**PST1** and **ECM33** encode two yeast cell surface GPI proteins important for cell wall integrity

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Pst1p was previously identified as a protein secreted by yeast regenerating protoplasts, which suggests a role in cell wall construction. ECM33 encodes a protein homologous to Pst1p, and both of them display typical features of GPI-anchored proteins and a characteristic receptor L-domain. Pst1p and Ecm33p are both localized to the cell surface, Pst1p being at the cell membrane and possibly also in the periplasmic space. Here, the characterization of pst1Δ, ecm33Δ and pst1Δ ecm33Δ mutants is described. Deletion of ECM33 leads to a weakened cell wall, and this defect is further aggravated by simultaneous deletion of PST1. As a result, the ecm33Δ mutant displays increased levels of activated Slt2p, the MAP kinase of the cell integrity pathway, and relies on a functional Slt2-mediated cell integrity pathway to ensure viability. Analyses of model glycosylated proteins show glycosylation defects in the ecm33Δ mutant. Ecm33p is also important for proper cell wall ultrastructure organization and, furthermore, for the correct assembly of the mannoprotein outer layer of the cell wall. Pst1p seems to act in the compensatory mechanism activated upon cell wall damage and, in these conditions, may partially substitute for Ecm33p.

**INTRODUCTION**

The cell wall is a highly dynamic structure that is required to maintain the osmotic integrity of fungal cells. It is also a determinant of cell morphology during vegetative growth or different developmental programmes, such as mating, sporulation and pseudohyphal growth. The fungal cell wall undergoes extensive changes, both in composition and shape, throughout the cell cycle and in response to different environmental cues. For this reason, it provides an excellent model to study cell morphogenesis (Cabib et al., 2001). In addition, due to its specificity to fungi, cell wall synthesis is an attractive target for selective antifungal therapy.

The fungal cell wall comprises three major polysaccharides: glucans, mannoproteins and chitin (Cid et al., 1995; Kapteyn et al., 1999a; Klis et al., 2002; Molina et al., 2000; Orlean, 1997). 1,3-β-glucan accounts for 40% of the dry weight of the cell wall, and together with chitin is responsible for the rigidity and integrity of the structure. This polymer can be linked by its non-reducing ends to 1,6-β-glucan and/or chitin (Kollár et al., 1995, 1997). 1,6-β-Glucan accounts for 10% of the cell wall dry weight and plays an important role in its organization, since it is the central molecule that links all the cell wall components together (Kapteyn et al., 1996, 1997; Kollár et al., 1997). Chitin represents only 2% of the cell wall dry weight, but is essential for cell wall integrity (Orlean, 1997). Mannoproteins contribute about 40% of the cell wall dry weight, and they constitute a filling material that is embedded in the glucan and chitin structural network. They can be extracted from the cell wall by different procedures, according to which they have been classified into two groups: (i) SDS- and reducing-agent-extractable mannoproteins are loosely associated with the cell wall or linked to this structure via disulfide bridges (Lipke & Ovalle, 1998); (ii) glucanase-extractable mannoproteins are covalently linked to cell wall glucans. Among these, a further distinction can be made. Glycosyl phosphatidylinositol (GPI)-dependent cell wall proteins are linked to 1,6-β-glucan via a GPI remnant (Kapteyn et al., 1996). In turn, this 1,6-β-glucan can be linked to 1,3-β-glucan or directly to chitin (Kapteyn et al., 1996, 1997; Kollár et al., 1997). PIR proteins (proteins with internal repeats) are covalently linked to 1,3-β-glucan (Kapteyn et al., 1999b). It has been speculated that this linkage could take place through their O-glycosidic chains, since PIR proteins can also be extracted from isolated cell walls by treatment with mild alkali, which breaks O-glycosidic bonds in a process called β-elimination (Mřáš et al., 1997), but recent data suggest...
that PIR proteins are attached to the cell wall via a transglutaminase-type reaction (Ecker et al., 2003).

In this work we have undertaken the characterization of two novel cell surface proteins involved in cell wall integrity which have been identified by in silico analysis as GPI proteins (Caro et al., 1997). Pst1p was identified as a protein secreted by Saccharomyces cerevisiae regenerating protoplasts (Pardo et al., 1999, 2000), that is, during the process of active construction of the cell wall, suggestive of its role in this event. Ecm33p is homologous to Pst1p, and a transposon insertion mutation in ECM33 has been shown to have cell-wall-related defects (Lussier et al., 1997). Here we show that the absence of Ecm33p, but not of Pst1p alone, leads to a defective cell wall with a compromised integrity. Moreover, simultaneous elimination of both proteins results in a more deleterious effect. Both Pst1p and Ecm33p localize to the cell surface, further supporting its role in cell wall maintenance or biosynthesis. We show also that ecn33Δ mutants have defects in protein glycosylation, as well as in mannoprotein anchoring to the polysaccharide fibrillar network of the cell wall. Whether these two roles are related remains to be determined.

METHODS

Plasmids and strains. Yeast strains and oligonucleotides used in this study are listed in Table 1 and Table 2, respectively. Plasmids generated in this study are listed in Table 3. The FY1679 ydr055w::KanMX4 disruptant (MY55) was obtained by gene replacement. The YDR055w/PST1 ORF plus adjacent sequences was amplified by PCR from strain FY1679 genomic DNA with primers UPA1 and LOA4 and subcloned into pUC19. Deletion of the YDR055w/PST1 ORF was accomplished by elimination of a StyI–StyI fragment and substitution for the KanMX4 module (Wach et al., 1994). Correct insertion of the cassette and deletion of the ORF were confirmed by PCR analysis of yeast colonies using two pairs of oligonucleotides binding either outside the target ORF or within the selection marker. PCR was carried out with the UPA1/K3 and LOA4/K2 pairs using Biotaq DNA Polymerase. ecn33Δ pst1Δ mutants were generated by genetic means, and the double deletion was confirmed by PCR.

Yeast genetics and phenotypic tests. Tetrad analyses were performed by standard micromanipulation procedures. Sonication tests were carried out as described previously (Ruiz et al., 1999). Sensitivities to Calcofluor White (CFW), Congo Red, hygromycin B, caffeine, sodium orthovanadate and SDS were tested by spotting cells onto plates. Exponential-phase cultures were adjusted to an OD600 of 0.5, and this sample plus three 10-fold serial dilutions was spotted onto yeast peptone glucose (YPD) plates supplemented with

Table 1. Yeast strains used in this study

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<th>Strain</th>
<th>Relevant genotype</th>
<th>Source/reference</th>
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<tr>
<td>FY679</td>
<td>MATα ura3-52, his3Δ200 trp1Δ63 leu2Δ1 ybi1·100 hsp150Δ:URA3</td>
<td>This work</td>
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<tr>
<td>FY679-28C</td>
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<td>B. Dujon, Institut Pasteur, Paris</td>
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Table 2. Oligonucleotide sequences

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<td>LOA4</td>
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<td>K2</td>
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<td>PNOT1</td>
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<tr>
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Table 3. Plasmids generated in this work

<table>
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<th>Plasmid</th>
<th>Vector</th>
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<td>YEp352</td>
<td>Contains PST1 ORF with upstream and downstream sequences</td>
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<tr>
<td>pCM190-PST1</td>
<td>pCM190</td>
<td>Contains PST1 ORF with downstream sequences after the tetracycline-repressible tetO promoter</td>
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<tr>
<td>pMIL1</td>
<td>YCplac111</td>
<td>Contains ps1 ORF (with the inserted NotI site) plus upstream and downstream sequences</td>
</tr>
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<td>pMIL4</td>
<td>YCplac111</td>
<td>Derived from pMIL1 by cloning of a NotI–NotI fragment containing 6 × c-myc in the NotI site</td>
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<td>pDR21</td>
<td>pRS316</td>
<td>Contains ecm33 ORF (with the inserted NotI site) plus upstream and downstream sequences</td>
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<td>pDR40</td>
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<td>Derived from pDR21 by cloning of a NotI–NotI fragment containing 6 × c-myc in the NotI site</td>
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the appropriate amount of the compound to be tested. Growth was scored after 2 to 3 days at 28 °C. The zymolyase sensitivity assay was performed as described previously (Lussier et al., 1997). The concentration of zymolyase 20T (ICN Biomedicals) was 12 µg ml⁻¹. Sensitivity to killer toxin K1 was tested as follows. S. cerevisiae killer strain T158C/S14a and strains to be tested were grown overnight to stationary phase. A 20 ml volume of methylene blue medium (3 % glucose, 1 % peptone, 1 % yeast extract, 2 % agar, buffered with 3 % sodium citrate, pH 4-7, and supplemented with 0-003 % methylene blue) maintained at 45 °C was inoculated with 15 µl of the culture to be tested, and poured into Petri dishes. Three 5 µl drops of the killer strain culture were spotted on each plate. Plates were incubated at 20 °C for 4 days, after which growth inhibition haloes were measured.

Cell wall association assays. Cell wall proteins were isolated essentially as described previously (Klis et al., 1998). Briefly, 50 ml cultures were grown in YPD medium to an OD₆₀₀ of 1. Cells were broken in a Fastprep fp120 (Bio 101) with the aid of 0-45 mm diameter glass beads (cell breakage was verified by phase-contrast microscopy). Cell walls were isolated by centrifugation, and the resulting pellet was washed six times with cold 1 M NaCl and four times with 1 M PMSF. Cell walls were extracted by boiling twice in SDS extraction buffer for 5 min. Only the first extract was kept, which was considered to be the SDS-extractable protein fraction. The pellet was then washed five times with water, and once more with 0-1 M sodium acetate, pH 5-2, 1 mM PMSF. Glucanase-extractable proteins were obtained by digestion of the resulting pellet with 0-5 mg zymolyase 20T ml⁻¹ in 10 mM Tris/HCl, pH 7-5, for 3 h at 37 °C.

Membrane association assays. Isolation of integral membrane proteins was carried out by solubilization with Triton X-114 (Serva) and subsequent phase separation (Bordier, 1981). The procedure used was that described previously by Condolmam et al. (1986, 1988).

Isolation of medium proteins. Medium proteins were isolated from 400 µl of an OD = 2 culture by TCA precipitation, as described by Klis et al. (1998).

Endoglucosaminidase H deglycosylation. N-deglycosylation of proteins was carried out by treatment with endoglucosaminidase H (Endo H; recombinant, Boehringer Mannheim) according to Klis et al. (1998).

Western blotting experiments. Western blotting was performed according to standard protocols (Ausubel et al., 1993). For Cwp1p and 1,3-β-glucan detection, filters were incubated in 50 mM sodium periodate, 100 mM sodium acetate, pH 4-5, for 30 min prior to the blocking step (Montijn et al., 1994). Cwp1p, 1,3-β-glucan and 1,3-β-glucan antisera were kindly provided by F. Klis. Gas1p antibodies were a gift from L. Popolo. p44/p42 (Thr202/Tyr204) MAP kinase antibodies were purchased from New England Biolabs. Pir2p antibody was a kind gift from M. Karakow. Cts1p antiserum was kindly provided by W. Tanner. Suc2p antibody was provided by S. Ferro-Novick. Carboxypeptidase Y (CPY) antiserum was a kind gift from M. Aebi.

Protein level quantification. Anti-phospho-p44/p42 MAPK (Thr202/Tyr204) antibody was used to detect dually phosphorylated Slt2p. To monitor the amount of Slt2p total protein, blots were stripped and reprobed with polyclonal anti-Slt2p antibodies (Martin et al., 1993). For protein quantification, blots were scanned with a GS900 Imaging Densitometer (Bio-Rad), and Slt2p–PP protein levels were quantified using the Molecular Analyst Software (Bio-Rad) and normalized to the total Slt2p protein level.

Construction of myc-tagged Pst1p and Ecm33p. A PST1–myc fusion was generated by modifying the coding sequence of PST1 by inserting a NotI restriction site via site-directed mutagenesis after base pair 442. Similarly, an ECM33–myc fusion was generated by modifying the ECM33 coding sequence by the insertion of a NotI restriction site via site-directed mutagenesis after base pair 92 of the exon. PCR products were verified by sequencing. Centromeric plasmids of myc-tagged PST1 and ECM33 under the control of their own promoters were constructed as follows. A PsrI–PsrI fragment containing the modified PST1 sequence with the NotI site plus upstream and downstream regulatory regions, was subcloned into YCplac111 to yield pMIL1. A SalI–SacII fragment containing the modified ECM33–NotI plus adjacent regions was subcloned into pRS316 to yield pDR21. A NotI–NotI DNA fragment encoding six copies of the c-myc epitope was obtained from p3291 (a kind gift from H. Martin) and ligated into the NotI restriction site in the modified PST1 and ECM33 sequences to yield in-frame fusions in plasmids pMIL4 and pDR40, respectively.

Indirect immunofluorescence. Cells were grown overnight in YPD medium to an exponential phase and processed according to the procedure described by Pringle et al. (1991). Pst1p–myc and Ecm33p–myc were detected by incubation with anti-c-myc monoclonal antibody 9E10 (BabCo) at 1 : 5000 dilution, and subsequent incubation with a Cy3-labelled anti-mouse IgG antibody (Sigma) at 1 : 1000 dilution. Samples were observed with a confocal MRC-1024 microscope (Bio-Rad).

Electron microscopy. Samples for transmission electron microscopy were prepared as described by Miret et al. (1992). Cells were observed with a JEOL JSM-6400 electron microscope.

RESULTS

Characteristics of Pst1p and Ecm33p

The gene product of ORF YDR055w was identified as a protein secreted by protoplasts of S. cerevisiae incubated under conditions of active regeneration of the cell wall (Pardo et al., 1999) and named Pst1p (protoplasts-secreted...
protein). Pst1p is a 444 amino acid protein with the typical features of GPI proteins targeted to the cell surface. It has an N-terminal signal peptide which is removed upon cleavage between Ala19 and Thr20 (M. Pardo, unpublished results), it is rich in serine and threonine, which are residues likely to be heavily O-glycosylated, and it displays a potential C-terminal domain for GPI anchor attachment. There are three other S. cerevisiae proteins that show significant degrees of similarity to Pst1p and display similar characteristics, the ECM33/YBR078W, SPS2/YDL052C and YCL048W gene products. The four proteins have been grouped in the so-called SPS2 family (Caro et al., 1997), named after the first described member. Overexpression of the 5’ end of SPS2 has been reported to inhibit sporulation (Percival-Smith & Segall, 1987). These proteins show the highest similarity in pairs: Pst1p is most similar to Ecm33p, showing 58% identity and 79% similarity over the whole protein; Pst1p shows 31% and 29% similarity to Sp2p and the product of YCL048w, respectively. Ecm33p is a 468 amino acid protein with the same features as Pst1p. Both proteins display a dibasic motif, which has been suggested to be a negative signal for incorporation of GPI proteins to the cell wall, upstream of the ω site for GPI anchoring (Caro et al., 1997; Hamada et al., 1998a).

Interestingly, both Ecm33p and Pst1p have a receptor L-domain in the N-terminal region (amino acids 50 to 91 in Ecm33p). This domain is characterized of some mammalian receptors, such as the type-1 insulin-like growth factor receptor (IGF-1R) and the insulin receptor (IR). Two L-domains from these receptors make up the bilobal ligand binding site (Garrett et al., 1998). The SPS2 family contains the only S. cerevisiae proteins where this domain has been detected.

The presence of proteins homologous to Ecm33p or to other members of the SPS2 family has been described in different fungal species. In CandidaDB, the Candida albicans genomics database (http://genolist.pasteur.fr/CandidaDB/), ECM33.3 and ECM33.1 have been identified as two genes homologous to ECM33. One of these genes codifies the Candida Pst1p homologue, which has recently been identified as a secretary protein in a heterologous genome-wide screen (Monteoliva et al., 2002). Similarly, at least two genes of this family have been detected in the Schizosaccharomyces pombe genome: SPAC23H4.19 and meu10. The latter is involved in the formation of the mature spore wall (Tougan et al., 2002). A random sequencing project of other species of hemiascomycetous yeasts has also identified sequences showing homology to both PST1 and ECM33 in several yeasts, namely Kluyveromyces lactis, Candida tropicalis, Pichia farinosa, Saccharomyces kluveri, Saccharomyces bayanus and Zygosaccharomyces rouxii (Souciet et al., 2000). Proteome analysis of GPI proteins from Aspergillus fumigatus has also identified a protein homologous to Ecm33p (Bruneau et al., 2001). This suggests that these two genes are conserved amongst fungi.

**Deletions of ECM33 and PST1 cause synergistic cell wall defects**

Interestingly, ECM33 was identified in a screen for transposon insertion mutants hypersensitive to the cell wall disturbing agent Calcofluor White (CFW; Lussier et al., 1997). To investigate the roles of PST1 and ECM33 in cell wall integrity, a search for cell wall-specific phenotypes was carried out on FY1679-derived strains with deletions in these genes. A haploid strain carrying a deletion in PST1 was constructed by replacing part of the ORF with the kan’ marker, and the ecm33Δ strain was obtained from EUROSCARF. ecm33Δ cells displayed several cell-wall-related phenotypes, as previously reported for the ecm33 transposon insertion mutation (Lussier et al., 1997). Deletion of ECM33 resulted in hypersensitivity to the cell wall disturbing agents CFW (Fig. 1a) and Congo Red, which interfere with the proper assembly of glucan and chitin microfibrils (Kopecka & Gabriel, 1992; Roncero & Durán, 1985). ecm33Δ was also hypersensitive to treatment with zymolyase, a mixture of cell wall hydrolytic enzymes. The incubation time needed to achieve a 40% decrease in optical density was 270 min for both wild-type and pst1Δ, whereas a similar effect was observed in the ecm33Δ strain after only 94 min of zymolyase treatment (Fig. 1b). K1 killer toxin binds to a 1,6-β-glucan and mannann receptor at the cell wall, and this binding is needed for toxin action (Bussey, 1991; Hutchins & Bussey, 1983). Mutants with altered levels of these polymers in the cell wall display different sensitivities to the toxin (Lussier et al., 1997). ecm33Δ was hypersensitive to K1 killer toxin. The diameter of the inhibition halo was 12 mm for the wild-type and pst1Δ strains, whereas ecm33Δ showed a halo of 15 mm. Deletion of ECM33 also resulted in hypersensitivity to hygromycin B (Fig. 1a), which is often related to glycosylation defects (Ballou et al., 1991; Dean, 1995; Kanik-Ennulat et al., 1995). We also found ecm33Δ to be hypersensitive to caffeine (Fig. 1a); sensitivity to caffeine was suppressible by osmotic stabilization of the medium with sorbitol (Fig. 1a), suggesting that the sensitivity observed was due to a defect in the cell wall (Ruiz et al., 1999). In all these assays, the pst1Δ mutant behaved as the wild-type strain (Fig. 1). Similar phenotypic tests conducted blindly in a ydr055wΔ strain generated within the EUROFAN project led to the same results (de Groot et al., 2001). In addition, deletion of ECM33, but not of PST1, resulted in sensitivity to sonication, which constitutes a mechanical stress to the integrity of the cell wall (Ruiz et al., 1999). The ecm33Δ mutant was three times more sensitive to this treatment than wild-type or pst1Δ mutant strains.

Interestingly, although no cell-wall-related phenotypes were observed in pst1Δ cells, ecm33Δ pst1Δ mutants were even more sensitive to some insults to the cell wall than the ecm33Δ single mutant. Mutants with deletions in both PST1 and ECM33 displayed a more severe sensitivity to sonication. ecm33Δ pst1Δ double mutants showed a 40% decrease in optical density only after 45 min of zymolyase treatment, that is, cell lysis occurred twice as quickly as in
the *ecm33*Δ single mutant (Fig. 1b). The double disruptants also showed enhanced sensitivity to K1 killer toxin (22 mm halo, compared to 15 mm in the *ecm33*Δ single mutant) and a slight but reproducible increase in sensitivity to hygromycin B. In contrast, sensitivity to CFW or Congo Red was similar in the *ecm33*Δ and *ecm33*Δ *pst1*Δ mutants (Fig. 1a).

All these phenotypes suggest that the *ecm33*Δ mutant has a weakened cell wall, which results in the sensitivity to the various cell wall damaging or disrupting agents. Although elimination of *PST1* by itself does not result in a detectable cell wall defect, it enhances some of the phenotypes in an *ecm33*Δ background. Given the high degree of similarity between Pst1p and Ecm33p, we tested whether overexpression of *PST1* could complement the phenotypes shown by the *ecm33*Δ mutant. High-copy plasmid expression of *PST1* under the control of its own promoter (YEp352-*PST1*) in an *ecm33*Δ background was not able to suppress the hypersensitivity to caffeine, CFW or Congo Red, whereas the sensitivity to hygromycin B and K1 killer toxin of the *ecm33*Δ mutant was partially alleviated (Fig. 1c). Transcription of *PST1* has been shown to vary along the cell cycle, whereas transcription of *ECM33* remains constant (Spellman et al., 1998). This difference in the transcription pattern could account for the observed lack of complementation. To rule out this possibility, *PST1* was introduced into the pCM190 plasmid and expressed under the control of the tetracycline-repressible *tetO* promoter, which induces high expression of the gene under its control (Gari et al., 1997). Overexpression of *PST1* in *ecm33*Δ cells growing in the absence of doxycycline showed essentially the same behaviour as high-copy plasmid expression with regard to sensitivity to the compounds cited above. These results demonstrate that high-level expression of *PST1* cannot fully compensate for the lack of Ecm33p, and although Pst1p and Ecm33p could have some similar or overlapping activities, they are not functionally redundant.

**The MAP kinase Slt2p is activated in *ecm33*Δ and *ecm33*Δ *pst1*Δ mutants**

Cell wall damage has been shown to trigger activation of the PKC pathway responsible for the maintenance of cell integrity and, as a consequence, to result in dual phosphorylation of Slt2p, the MAP kinase which mediates this signalling cascade (de Nobel et al., 2000). Slt2p activation was investigated in the deletion mutants *pst1*Δ, *ecm33*Δ and *ecm33*Δ *pst1*Δ by Western blotting with a phospho-p44/p42 MAP kinase antibody which recognizes the activated form of Slt2p (Martín et al., 2000). The strains were grown at 24 °C, a temperature at which only a basal level of Slt2p activation is usually detected in a wild-type strain (Martin et al., 2000). Under these conditions, the *pst1*Δ mutant was indistinguishable from the wild-type, but *ecm33*Δ showed a threefold increase in the levels of dual-phosphorylated Slt2p compared to the wild-type.
strain (Fig. 2). Moreover, these levels were increased sevenfold in the ecm33Δ pst1Δ double mutants (Fig. 2), indicative of an even more severe cell wall defect. The gas1Δ mutant was used as a control for Slt2p activation, showing a 10-fold increase in the levels of dual-phosphorylated Slt2p (Fig. 2, right-hand lane).

To determine if activation of the Slt2p-mediated signalling pathway is required for survival of the ecm33Δ mutant, we constructed a heterozygous ecm33Δ slt2Δ diploid and induced sporulation. Analysis of the spore progeny showed that simultaneous deletion of ECM33 and SLT2 results in synthetic lethality. A systematic lethal study has recently uncovered this relationship (Tong et al., 2004). This result implies that the ecm33Δ mutant relies on a functional Slt2p-mediated signalling cascade to ensure the maintenance of a partially functional cell wall, and hence viability. This situation is similarly encountered by other mutant strains with a compromised cell wall integrity, namely gas1Δ (Turchini et al., 2000), fks1Δ (Garrett-Éngele et al., 1995) and kre6Δ mutants (Roemer et al., 1994).

**ecm33Δ cells have a disorganized cell wall structure**

To investigate the morphology of the ecm33Δ cell wall in more detail, we observed these cells by thin-section electron microscopy. The yeast cell wall normally displays a homogeneous layered structure, with an electron-transparent inner layer (mainly 1,3-β-glucan and chitin) and an electron-dense outer layer, which corresponds to mannoproteins (Fig. 3a, top panel). ecm33Δ cells showed several cell wall ultrastructure alterations. The cell wall of ecm33Δ cells was irregular in thickness, and a clear disorganization of the layered structure was observed (Fig. 3a, bottom). The most severe defect was observed in the mannoprotein outer layer, which was very thin or even absent in many regions of the cell surface. The electron-transparent inner layer presented regions more transparent to electrons. We also found that the cell wall of ecm33Δ cells showed increased CFW staining compared to wild-type (Fig. 3b), suggesting that it is enriched in chitin, a phenotype often observed in cell wall-defective mutants, in which increased chitin deposition compensates for the weakened cell wall (Kapteyn et al., 1999a).

The defect in the mannoprotein outer layer might be due to a defect in its assembly to the cell wall structure. Several cell wall mutants defective in mannoprotein assembly secrete increased levels of cell wall components into the growth medium (Kapteyn et al., 1999b). We therefore explored whether this was the case in ecm33Δ and ecm33Δ pst1Δ cells. The culture supernatants were precipitated and analysed by Western blotting with antibodies against several cell wall components. Cwp1p was used as a model for GPI-anchored cell wall proteins. We only detected a slight increase in Cwp1p secretion to the culture medium in pst1Δ, ecm33Δ and ecm33Δ pst1Δ cells compared to the wild-type strain (Fig. 4). Pir2p was used as a model for PIR proteins, which are linked to the cell wall directly through 1,3-β-glucan (Kapteyn et al., 1999b). In wild-type and pst1Δ strains, a major band of 150 kDa was detected, the intensity of which was reduced in ecm33Δ and ecm33Δ pst1Δ double mutants. A slower minor form was increased in these mutants (Fig. 4). Therefore, there are both qualitative and quantitative changes in the secreted Pir2p in the ecm33Δ strains, which could be due to differential glycosylation or attachment to glucans.

We also observed an increased secretion of protein-linked 1,6-β-glucan in ecm33Δ and ecm33Δ pst1Δ mutants (data not shown). This result suggests that there is a defect in the linkage of 1,6-β-glucan-linked proteins to the cell wall, presumably to 1,3-β-glucan or chitin, leading to the presence of these proteins in the growth medium. Several other cell-wall-defective mutants have also been shown to secrete increased levels of mannoproteins and β-glucans into the culture medium (de Groot et al., 2001; Kapteyn et al., 1999b; Ram et al., 1998; Richard et al., 2002).

**ecm33Δ mutants show defects in protein N-linked glycosylation**

Yeast mutants with defects in protein glycosylation, either N- or O-glycosylation, or GPI synthesis exhibit hypersensitivity to the antibiotic hygromycin B, which is often accompanied by increased resistance to vanadate (Ballou et al., 1991; Dean, 1995). As described above, the ecm33Δ mutant displayed hypersensitivity to hygromycin B, and this phenotype was slightly enhanced in ecm33Δ pst1Δ double mutants (see Fig. 1a). In view of this sensitivity, we explored whether the deletion of ECM33 or PST1 affected glycosylation of proteins or not. Invertase was used as a model protein to analyse N-glycosylation. Secreted invertase contains 9 or 10 sugar chains on asparagine residues and
Western blot analysis of cell extracts showed that glycosylated invertase produced by *ecm33Δ* cells and by *ecm33Δ pst1Δ* double mutants migrated slightly faster than that of wild-type or *pst1Δ* cells (Fig. 5a). Wild-type invertase showed an *M*<sub>r</sub> between 140 and 240 kDa, while invertase produced by *ecm33Δ* and *ecm33Δ pst1Δ* double mutants migrated between 100 and 170 kDa. The difference in *M*<sub>r</sub> was due to N-linked sugars, since treatment of the samples with Endo H, which specifically removes N-linked sugar chains, yielded the same de-N-glycosylated species of 62 kDa for the invertase synthesized by both wild-type and mutant strains (Fig. 5b). The slight shift in *M*<sub>r</sub> between glycosylated invertase produced by wild-type and mutant strains suggested that it could be due to the presence of a shorter outer chain in the latter. We further investigated this by analysing another model glycoprotein, carboxypeptidase Y (CPY). Vacular CPY contains four N-linked oligosaccharides, which consist of a core part that is not elongated with an outer chain (Ballou, 1990). Western blotting experiments showed that all wild-type, *pst1Δ*, *ecm33Δ* and *ecm33Δ pst1Δ* mutants produced a predominant CPY glycoform of 61 kDa (Fig. 5c). This experiment confirms that transfer of the core part of N-linked sugars is not affected in the mutants tested. Therefore, these results suggest that elongation of the N-linked outer chain is partially defective in *ecm33Δ* and *ecm33Δ pst1Δ* mutants. To test if this was a general effect, a second protein was studied. Western-blot analysis of a cell surface protein fraction with Gas1p antibodies also showed a slight decrease in the *M*<sub>r</sub> of glycosylated Gas1p synthesized by *ecm33Δ* compared to wild-type cells (data not shown).

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**Fig. 3.** Deletion of *ECM33* alters cell wall structure and composition. (a) Electron microscopy analysis of the cell wall. Details of different zones of the *ecm33Δ* cell wall are shown. Arrows show regions more transparent to electrons. (b) Calcofluor White staining of wild-type (WT, top) and *ecm33Δ* mutant (bottom).

**Fig. 4.** Release of cell wall components into the culture medium. Culture supernatants were precipitated with TCA, and proteins were resolubilized in SDS loading buffer. Molecular mass (kDa) is shown to the right of the panels. WT, wild-type.
incorporation of GPI proteins to the cell wall (Caro et al., 1997). Specific amino acid residues at positions ω-2 and ω-5 have also been shown to determine incorporation of GPI proteins to the cell wall (Hamada et al., 1998a, 1999; Terashima et al., 2003). However, other studies have suggested that all GPI proteins are targeted to the cell wall (de Sampaio et al., 1999). Both Pst1p and Ecm33p contain the dibasic motif upstream of the ω site (Caro et al., 1997). A fusion protein containing the signal peptide from invertase, guar α-galactosidase, the HA epitope, and the last 40 amino acids of Pst1p has been shown to be extractable with phosphoinositol-phospholipase C from isolated membrane proteins, suggesting that it is attached to the cell membrane via GPI (Hamada et al., 1998b).

In order to examine their subcellular localization, both Pst1p and Ecm33p were tagged internally with six copies of the c-myc epitope. N- and C-terminal fusions of the tag were avoided in order to prevent elimination of the epitope by signal peptide or GPI anchor processing. Both fusion constructs were expressed from a centromeric vector under the control of their own promoters in the corresponding pst1Δ or ecm33Δ strains. To assess the functionality of Pst1p–myc, the fusion was expressed in the pst1Δ ecm33Δ double mutant, and the level of activated Slt2p was found to be the same as in the single ecm33Δ mutant (data not shown). Immunofluorescence studies on pst1Δ cells expressing the tagged protein showed that Pst1p–myc was found both in budded and unbudded cells, and localized mainly to the cell surface. In budded cells, Pst1p–myc was concentrated at the surface of the buds. Confocal planes photographed with a 0.5 μm Δz confirmed that distribution of Pst1p—myc-associated immunofluorescence was mainly at the cell periphery (Fig. 6).

Fractionation and Western blotting experiments were carried out to investigate the precise location of Pst1p. Cell walls were isolated from pst1Δ cells expressing Pst1p–myc, and SDS-extractable and glucanase-extractable proteins were obtained. Pst1p–myc was found to be present among the proteins extractable with SDS under reducing conditions (Fig. 7a), suggesting its localization at the cell wall as a non-covalently-bound protein or its linkage to this structure via disulfide bridges. We were not able to detect any zymolyase-extractable Pst1p–myc. Cwp1p, a glucanase-extractable GPI protein, was used as an internal control of the zymolyase-extracted fraction. Since some of the proteins extractable with SDS from isolated cell walls are actually plasma membrane constituents (Klis, 1994), we tested whether Pst1p–myc was present in cell membranes. Integral membrane proteins were isolated, and Pst1p–myc was detected in similar proportion both in the aqueous and in the detergent phases (Fig. 7b). These results suggest that Pst1p–myc is present both in the cell membrane and as a soluble protein, probably in the periplasmic space. Gas1p, a GPI protein localized at the plasma membrane, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a soluble protein, were used as controls for the purity of the detergent and aqueous fractions, respectively.

**Fig. 5.** Deletion of ECM33 affects N-glycosylation of proteins. Western blotting analysis of (a) invertase, (b) invertase after Endo H treatment, (c) carboxypeptidase Y, (d) chitinase. Labelling of lanes in (d) is as in panel (c). WT, wild-type.

Elongation of the outer chain starts in the Golgi where, in addition, some O-mannosylation occurs. To explore whether the ecm33Δ mutants suffered a general Golgi glycosylation defect or not, we also tested O-glycosylation of proteins by Western blotting using chitinase as a model protein. We did not detect any difference between chitinase secreted by wild-type cells and chitinase produced by ecm33Δ, pst1Δ and ecm33Δ pst1Δ mutants (Fig. 5d). Similar results were obtained with Pir2p (data not shown). Hence, the defect in the addition of sugar chains observed in the mutant strains affects only N-glycosylation and not O-glycosylation.

**Pst1p and Ecm33p localize to the cell surface**

In *S. cerevisiae*, GPI proteins can be localized not only at the plasma membrane, but also at the cell wall. A dibasic motif immediately upstream of the GPI anchor attachment site (ω site) has been proposed to be a negative signal for incorporation of GPI proteins to the cell wall (Caro et al., 1997). Specific amino acid residues at positions ω-2 and ω-5 have also been shown to determine incorporation of GPI proteins to the cell wall (Hamada et al., 1998a, 1999; Terashima et al., 2003). However, other studies have suggested that all GPI proteins are targeted to the cell wall (de Sampaio et al., 1999). Both Pst1p and Ecm33p contain the dibasic motif upstream of the ω site (Caro et al., 1997). A fusion protein containing the signal peptide from invertase, guar α-galactosidase, the HA epitope, and the last 40 amino acids of Pst1p has been shown to be extractable with phosphoinositol-phospholipase C from isolated membrane proteins, suggesting that it is attached to the cell membrane via GPI (Hamada et al., 1998b).

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**Fig. 6.** Cellular localization of Pst1p–myc. Indirect immunofluorescence was performed on *pst1Δ* cells bearing the pMIL4 plasmid. Confocal analysis of eight z slices (0–5 μm Δz) showed the preferential localization of Pst1p–myc to the cell surface.

![Image of cellular localization](image-url)

**Fig. 7.** Pst1–myc is present in cell wall and membrane fractions. Immunoblot analysis of cell wall (a) and membrane protein fractions (b) from cells expressing Pst1p–myc. T, total extract; A, aqueous phase; D, detergent phase; SDS, SDS-extractable cell wall protein fraction; ZYM, zymolyase-extractable cell wall protein fraction. For both panels, lanes labelled (−) correspond to the control plasmid pMIL1, and lanes labelled (+) correspond to the fusion construct plasmid pMIL4.

![Image of immunoblot analysis](image-url)
Several tagged versions of Ecm33p were constructed, but their expression failed to complement the hygromycin B and Congo Red phenotypes displayed by the ecm33Δ mutant. These chimeric proteins were thus judged non-functional and not used for further localization studies. Terashima et al. (2003) have recently shown that an HA-tagged version of Ecm33p which is able to restore growth at high temperature in ecm33Δ cells fractionates to the plasma membrane in a GPI-dependent manner.

**DISCUSSION**

**ecm33Δ and pst1Δ show synergistic cell wall defects**

Pst1p has been identified as a protein secreted by regenerating protoplasts of *S. cerevisiae* which shows the typical features of GPI proteins, suggestive of a role in cell wall construction (Pardo et al., 1999). Ecm33p displays the same features, and very high similarity to Pst1p, and both have been included in the SPS2 protein family, together with Sps2p and the product of the gene YCL048w (Caro et al., 1997). In this work, we have analysed the consequences of the loss of both Pst1p and Ecm33p. Deletion of *ECM33* resulted in several phenotypes indicative of a weakened cell wall, such as heightened sensitivities to different cell wall disturbing agents. By contrast, deletion of *PST1* alone did not result in any cell wall defect. Nevertheless, loss of Pst1p in an ecm33Δ background enhanced several of the phenotypes displayed by the single mutant, in particular the glucan- and mannan-related phenotypes, suggesting a more severe cell wall defect in the double mutant. Despite their high degree of similarity, *PST1* and *ECM33* do not appear to be redundant, since overexpression of Pst1p did not suppress all the phenotypes of ecm33Δ cells, but only slightly alleviated some of them. Taken together, all these results point to Ecm33p as a central protein in cell wall organization during vegetative growth, lack of which leads to diverse consequences related to all cell wall polymers. On the other hand, Pst1p, despite its sequence similarity, seems to have an important role in the compensatory mechanism.

Transcription of *PST1* is induced upon activation of the Slt2p-mediated MAP kinase cascade responsible for cell wall integrity (Jung & Levin, 1999). This signalling pathway is activated under various cell-wall-stressing conditions, triggering a compensatory mechanism to ensure cell wall integrity (Martin et al., 2000). Indeed, the ecm33Δ strain showed increased activation of the Slt2p-mediated cascade, in agreement with the observed cell wall defect. Furthermore, this integrity pathway proved to be required for viability of ecm33Δ, since simultaneous deletion of *ECM33* and *SLT2* resulted in synthetic lethality. Thus, the ecm33Δ mutant relies on a functional compensatory repair mechanism to ensure its survival. The enhanced severity of the phenotypes of the ecm33Δ pst1Δ double mutant compared to the single ecm33Δ mutant could therefore result from a partially defective repair mechanism. This points to Pst1p as one of the proteins involved in this repair mechanism activated in response to cell wall damage. This has also been suggested after genome-wide expression studies of several cell wall mutants. *PST1* transcription levels have been found to be increased in gas1Δ, mnn9Δ, fks1Δ, knr4Δ and kre6Δ mutants, and thus *PST1* has been included in the recently defined ‘cell wall compensatory-dependent gene’ cluster (Lagorce et al., 2003; Terashima et al., 1999).

Despite its putative role in the compensatory repair mechanism, high-copy-number expression of *PST1* was not able to suppress the synthetic lethality of *ECM33* and *SLT2* (M. Pardo, unpublished results). *CRH1*, another cell-wall-related protein-encoding gene (Rodríguez-Peña et al., 2000) whose transcription is also induced upon activation of the cell integrity pathway (Jung & Levin, 1999), did not suppress this lethality either (M. Pardo, unpublished results). Hence, individual downstream targets of the cell integrity pathway, or elevated levels of an individual cell wall protein, do not have the ability to compensate for the defect caused by loss of Ecm33p but, rather, a more complex response is needed to guarantee viability with a weakened cell wall.

Like ecm33Δ pst1Δ mutants, fks1Δ pst1Δ double mutants are fully viable, and so are gas1Δ pst1Δ and kre6Δ pst1Δ double mutants. Furthermore, deletion of *PST1* in an fks1Δ or gas1Δ background does not result in increased sensitivity to caffeine, CFW and Congo Red (M. Pardo, unpublished results).

**Ecm33p affects the anchoring of mannoproteins to the cell wall**

*ecm33Δ* showed an altered pattern of cell wall components secreted into the medium. Levels of 1,6-β-glucan linked proteins were increased in the *ecm33Δ* growth medium compared to those of the wild-type or *pst1Δ* strains, as observed by detection of 1,6-β-glucan and Cwp1p, a GPI-anchored protein that has been shown to carry 1,6-β-glucan side chains. This suggests a defect in the linkage of these proteins to the cell wall structure. Pir2p levels were also altered, with the ratio between a slower and a quicker form being reversed. This shift could be due to an increase in the amount of 1,3-β-glucan linked to Pir2p. The ecm33Δ mutant has also been shown by dot blot to secrete increased levels of 1,3-β-glucan into the growth medium (de Groot et al., 2001). Interestingly, Meu10p, a *Sch. pombe* homologue of the SPS2 family, seems to be required for proper 1,3-β-glucan localization in the spore wall (Tougan et al., 2002). In *meu10Δ* spores, 1,3-β-glucan is scattered throughout the cell wall instead of forming a well-defined inner layer. Several other cell-wall-defective mutants also show increased levels of mannoproteins and β-glucans in the culture medium (Kapteyn et al., 1999b; Ram et al., 1998; Richard et al., 2002), and in fact this phenotype has been used to identify putative assembly enzymes involved in cell wall construction (de Groot et al., 2001).
A major defect in the assembly of mannoproteins onto the cell wall structure was confirmed by electron microscopy, whereby the outer layer of mannoprotein was absent in some areas of the cell wall in ecm33A cells. ECM33 has been identified in a screen looking for genes required for proper apical growth (Bidlingmaier & Snyder, 2002). We also observed that cycling ecm33Δ cells looked rounded and more swollen that wild-type cells, probably a consequence of the inability of the weakened cell wall to maintain a proper morphology.

**Mutant ecm33Δ has N-glycosylation defects**

Hypersensitivity to the antibiotic hygromycin B, which is usually accompanied by resistance to vanadate, is a common phenotype amongst mutants with defects in any type of glycosylation (Dean, 1995). The ecm33A mutant showed hypersensitivity to hygromycin B, but did not display resistance to vanadate (our unpublished observations). This phenotype correlated with an increased mobility of the invertase produced by the mutant strain compared to that of the wild-type. The mobility shift was due to N-linked sugars, suggesting that ecm33Δ has a defect in N-glycosylation of proteins. Moreover, given that the addition of the core of N-linked sugars to CPY is not affected in ecm33A, we suggest that the defect is probably in the N-linked outer chain. In agreement with our results, ecm33Δ cells have lowered levels of sugar-linked phosphate at the cell surface (Conde et al., 2003). Conde et al. (2003) identified a variety of mutants with altered mannosylphosphate content in the cell wall which define genes acting in different cellular processes, suggesting that several pathways may be involved in modulating the amount of mannosylphosphate in the cell wall.

Ecm33p overexpression suppresses the temperature-sensitive growth phenotype of gpi13, las21/gpi7 and mcd4 mutants (Toh-E & Oguchi, 2002). The products of these three genes are involved in the transfer of ethanolaminephosphate to mannoses in the GPI structure. This suggests that Ecm33p might perform a similar biochemical function, maybe transferring a modification onto the mannose backbone of N-glycosylation chains. Although it is thought that GPI addition occurs in the Golgi, the bulk of Gpi7p is located at the plasma membrane. However, there is a smaller minor form in the endoplasmic reticulum, which transfers phosphatidylinositol to the second mannose of GPI (Benachour et al., 1999). We have observed that Ecm33p also has two forms, a major high-molecular-mass band and a minor smaller one (M. Pardo, unpublished results). The minor form of Ecm33p could be involved in some kind of group transfer in early secretory compartments. Alternatively, suppression could be due to an unrelated activity that compensates the GPI defects of the mutants in an indirect way. The correct O-glycosylation of two model proteins, Cts1p and Hsp150p, ruled out the existence of a general Golgi glycosylation defect in the ecm33A mutant, such as that exhibited by mnn1, mnn3 or kre2 mutants, which are defective in both N- and O-glycosylation (Orlean, 1997). Therefore, ECM33 seems to be involved, directly or indirectly, in the correct formation of N-linked sugar outer chains.

Since mannoproteins account for a significant proportion of the cell wall dry weight, and since some cell wall biosynthetic enzymes are N-glycosylated, the failure of their anchoring to the cell surface could lead to a defective cell wall. This is also the case with other mutants with defects in N- and/or O-glycosylation, which also display cell wall defects (Orlean, 1997). Some evidence has arisen which supports the attachment of 1,6-β-glucan to protein N-glycosyl chains (reviewed by Shahinian & Bussey, 2000). It is possible that the defects in N-glycosylation displayed by ecm33Δ have an effect on the anchoring of some proteins to 1,6-β-glucan, the central core of the cell wall network, and thus on their assembly into the cell wall and the integrity of this structure (Chavan et al., 2003).

**Pst1p and Ecm33p localize at the cell surface**

The protein sequences of Pst1p and Ecm33p show features that lead to the prediction for both of a cell surface localization either at the cell wall or at the plasma membrane. Confocal microscopy analysis of pst1Δ cells expressing a tagged version of Pst1p showed that Pst1p–myc localized primarily to the cell surface. In budded cells, Pst1p–myc was concentrated in the buds. Since this is where active cell wall construction is taking place, Pst1p may be needed at the bud to ensure cell wall integrity as the cell wall undergoes some degradation to allow new cell wall material to be added. PST1 does not seem to be specifically expressed in daughter cells (Colman-Lerner et al., 2001), suggesting that another mechanism must be responsible for the preferential localization of Pst1p at the buds. Pst1p–myc was extractable from isolated cell walls with SDS and a reducing agent, which means that it could be linked to the cell wall structure via non-covalent associations or disulfide bridges to other cell wall proteins. Pst1p–myc was also found in the detergent phase after extraction of membrane proteins, which demonstrates that it is an integral membrane protein. All these data are in agreement with the fractionation data of Hamada et al. (1998b) for a fusion protein containing a signal peptide from invertase, guar α-galactosidase, the HA epitope and the last 40 amino acids from Pst1p. Summing up, Pst1p–myc is localized at the cell surface as a membrane protein, possibly via its GPI moiety, and at the periplasmic space as a soluble protein, probably loosely associated with the cell wall.

Several N-terminal-tagged versions of Ecm33p were constructed, but they did not complement the phenotypes of the ecm33A mutant. This lack of functionality could be due to improper localization of the fusion proteins. Immunofluorescence analysis of ecm33A cells expressing Ecm33p–myc showed that the fusion protein was distributed in the cytoplasm, in contrast to the predicted cell surface localization (our unpublished observations). Another possibility is that insertion of the epitope either prevents the proper
folding of the nearby L domain or interferes with a putative catalytic site. Two different HA-tagged versions of Ecm33p have been shown to be localized to the nuclear rim and ER and to the cell periphery (Kumar et al., 2000; Ross-Macdonald et al., 1999), which together with the prediction by the protein sequence strongly suggests that the final localization of Ecm33p is the cell periphery. It is noteworthy that these clones carry the epitope in the C-terminal region of the protein, suggesting that the N-terminal region, where our non-functional construct bore the epitope, might be important for the localization and/or function of Ecm33p.

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Yeast GPI proteins involved in cell wall integrity


