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Identification of Three Mannoproteins in the Cell Wall of Saccharomyces cerevisiae

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Three glucanase-extractable cell wall proteins from Saccharomyces cerevisiae were purified, and their N-terminal amino acid sequences were determined. With this information, we were able to assign gene products to three known open reading frames (ORFs). The N-terminal sequence of a 55-kDa mannoprotein corresponded with the product of ORF YKL096w, which we named CWP1 (cell wall protein 1). A 80-kDa mannoprotein was identified as the product of the TIP1 gene, and a 180-kDa mannoprotein corresponded to the product of the ORF YKL444, which we named CWP2, CWP1, TIP1, and CWP2 encode proteins of 239, 210, and 92 amino acids, respectively. The C-terminal regions of these proteins all consist for more than 40% of serine/threonine and contain putative glycosylphosphatidylinositol attachment signals. Furthermore, Cwp1p and Tip1p were shown to carry a β1,6-glucose-containing side chain. The cwp2 deletion mutant displayed an increased sensitivity to Congo red, calciofluor white, and Zymolyase. Electron microscopic analysis of the cwp2 deletion mutant showed a strongly reduced electron-dense layer on the outside of the cell wall. These results indicate that Cwp2p is a major constituent of the cell wall and plays an important role in stabilizing the cell wall. Depletion of Cwp1p or Tip1p also caused increased sensitivities to Congo red and calciofluor white, but the effects were less pronounced than for cwp2Δ. All three cell wall proteins show a substantial homology with Srp1p, which also appears to be localized in the cell wall. We conclude that these four proteins are small structurally related cell wall proteins.

The two major components of the cell wall of the yeast Saccharomyces cerevisiae are glucan, which constitutes the inner layer of the cell wall, and mannoproteins, which are embedded in and cover this glucan layer. Chitin is a minor component of the cell wall (13, 18). The mannoproteins can be divided into two groups, the sodium dodecyl sulfate (SDS)-extractable mannoproteins and the glucanase-extractable mannoproteins, which are solubilized by glucanase digestion of the glucan layer. Several glucanase-extractable mannoproteins have been identified. These proteins have two characteristics in common: their C-terminal regions are rich in serine and threonine, and they all contain putative glycosylphosphatidylinositol (GPI) attachment signals. Two of these proteins, α-agglutinin (22) and the core subunit of a-agglutinin (33), are involved in mating. The third, which is involved in flocculation, is the product of the FLO1 gene (42). Because of the high serine and threonine content of their C-terminal regions, these proteins are probably heavily O glycosylated, which could give the protein a rod-like structure (15). The presence of a GPI anchor has been demonstrated only for the intracellular precursor form of α-agglutinin (47). The glucanase-extractable mannoproteins are proposed to be covalently linked to glucan (29, 38, 45). Several groups have investigated which part of the protein is responsible for anchoring the protein to the cell wall. Schreuder et al. (36) showed that a fusion protein consisting of the invertase signal sequence, the guar α-galactosidase coding sequence, and the C-terminal half of α-agglutinin was incorporated into the cell wall and was released only by glucanase extraction of isolated cell walls. In contrast, a control protein lacking the α-agglutinin part was released into the medium. Recently, Van Berkel et al. (44) showed that in a similar construct, addition of only the C-terminal 30 amino acids of α-agglutinin is sufficient for incorporation of the chimeric protein into the cell wall. Thus, the C-terminal stretch of 30 amino acids of α-agglutinin contains all information necessary to incorporate a protein into the cell wall. In a second experiment, Wojciechowicz et al. (47) showed that removal of the C-terminal hydrophobic sequence allowed secretion of active α-agglutinin in the medium. This finding indicates that a GPI attachment signal is essential for incorporation of a protein into the cell wall. However, the proteins encoded by KRE1 (4) and GPP1 (30) are both GPI anchored but are not known to be associated with the cell wall; thus, it seems that a GPI anchor alone is not sufficient to locate a protein in the cell wall. It is not clear in which way the proteins are covalently linked to the cell wall. Recently, a carbohydrate side chain containing β1,6-glucose residues has been shown to be attached to the glucanase-extractable proteins (24, 45), and it has been suggested that this chain is involved in cell wall anchorage (8, 44).

To gain further insight into the yeast cell wall structure, we aimed to isolate the major glucanase-extractable cell wall proteins of S. cerevisiae. Here we report the identification of three open reading frames (ORFs) encoding glucanase-extractable cell wall mannoproteins. They all have a serine- and/or threonine-rich C-terminal domain and contain putative GPI attachment signals. The effect of deletion of these cell wall protein genes was investigated.
In addition to their described by Becker and Guarente (2). In addition to their described by Sambrook et al. (35). Propagation of plasmids, and competent cells were prepared by the method of N-terminally sequenced, as a soluble sample, by the Edman protocol, the cells were incubated for 10 min at 30°C in a freshly prepared N,N-diethythiolethanic acid (HEDPS)-dithiothreitol buffer (20 mM HEDES and 25 mM dithiothreitol in YEPD) prior to the first washing step. Furthermore, cells were resuspended in ice-cold 10% glycerol instead of 1 M sorbitol. For each electrophoresis (800 V, 25 μF, and 1.1 kV), 50 μl of the suspended cells was mixed with a minimal volume of DNA (2 to 7 μl). Transformsants were selected on MM plates lacking leucine. For chromosomal DNA isolation, S. cerevisiae strains from a 4-ml culture were harvested 17 h after inoculation, and total DNA was isolated as described by Johnston (16).

**PCR amplification.** The PCR amplification (Perkin-Elmer Cetus DNA Thermal Cycler) was carried out in 100 μl of 10 mM Tris-HCl pH 8.3–50 mM KCl–1.5 mM MgCl₂–0.001% gelatin, with 0.2 mM each dNTP, trichloroacetic acid, 100 pmol of the DNA oligonucleotides, ca. 0.25 μg of HindIII-digested chromosomal DNA of strain TM2683-20 (or 2 ng of plasmid DNA), and 1 U of AmpliTaq polymerase. In PCRs using degenerate primers, a ramp time of 2 min between the annealing temperature (1.5 min at 37°C) and the extension temperature (1 min at 72°C) was used in the first three cycles. The following 27 cycles were for 1 min at 95°C, 1 min at 50°C, and 0.5 min at 72°C. In the second round of PCR with degenerate primers, 3 cycles of 1 min at 95°C, 1 min at 50°C, and 0.5 min at 72°C were performed. In PCRs creating deletion fragments, incubation parameters were set as follows: 25 cycles of 1 min at 95°C, 1.5 min at 54°C, and 2 min at 72°C.

**Construction of deletion fragments.** All deletions were created by splicing by overlap extension as described for the construction of hybrid genes by Horton et al. (14). For each deletion fragment, four oligonucleotides (Table 1) were designed to amplify approximately 500 bp on each side of the coding sequence of the ORF of interest in the left-hand fragment or in the 5′-oligonucleotide of the right-hand fragment were complementary over a 20-bp stretch, and this sequence contained a BamHI recognition sequence. One picomole, instead of 100 pmol, of each of these two oligonucleotides was added during the PCR. The 5′ oligonucleotide of the left-hand fragment contained an EcoRI recognition sequence, and the 3′ oligonucleotide of the right-hand fragment contained a HindIII recognition sequence. As CWPI is located directly upstream of CWP2 on chromosome XI, it was also possible to create a double mutant (cwp2 Δ cwp2Δ) in one replacement. For this purpose, a hybrid fragment of the left-hand fragment of CWPI and the right-hand fragment of CWP2 was created. After PCR amplification, the hybrid fragments containing the left- and right-hand fragments were cloned into EcoRI-HindIII-digested pTZ19R (23). The LEU2 gene, isolated from YEP-D (3) by BamHI digestion, was cloned into the BamHI site of the resulting plasmids. Deletion fragments were amplified from plasmid with the 5′ oligonucleotide of the left-hand fragment and the 3′ oligonucleotide of the right-hand fragment and used for one-step gene disruption of S. cerevisiae (32).

**Analysis of deletion mutants.** Prior to the analysis, the disruptions of the ORFs in S. cerevisiae were confirmed by Southern analysis (39) (data not shown). Congo red and calcofluor white sensitivity was monitored by streaking out cells, grown in 2-hydroxyethylpiperazine-N,N′-ethanesulfonic acid (HEPES)-dithiothreitol buffer (20 mM HEDES and 25 mM dithiothreitol in YEPD) for 17 h after inoculation, and total DNA was isolated as described by Johnston (16).

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cells) of Zymolyase 20T per ml. Subsequently, the decrease of the OD$_{600}$ was monitored for several hours.

**Electron microscopy.** Cells were grown in MM (OD$_{600}$ = 0.5), harvested, washed two times with distilled H$_2$O, fixed in 1.5% KMnO$_4$, and incubated at room temperature for 20 min. Fixed cells were embedded in low-melting-point agar and subsequently immersed in 30% aqueous N,N-dimethylformamide for 30 min and cryofixed in liquid propane. The frozen cells were freeze-substituted for 2 days at −90°C in anhydrous methanol (CS auto; Reichert-Jung). After the temperature was gradually increased (5°C/h) to room temperature, the methanol was replaced by anhydrous acetone and infiltrated with Spurr’s epoxy resin (40). After 24 h of polymerization at 60°C, 80-nm sections were cut with a diamond knife on an ultramicrotome (Reichert-Jung). Sections were mounted on 0.7% agar and subsequently immersedin30% aqueous N$_2$-dimethylformamide for 30 min and cryofixed in liquid propane. The frozen cellswere freeze-substituted for 2 days at −90°C in anhydrous methanol (CS auto; Reichert-Jung). After the temperature was gradually increased (5°C/h) to room temperature, the methanol was replaced by anhydrous acetone and infiltrated with Spurr’s epoxy resin (40). 24 h of polymerization at 60°C, 80-nm sections were cut with a diamond knife on an ultramicrotome (Reichert-Jung). Sections were mounted on 0.7% pialloform-coated, carbon-evaporated one-hole copper grids and dried for 16 h. Subsequently, the sections were viewed on a Philips EM420 electron microscope at an operating voltage of 80 kV. Fifty independent measurements of the thickness of the electron-dense outer layer of the cell wall were carried out on median sections.

**RESULTS**

Isolation and N-terminal sequencing of glucanase-extractable mannoproteins. It has been demonstrated that the glucanase-extractable mannoproteins often contain a carbohydrate side chain containing β1,6-glucose residues (24, 45). A polyclonal antiserum raised against β1,6-glucan (provided by Roy Montijn, University of Amsterdam) recognized four glucanomannoproteins released from the cell walls by the mollusk laminarinase extraction procedure. These four protein bands were excised from the Western blot and N-terminally sequenced.

The N-terminal sequence of the protein band with an estimated molecular mass of 55 kDa (Table 2) proved to be 100% homologous with the YKL096w gene product present in the yeast DNA sequencing data bank. This ORF, previously sequenced by Pallier et al. (28), is located on chromosome XI and represents a transcript capable of encoding 239 amino acids. It contains a putative GPI attachment signal at the C-terminal part of the protein, is rich in serine and threonine (11%). A signal sequence is present at the N-terminus, as the determined N-terminal sequence starts exactly after the predicted signal peptidase cleavage site (46). We named this gene CWP1 (cell wall protein 1).

The N-terminal sequence of the protein band of approximately 80 kDa (Table 2) corresponded with the TIP1 gene product, identified by Kondo and Inouye (19) as a temperature-inducible protein by differential gene expression analysis. This protein consists of 210 amino acids and contains a putative GPI attachment signal, and the C-terminal part of the protein is rich in serine (35%) and threonine (11%). A signal sequence is present at the N-terminus, as the determined N-terminal sequence starts exactly after the predicted signal peptidase cleavage site (46). We named this gene CWP1 (cell wall protein 1).

When the SDS-extracted cell walls were digested with laminarinase isolated from *Trichoderma* species, one predominant band of 180 kDa, which did not react with the anti β1,6-glucan antiserum, was detectable by silver staining (data not shown). This protein band was also N-terminally sequenced (Table 2). No striking homology was found between this sequencing and proteins in the *S. cerevisiae* gene bank. On the basis of this sequence, degenerate probes were designed for PCR. The sense primer 5' CA AAG CTT GCT GCT/C ATT GCT/C CA 3' was based on amino acids 4 to 8 and contained an additional HindIII restriction site. The antisense primer 5' TC GAA TTC AC TGG A/GGC A/GGC 3' was based on amino acids 28 to 31 and contained an additional EcoRI restriction site. The PCR gave, among others, a product of the predicted size which hybridized with an internal oligonucleotide (5' GAT GGT CAA ATT/C INN GCT GCT 3') based on amino acids 11 to 17. The PCR product was isolated and used as a template for a second round of PCR. The products of this second amplification were cloned in puC18, and the inserts of several plasmids were shown to hybridize with the internal oligonucleotide. One such clone had the capacity to code for an amino acid sequence identical to the N-terminus of the protein encoded by a small ORF, YKL444 (CWP2), sequenced by Pallier et al. (28). In many cases, threonine residues deduced from the DNA sequence had been misidentified during the amino acid analysis, but closer inspection of the amino acid sequence data showed small threonine peaks in these places. Misidentification of these amino acids is possibly caused by glycosylation of the threonine residues. The ORF CWP2 is located on chromosome XI, codes for a protein consisting of 92 amino acids, has a putative GPI attachment signal, and is rich in serine (15%) and threonine (26%) in the C-terminal part. The determined N-terminal sequence starts at the predicted site for signal sequence cleavage; thus, a signal sequence is also present on this protein. This ORF was not included in the analysis of chromosome XI, as it encodes a protein of less than 100 amino acids.

**Structural analysis of the deduced protein sequences encoded by CWP1, TIP1, and CWP2.** Translation of the three identified ORFs into their amino acid sequences reveals that they have several characteristics in common. First, they all contain an N-terminal signal sequence for import into the endoplasmic reticulum (46). Second, their C-terminal halves are all rich in serine and/or threonine, which implies that these domains are highly O glycosylated. For the cell wall protein α-agglutinin, Wojciechowicz et al. (47) postulated that this structure facilitates the exposure of the N-terminal binding domain on the surface of the cell wall. Third, the deduced C-terminal amino acid sequences of these ORFs contain putative GPI attachment signals. Nuoffer et al. (27) analyzed the sequence requirements for peptide cleavage and glycolipid addition for Gas1p of *S. cerevisiae* and showed that asparagine is the most efficient anchor attachment site and that the two adjacent amino acids to the carboxyl-terminal should have relative short side chains. The deduced protein sequences of Cwp1p, Tiplp, and Cwp2p all contain an asparagine followed by glycine and alanine near the carboxyl termini (Fig. 1); the necessary polar and hydrophobic regions (6, 7) are also present, and although no specific sequence is required (5), we
find a high degree of homology between the hydrophobic regions (Fig. 1).

**Disruption of the CWP1, TIP1, CWP2, and SRP1 genes.** Disruption mutations in the newly identified ORFs were constructed as described in Materials and Methods. To confirm that each identified ORF encodes the mannoprotein that was N-terminally sequenced, glucanase-extractable mannoproteins were isolated from cell walls of each deletion mutant. Comparison of the protein patterns of the parent strain and the deletion mutants confirmed that the identified ORFs code for the expected glucanase-extractable mannoproteins. In a Western analysis with the anti-1,6-glucan antiserum of the Tip1p gene product has high homology with Tip1p (19), the parent strain and the mutant strain were subjected to a cold shock. (B) Endoglycosidase H-treated mannoproteins isolated from SU50 cwp2Δ and the parent strain were separated on an SDS–2 to 20% polyacrylamide gel and silver stained to reveal the proteins.

**TABLE 3. Phenotypic characteristics of exponentially growing cells of the deletion mutants**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sensitivity to:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Calcofluor white</td>
</tr>
<tr>
<td>Wild type</td>
<td>++</td>
</tr>
<tr>
<td>cwp1Δ</td>
<td>++</td>
</tr>
<tr>
<td>cwp2Δ</td>
<td>+++</td>
</tr>
<tr>
<td>cwp1Δ cwp2Δ</td>
<td>+++</td>
</tr>
<tr>
<td>tip1Δ</td>
<td>++</td>
</tr>
<tr>
<td>srp1Δ</td>
<td>++</td>
</tr>
</tbody>
</table>

* +, inhibitory concentration = 0.0 mg/ml; ++, inhibitory concentration = 1.0 mg/ml; ++++, inhibitory concentration = 1.0 mg/ml.

* Zymolyase concentration was 30 μg/ml; incubation lasted for 2.5 h. wt, wild-type sensitivity; sensitive, increased sensitivity relative to the wild type.
layer of cells of the cwp2Δ mutant strain and cells of the parental strain was observed (data not shown).

The deletion mutants did not show increased sensitivity to low pH (pH 2 to 4) or high salt concentrations (NaCl, 5 to 20%) (data not shown).

DISCUSSION

Structure of CWP1, TIP1, CWP2, and SRP1 and their deduced protein sequences. In this report, three ORFs encoding related glucanase-extractable mannoproteins have been identified. Translation of these ORFs into their deduced amino acid sequences revealed that they share several characteristics which are also present in the deduced amino acid sequence of SRP1, suggesting that Srp1p is a potential cell wall protein. For this reason, Srp1p was included in our analysis. The similarities between Tip1p and Srp1p have already been described by Kondo and Inouye (19). In the N-terminal signal sequences of Cwp1p and Cwp2p, there is a very homologous stretch of 15 amino acids. Furthermore, the element ISQIT DGQIQA is found in Cwp1p (amino acids 197 to 207), Cwp2p (amino acids 26 to 36), and Srp1p (amino acids 213 to 223). This element displays a high degree of similarity with the 11-amino-acid tandem repeats found in the secretory glycoprotein encoded by the HSP150 gene (34). The C-terminal 25 amino acids of Cwp1p, Cwp2p, Srp1p, and the C-terminal 12 amino acids of Tip1p containing (part of) the GPI attachment signal are also very homologous. The four most C-terminal amino acids, predicted to remain on Cwp1p, Cwp2p, Srp1p, and Tip1p (QTEN) after cleavage of the GPI attachment signal, might act as an element directing proteins to their correct place in the cell wall. Munoz-Dorado et al. (25) identified two elements (ATTTGG) in the promoter region of TIP1 which are located in the region responsible for induction of the gene after a cold shock. In E. coli, this DNA sequence is recognized by a cold shock protein (CspA [17]). The promoter region of the CWP1 gene also contains two of these elements (positions −334 and −669 relative to ATG).
Comparison of the molecular weight of Cwp2p determined by SDS-PAGE and its predicted molecular weight, calculated from the deduced amino acid sequence of the mature protein, reveals a substantial difference. This discrepancy cannot be accounted for by glycosylation, as Cwp2p contains no consensus sequence for N glycosylation and even O glycosylation of all serine and threonine residues would lead to a lower molecular weight than observed by SDS-PAGE. Furthermore, the Cwp2p mannoprotein was not recognized by the β,1,6-glucan antiserum, indicating that a β,1,6-glucose-containing side chain is not present on this protein. This implies that the mannoprotein results from a change in the cell wall structure and aggravates the effects of cell wall defects (11, 20, 31), increased sensitivities toward both compounds were found. The fact that the cwp2Δ mutant is more sensitive to these compounds than the other three mutants could be explained by a greater abundance of Cwp2p in the cell wall, causing a greater perturbation to the cell wall upon deletion.

Functional analysis of deletion mutants. The cwp1Δ, tip1Δ, cwp2Δ, and srr1Δ deletion mutants grew as well as the parent strain in both minimal and rich media. Under these conditions, the products of the deleted genes are therefore not essential for growth. However, when the deletion mutants were grown in the presence of calcifluor white or Congo red, both of which cause a change in the cell wall structure and aggravate the effects of cell wall defects (11, 20, 31), increased sensitivities toward both compounds were found. The fact that the cwp2Δ mutant is more sensitive to these compounds than the other three mutants could be explained by a greater abundance of Cwp2p in the cell wall, causing a greater perturbation to the cell wall upon deletion.

Zlotnik et al. (49) showed that lysis of the cell wall of S. cerevisiae with Z-glucanase requires a previous incubation with Z-protease. This finding implies that the mannoproteins are involved in protecting the structural glucan from the action of the glucanase. Incubation of the deletion mutants with Zymolase, which contains Z-glucanase and Z-protease, showed an increased sensitivity of the cwp2Δ mutant and the cwp1Δ cwp2Δ double mutant in exponentially growing cells. Electron microscopic analysis showed that the depletion of Cwp2p corresponds with a considerable decrease in thickness of the electron-dense layer around the glucan layer in this mutant. In the other deletion mutants, no increased Zymolase sensitivity was found. On the basis of their sequence similarity, it is possible that these proteins can substitute for each other’s function. Another possibility is that these cell wall proteins are less important for the stability of the cell wall. Stationary-phase cells of the mutants showed no enhanced Zymolase sensitivity. This could imply that other cell wall proteins become more abundant in the stationary phase.

Through analysis of cell wall glucanmannoproteins, we have been able to assign gene products to two ORFs identified in the yeast genomic sequencing program. In addition, we have identified the TIP1 gene product as a cell wall component. These genes encode cell wall proteins in S. cerevisiae.

ACKNOWLEDGMENTS

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