Increased mortality of *Saccharomyces cerevisiae* cell wall protein mutants

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The yeast cell wall contains an unusually high number of different mannoproteins. The physiological role of most of them is unknown and gene disruptions leading to depletion of different proteins do not affect major functions of the wall. In this work the phenotype of different single and multiple cell wall protein mutants was observed at the level of individual cells. It was found that the lack of the non-covalently bound wall proteins Scw4p, Scw10p and Bgl2p increases the mortality of *Saccharomyces cerevisiae* cells grown exponentially under standard laboratory conditions, as assayed by methylene blue staining. Mutation of *SCW11*, however, suppressed the phenotype of *scw4scw10*, or *scw4scw10bgl2*, indicating that Scw4p, Scw10p and Bgl2p act synergistically while Scw11p has an activity antagonistic to that of the other three proteins. Mutants lacking major covalently bound proteins, either all four described Pir-proteins or the five most abundant glycosylphosphatidylinositol (GPI)-anchored proteins (Ccw12p, Ccw13p/Dan1p, Ccw14p/Icwp1p, Tip1p and Cwp1p), also had increased mortalities, the first somewhat more and the latter less than that of *scw4scw10bgl2*. In all cases the observed phenotype was suppressed by the addition of an osmotic stabilizer to the growth medium, indicating that cells died due to decreased osmotic stability. If cells were grown to stationary phase, Scw-mutants showed only slightly increased mortality, but mutants lacking Pir- or GPI-anchored proteins had significantly increased sensitivity, suggesting that their physiological function is primarily expressed in stationary-phase cells. In many cases structures consisting of a living *ccw5ccw6ccw7ccw8* (multiple Pir-protein mutant) mother with two methylene blue-stained daughters could be seen.

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INTRODUCTION

The mechanical and osmotic stability of most plant and micro-organism cells is achieved by their cell walls, constructed of different polysaccharide moieties to which various proteins are attached. In yeasts, the basic network component is 1,3- β -glucan and it is responsible for the unusually high mechanical stability and cell turgor (Eamus & Jennings, 1986). Two other polysaccharides, 1,6- β -glucan and chitin, are linked to 1,3- β -glucan (Fleet, 1991) and have functions other than preserving cellular shape and firmness. A variety of different wall proteins, many of which are highly mannosylated, are specific for yeast cell walls. Most of them are located externally to the 1,3- β -glucan network, resulting in an outer mannan layer. Yeasts have evolved three different ways of attaching proteins to the polysaccharide moiety. Some proteins are bound to 1,3- β -glucan non-covalently (Valentin *et al.*, 1984; Cappellaro *et al.*, 1998), some are attached covalently through glycosylphosphatidylinositol (GPI) anchoring to 1,6- β -glucan (Montijn *et al.*, 1994; Klis, 1994) and the third group, consisting of

members of the so-called Pir-protein family, is also covalently attached directly to 1,3- β -glucan by a mechanism still not completely understood (Mrša *et al.*, 1997; Mrša & Tanner, 1999). In some cases it has been shown that the proper localization of a protein is a prerequisite for its function (Terashima *et al.*, 2003). Different biochemical approaches have been used to identify and characterize proteins extracted from the cell wall. Non-covalently attached proteins can be released using hot SDS or DTT (Valentin *et al.*, 1984; Cappellaro *et al.*, 1998), while covalently bound proteins can be extracted either by different glucanase preparations (Montijn *et al.*, 1994; Klis, 1994) or by a mild alkali treatment (Mrša *et al.*, 1997).

Since 1989, and the first report on cloning and partial characterization of a *Saccharomyces cerevisiae* integral cell wall protein, Bgl2p (Klebl & Tanner, 1989), later characterized *in vitro* as endoglucanase (Mrša *et al.*, 1993), these efforts have resulted in the identification, cloning, characterization and often disruption of corresponding genes of over 30 different proteins isolated from the walls of many different yeasts, particularly *S. cerevisiae* (Van Der Vaart *et al.*, 1995; Cappellaro *et al.*, 1998; Mrša *et al.*, 1997; Rodriguez-Pena *et al.*, 2000). In all cases the disruptants

Abbreviations: CFW, Calcofluor White; GPI, glycosylphosphatidylinositol; MTT, 3-(4,5-dimethylthiazoyl-2-yl) 2,5-diphenyltetrazolium bromide.

reported survived the lack of the depleted protein and required no osmotic stabilization for growth (Cappellaro *et al.*, 1998; Mrša & Tanner, 1999; Moukadiri *et al.*, 1997; Mrša *et al.*, 1999; Rodriguez-Pena *et al.*, 2000). Since a significant genome redundancy was observed, particularly in *S. cerevisiae*, multiple gene deletions have been performed. These, however, had little apparent effect on cellular functions and no physiologically significant phenotype was observed (Mrša & Tanner, 1999), except for increased sensitivity to some chemical drugs like Calcofluor White (CFW) or Congo Red, and sometimes changed growth behaviour. As a consequence, the present understanding of the physiological functions of yeast cell wall proteins is rather poor, which is particularly intriguing bearing in mind their number.

In this work, additional phenotypic characteristics of different single and multiple cell wall protein mutants lacking either non-covalently or covalently attached proteins, respectively, were observed at the level of individual cells in an attempt to gain further insight into their significance for the cell.

METHODS

Strains and media. *Saccharomyces cerevisiae* strains used in this work are listed in Table 1. All strains were grown in standard YPD medium (1 % yeast extract, 2 % peptone, 2 % glucose).

Zymolyase sensitivity. Yeast cells were harvested at an OD₆₀₀ of 2.0–3.0, washed once with distilled water and once with 0.01 M sodium phosphate buffer, pH 7.5. Washed cells were resuspended in

the same buffer and the cell density was adjusted to an OD₆₀₀ of about 0.6. Zymolyase 100T solution was added to 3 ml portions of cell suspension at a final concentration of 3 µg ml⁻¹. The mixture was incubated at 30 °C with shaking and the OD₆₀₀ value was followed.

CFW staining. Cells were grown to mid-exponential phase and fixed by the addition of formaldehyde to a final concentration of 3.7 %. Cells were subsequently incubated for 1 h at 30 °C, washed with 0.05 M phosphate buffer, pH 8.0, and resuspended in the same buffer. CFW (Sigma) was added to a final concentration of 2 µg ml⁻¹. After 15 min incubation at room temperature, cells were washed in phosphate buffer. Chitin in the cell wall was visualized by fluorescence microscopy.

Nuclear DNA staining. Exponential-phase cells were fixed by the addition of formaldehyde to a final concentration of 3.7 % and incubated for 1 h at 30 °C. After that cells were washed in 0.05 M phosphate buffer, pH 8.0, and fixed with 70 % ethanol for 20 min at room temperature. Cells were washed once and resuspended in the same buffer. Nuclear DNA was stained with 4,6-diamino-2-phenylindole (DAPI) by adding the dye into the cell suspension to a final concentration of 1 µg ml⁻¹. After 15 min incubation at room temperature, cells were washed in phosphate buffer. Samples were observed by fluorescence microscopy.

Methylene blue staining. Cells were grown in standard YPD medium or in YPD medium with 1 M sorbitol. The cell suspension was mixed with an equal volume of methylene blue and incubated for 5 min at room temperature. Dead, blue-coloured cells were scored (Smart *et al.*, 1999). Typically, about 1000 cells were counted for each strain in each experiment, and every experiment was repeated at least three times. Mean values, together with the obtained ranges, are presented in diagrams.

MTT assay. Cells were grown in standard YPD medium to early exponential or stationary phase and washed with water. Cell density

Table 1. Yeast strains used in this work

Strain	Genotype	Reference
SEY6210	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL</i>	Robinson <i>et al.</i> (1988)
VMY5	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL ccw5:: (kanMX)</i>	Mrša & Tanner (1999)
VMY567	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL ccw5:: (kanMX)</i> <i>ccw6:: (kanMX) ccw7:: (kanMX)</i>	Mrša & Tanner (1999)
VMY5678	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL ccw5:: (kanMX)</i> <i>ccw6:: (kanMX) ccw7:: (kanMX) ccw8:: URA3</i>	Mrša & Tanner (1999)
MSP66A	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL scw4:: LEU2</i>	Cappellaro <i>et al.</i> (1998)
MSP66B	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL scw10:: HIS3</i>	Cappellaro <i>et al.</i> (1998)
MSP02G	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL scw4:: LEU2</i> <i>scw10:: HIS3</i>	Cappellaro <i>et al.</i> (1998)
MSP66C	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL scw11:: TRP1</i>	Cappellaro <i>et al.</i> (1998)
MSP029	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL bl2:: URA3</i>	Cappellaro <i>et al.</i> (1998)
MSP03G	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL scw4:: LEU2</i> <i>scw10:: HIS3 scw11:: TRP1</i>	Cappellaro <i>et al.</i> (1998)
MSP04G	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL scw4:: LEU2</i> <i>scw10:: HIS3 scw11:: TRP1 bgl2:: URA3</i>	Cappellaro <i>et al.</i> (1998)
MSP05G	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL scw4:: LEU2</i> <i>scw10:: HIS3 bgl2:: URA3</i>	Cappellaro <i>et al.</i> (1998)
MEY12A	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL ccw12:: URA3</i>	Mrša <i>et al.</i> (1999)
MEY5	MAT α <i>ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL ccw12:: URA3</i> <i>ccw13:: TRP1 ccw14:: HIS3 tip1:: LEU2 cwp1:: (kanMX)</i>	M. Ecker & others, unpublished

was adjusted to an OD₆₀₀ of 2 (corresponding to approx. 2×10^7 cells ml⁻¹). Cells from 1 ml suspension were harvested and resuspended in 0.4 ml 10% MTT [3-(4,5-dimethylthiazolyl-2-yl) 2,5-diphenyltetrazolium bromide]. The mixture was incubated at room temperature with shaking for 2 h. After that cells were harvested and resuspended in 1 ml acid 2-propanol (0.04 M HCl in 2-propanol). The suspension was agitated for 10 min, then centrifuged at 8000 r.p.m. The OD₅₄₀ of the supernatant was then measured (Garlier & Thomasett, 1986).

Transformation of *ccw5ccw6ccw7* with CCW5. Mutant strain *ccw5ccw6ccw7* was transformed with the CCW5 gene cloned into the *Sma*I restriction site of yeast episomal plasmid YEp351. Transformation was accomplished by the standard lithium-acetate method.

RESULTS AND DISCUSSION

Since the first systematic screenings for yeast cell wall proteins, nearly 50 different protein species have been found and this has changed our perspective with regard to the wall as the outermost cellular organelle. For many of these proteins, the corresponding genes have been disrupted and characterization of mutant phenotypes has been performed, usually in an effort to give some idea about their physiological functions. Yeast growth is, however, seriously affected only when Gas1p is depleted, which is in fact not a classical cell wall protein since it remains anchored to the plasma membrane, although its activity is apparently connected to the cell wall (Popolo & Vai, 1999; Mouyna *et al.*, 2000). For all other wall proteins, no clear damage to the cell has been reported as a result of their depletion which has raised general questions as to the requirements for these proteins and the evolutionary factors which have led to at least three different methods for their incorporation into the cell wall.

To investigate the role of yeast cell wall proteins of each of the three classes, namely SDS-extractable (Scw-proteins), glucanase-extractable (GPI-anchored proteins) and NaOH-extractable (Pir) proteins, different mutants were constructed lacking the most prominent and abundant of them (Table 1). Some of these mutants, such as the one lacking all four known Pir-proteins, have already been described and some of their phenotypic characteristics have been reported (Mrša *et al.*, 1997; Mrša & Tanner, 1999). However, these mutants could not help us to estimate the physiological significance of these proteins. Therefore, we decided to investigate properties such as morphological changes and survival capacity of these mutants under physiological conditions. First, the mortality of the mutants grown under usual laboratory aerobic conditions in YPD medium was investigated. The number of dead cells was estimated by methylene blue staining (Smart *et al.*, 1999). Other methods currently used for this purpose, including simple counting of colony forming units, were found to be inappropriate, since some of the mutants exhibited clumpy growth. In most clumps both dead and living cells were found, thus direct microscopic investigation was required to obtain accurate data. To avoid the possibility of artefacts potentially caused by the staining procedure, the number of dead

cells was also estimated by the MTT assay. Unlike methylene blue, MTT stains living cells and the assay is based on a different principle (Garlier & Thomasett, 1986; Zheng & Ben, 1992), but the data obtained by both methods were essentially the same. Results presented in Fig. 1 show that some mutants show much higher mortality in 'normal' laboratory cultivation. Among different Scw (non-covalently linked, SDS-soluble wall proteins; Fig. 1a) mutant *scw4* had a somewhat increased mortality compared to wild-type, *scw10* or *scw11*. All three proteins are homologues of known glucanases or transglucosidases and particularly SCW4 and SCW10 share a very high degree of identity (Cappellaro *et al.*, 1998). Therefore, it was not surprising that an *scw4scw10* double mutant had a significantly increased mortality, reaching about 5% dead cells in culture. Unexpectedly, an additional *scw11* mutation led

Fig. 1. Mortality of different cell wall protein mutants grown to exponential phase. (a) Non-covalently linked proteins; (b) Pir-proteins; (c) GPI-anchored proteins. Open bars, -sorbitol; grey bars, +sorbitol.

to suppression of the observed phenotype, indicating that Scw11p has an activity antagonistic to that of Scw4p and Scw10p. Further deletion of another known endoglucanase gene, *BGL2*, led to a further increase in the mortality rate and, consistently, the highest number of dead cells was found in a triple *scw4scw10bgl2* mutant.

The results indicate that proteins like Scw4p, Scw10p and Bgl2p are synergistically involved in building up the wall, while some others (Scw11p in this study) act antagonistically to the first group. This allows a dynamic equilibrium of different activities in the yeast cell wall which may be responsible for the remarkable properties of this cellular structure, having extreme firmness and rigidity and allowing high osmotic pressures, but still being able to grow and change (Eamus & Jennings, 1986). Such a model would also explain why there is little, if any phenotype for single *scw* mutations, and the fact that no enzymic activity could be demonstrated for Scw4p and Scw10p *in vitro* indicates that they require more complex substrates such as the defined moieties of the cell wall (Cappellaro *et al.*, 1998). Rodriguez-Pena *et al.* (2000) described another group of cell wall proteins also homologous to different glucanases/transglucosidases. It would therefore be interesting to compare their influence on cell wall structure to that of the Scw proteins studied here. Also, additional combinations of mutants would be required to see which of these proteins are involved in building up the wall and which are involved in loosening the structure.

As shown in Fig. 1(b), depletion of Pir-proteins from the cell wall had an even more pronounced effect on the mortality of cells. A single *ccw5* mutation causes a significant increase in the number of dead cells and, as reported for other mutant features (Mrša & Tanner, 1999), the phenotype was more pronounced when more members of this protein family were depleted, reaching nearly 8 % dead cells in a *ccw5ccw6ccw7ccw8* mutant. A decreased growth rate of about 20–30 %, which could be a result of increased mortality, has been reported for Pir-protein mutants (Mrša *et al.*, 1997), but the accurate estimation of growth rates of some cell wall protein mutants is often made difficult by their clumpy growth.

The influence of GPI-anchored proteins on the viability of cells was also investigated. Since there is no apparent protein family in this group of proteins, although in some cases pairs of partially homologous proteins can be observed, a mutant lacking the five proteins reported to be the most abundant in the wall was constructed. It was found that this mutant also had an increased mortality compared to wild-type, although not as high as for the other two groups of wall proteins (Fig. 1c).

To investigate the cause of death of cells in culture, cells were grown in medium containing sorbitol as osmotic stabilizer. Fig. 1 shows that in all cases significant suppression of the phenotype was achieved, suggesting that mutant cells died due to their osmotic instability.

Electrophoretic analysis of proteins from the culture medium supported this finding, since in mutants with higher mortality more intracellular proteins were found in the medium (not shown). Thus, although the lack of single cell wall proteins showed no pronounced phenotype, mutations of entire protein families or groups of proteins affected the main physiological function of the wall, as observed by the staining of individual cells.

Under natural physiological conditions yeast cells probably stay in stationary phase for most of the time. Therefore, the importance of different groups of wall proteins for cell viability was also estimated when cells were allowed to enter the stationary phase of growth (typically after 48–100 h cultivation in YPD). As shown in Fig. 2, the mortality rate of *scw* mutants was only slightly increased in stationary cells. In contrast, the lack of Pir-proteins, as well as

Fig. 2. Mortality of different cell wall protein mutants grown to stationary phase. (a) Non-covalently linked proteins; (b) Pir-proteins; (c) GPI-anchored proteins. Open bars, –sorbitol; grey bars, +sorbitol.

GPI-anchored proteins, had a much more pronounced effect, leading to up to 25 % of dead cells in the culture. These results indicate that the functions of these proteins are related to this growth phase or at least that the disabilities of mutants become critical only when cells enter stationary phase. Pir-proteins belong to the same family and share high sequence homologies (Mrša *et al.*, 1997). They also seem to have similar physiological role(s) as indicated previously (Mrša & Tanner, 1999) and in this study by the cumulative effect of multiple mutations and by the fact that transformation of the multiple mutants with just one of the wild-type genes (*CCW5* expressed from a high-copy-number plasmid) significantly restored wild-type characteristics. In contrast, the selection of mutated GPI-anchored proteins was done simply by their relative abundance in the wall as they do not seem to belong to any particular protein family. Therefore, their individual contribution in the overall phenotype may be different. An example is *Ccw12p* which seems to have very little impact on exponential-phase cells but is very important for cells in the stationary phase.

Again, in all cases the addition of sorbitol repressed the phenotype, showing that the cause of death was osmotic instability of cells.

To prove that the observed phenotype was indeed a direct result of the depletion of wall proteins, the *ccw5ccw6ccw7* mutant was transformed with a plasmid carrying *CCW5*. As shown in Fig. 2(b) (*ccw5ccw6ccw7* + *CCW5*), significant reversion to wild-type characteristics was achieved, indicating that members of the Pir-family can functionally complement each other.

An interesting observation was made upon closer observation of mutants lacking Pir-proteins. In many cases,

particularly if cells were grown in minimal (yeast nitrogen base) medium, mother cells with two daughters attached could be seen. Usually, as shown in Fig. 3, both daughters stained with methylene blue, while the mother was still alive (in our laboratory jargon we called such structures ‘Mickey Mouse cells’ for reasons obvious in Fig. 3). No mothers with more than two daughters were observed in the culture and the phenomenon was specific for Pir-protein mutants. It was observed neither in other mutants nor in very rare cases when dead wild-type cells were found in the medium (Fig. 3). Staining with DAPI showed that all cells, including the dead daughters, contained nuclei, so death occurred at a later stage of development and was not connected to formation or transport of new chromosomes. The fact that mother and daughters of multiple Pir-protein mutants showed different staining indicated that the septum between the cells was already complete, preventing diffusion of mother enzymes to daughter cells, but detachment of daughters could not take place. CFW staining corroborated that since the chitin septum between the cells could clearly be seen and the staining pattern, as well as intensity, was very similar to that of the wild-type (not shown), no increase in the amount of chitin was observed in the mutant.

One of the apparent phenotypes of multiple *pir* mutants is clumpy growth. It can result from the inability of mature daughter cells to detach from the mothers, like in the *cts1* mutant (lacking chitinase), or it may be due to changed chemical properties of the cell surface, resulting in non-specific cell–cell interactions. To differentiate between these possibilities, the *ccw5ccw6ccw7ccw8* mutant was treated with small concentrations of Zymolyase, causing partial spheroplasting of cells (in a preliminary experiment the sensitivity of the wild-type and mutant to Zymolyase was tested,

Fig. 3. Wild-type and Pir-protein mutant stained with methylene blue.

but no difference was detected). This procedure efficiently broke the clumps into individual cells. The same effect was achieved with 3 M urea, or 0.5 % SDS. However, 'Mickey Mouse' structures withstood all of these treatments and were only disrupted when total protoplasting was accomplished.

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