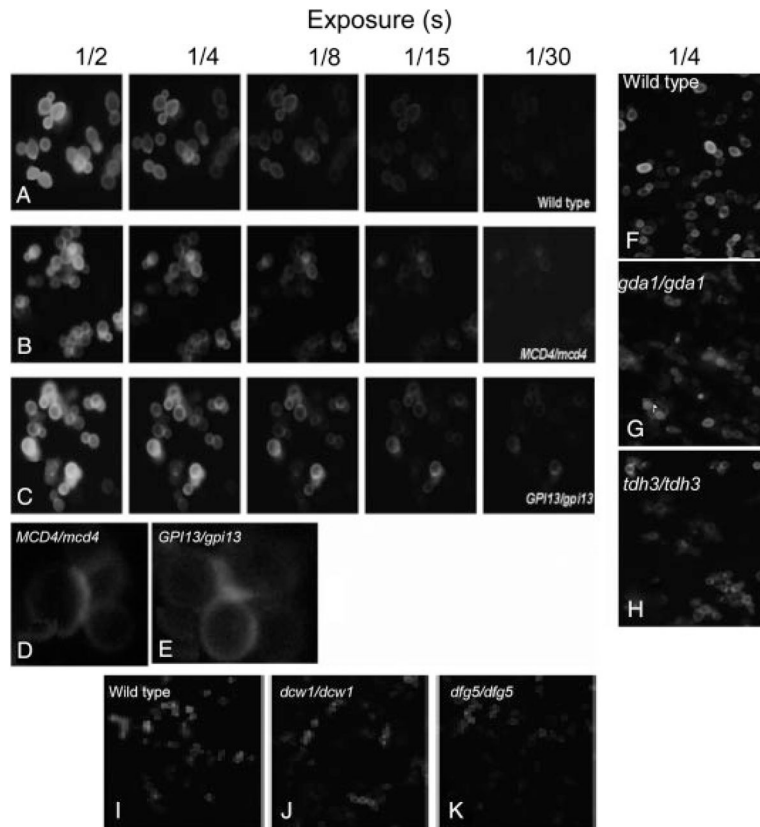
Figure 5.
Cell surface GFP fluorescence of BY4743 and selected mutants. Fluorescence exposure series for wild-type (A), MCD4/mcd4 (B) and GPI13/gpi13 (C). Also shown are details of septum regions for MCD4/mcd4 (D) and GPI13/gpi13 (E). On the right are matched exposures of BY4743 (F), gda1/gda1 (G) and tdh3/tdh3 (H). At the bottom are matched exposures of (I) BY4743, (J) dcw1/dcw1 and (K) dfg5/dfg5