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DOI
10.1128/jb.178.4.1162-1171.1996

Publication date
1996

Published in
Journal of Bacteriology

Citation for published version (APA):
CWH41 Encodes a Novel Endoplasmic Reticulum Membrane N-Glycoprotein Involved in β1,6-Glucan Assembly

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Received 12 September 1995/Accepted 7 December 1995

CWH41 encodes a novel type II integral membrane N-glycoprotein located in the endoplasmic reticulum. Disruption of the CWH41 gene leads to a K1 killer toxin-resistant phenotype and a 50% reduction in the cell wall β1,6-glucan level. CWH41 also displays strong genetic interactions with KRE1 and KRE6, two genes known to be involved in the β1,6-glucan biosynthetic pathway. The cwh41Δ kre6Δ double mutant is nonviable; and the cwh41Δ kre1Δ double mutation results in strong synergistic defects, with a severely slow-growth phenotype, a 75% reduction in β1,6-glucan level, and the secretion of a cell wall glucomanoprotein, Cwp1p. These results provide strong genetic evidence indicating that Cwh41p plays a functional role, possibly as a new synthetic component, in the assembly of cell wall β1,6-glucan.

KRE5 encodes a putative soluble endoplasmic reticulum (ER) protein that has extensive sequence homologies with a Drosophila ER glucosyltransferase (32, 37). The kre5Δ-null mutant failed to make any detectable amount of the polymer, indicating that Kre5p is essential for β1,6-glucan synthesis. Kre6p and Skn1p are a pair of functional homologs (43–45). They are both type II integral membrane glycoproteins, and the Kre6p protein has been localized in the Golgi apparatus. The C-terminal domains of Kre6p and Skn1p display significant sequence similarities to two glucan-binding proteins. Deletion of both KRE6 and SKN1 genes caused a major reduction (70 to 80%) in β1,6-glucan levels, consistent with both Kre6p and Skn1p playing direct functional roles in the synthesis of β1,6-glucan. KRE1 codes for a serine- and threonine-rich protein located on the cell surface (1). Disruption of the KRE1 gene leads to a 40% reduction in the level of β1,6-glucan. Polysaccharide structural analysis indicated that the β1,6-glucan made by the kre1Δ-null mutant had a reduced polymer size and contained fewer β1,6-linked residues than did the wild-type polymer. These results suggested that Kre1p may play a role to add or extend β1,6-linked side chains at the cell surface. KRE9 is the structural gene for a small serine- and/or threonine-rich protein (2, 3). Loss of function of the KRE9 gene led to a dramatic reduction (80%) in β1,6-glucan level, indicating that Kre9p plays an important role in the synthesis of the polymer. The Kre9p protein is O glycosylated, and when overproduced, it can be detected in the extracellular medium. KRE11 codes for a 63-kDa cytosolic protein, and disruption of the gene caused a 50% reduction in β1,6-glucan level (3). Based on the genetic analysis and molecular characterization of these KRE genes, a working model for β1,6-glucan synthesis has been proposed (3, 44). This model suggests that the polymer is made within the secretory pathway in a stepwise process which includes a Kre5p-dependent ER step involved in the initiation of the polymer synthesis, a Kre6p- and Skn1p-dependent Golgi step for further elaboration of the polymer, and a Kre1p-dependent cell surface step required for side chain addition and maturation of the β1,6-glucan polymer. Because the Kre11p protein is localized in the cytosol, it has been suggested that Kre11p plays a regulatory function in β1,6-glucan assembly.

We have previously reported the isolation of the cwh41
mutant and showed that the cwh41 strain displayed phenotypes characteristic of β1,6-glucan defects: it was resistant to K1 killer toxin and had a reduced glucose/mannose ratio, suggesting a lower cell wall glucose content (41). To gain a better understanding of its in vivo function, we cloned the CWH41 gene by functional complementation and examined the effects of the cwh41Δ-null mutation on cell wall β1,6-glucan assembly. Here we report that the CWH41 gene encodes a novel type II integral membrane N-glycoprotein located in the ER. Disruption of the CWH41 gene leads to a K1 killer-resistant phenotype and a 50% reduction in the cell wall β1,6-glucan level. We demonstrate that the cwh41Δ mutant displayed strong synergistic defects with krelΔ- or krel6A-null mutations, and we also show that the cwh41Δ krelΔ double mutation resulted in the secretion of Cwp1p, a glucosamminoprotein usually anchored covalently within the cell wall matrix. Together, these results provide evidence indicating that CWH41 is involved in the assembly of cell wall β1,6-glucan.

### MATERIALS AND METHODS

**Yeast and bacterial strains and growth media.** The *S. cerevisiae* strains used in this study are listed in Table 1. Growth conditions and media for yeast cells were as described previously (6). Standard procedures were used for genetic crosses, sporulation of diploids, and dissection of tetrads (50). Yeast transformations were performed as described previously (6). Standard procedures were used for genetic crosses, and dissection of tetrads (50). Yeast transformations were performed as described previously (6).

**Plasmids.** A pRS316-based yeast genomic DNA library (provided by C. Boone, Simon Fraser University, Burnaby, British Columbia, Canada) was used to clone the CWH41 gene. The centromeric vector pRS316 was used to subclone the CWH41 gene. The 2µ-based vector YEp351 was used to overexpress the native CWH41 gene or the epitope-tagged CWH41-HA gene. pBluescript II vectors were used for recombinant DNA constructions.

**DNA purification and recombinant DNA techniques.** Yeast DNA was isolated by the procedure of Hoffman and Winston (18). Plasmid DNA was prepared from *E. coli* as described by Sambrook et al. (48). Restriction endonucleases, Klenow and T4 DNA polymerases, shrimp alkali phosphatase, and T4 DNA ligase were purchased from Bethesda Research Laboratories Inc. (Gaithersburg, Md.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), Stratagene Cloning Systems (La Jolla, Calif.), or U.S. Biochemicals (Cleveland, Ohio) and were used according to the instructions of the manufacturers. Radioactive probes for DNA-DNA and RNA-DNA hybridizations were labeled with [α-32P]dCTP (Amersham Canada Limited, Oakville, Ontario) by using the Oligolabeling kit from Pharmacia.

**DNA sequencing.** The CWH41 DNA sequence was determined by a combination of nested deletion and oligonucleotide primer walking. The 4.8-kb HindIII subclone was inserted into the pBluescript II KS+ vector, and nested deletions were constructed with the pBluescript II Eco/Mung kit from Stratagene Cloning Systems. The nucleotide sequence of both DNA strands was determined by the dideoxy-chain termination method of Sanger et al. (49) with the Sequenase kit (U.S. Biochemicals) with [α-32P]dATP as a substrate (Amersham Canada). The coding region of the CWH41 gene and replaced with a 1.8-kb BamHI fragment containing the HIS3 gene. After digestion by HindIII, the DNA fragment carrying the cwh41Δ::HIS3 deletion construct was purified and used to transform a wild-type diploid strain by selecting for histidine prototrophy. Southern blot analysis (51) of genomic DNA from the resulting transformants indicated that disruptions were at the CWH41 locus (data not shown).

**Isolation of cell walls.** After breaking of the cells with glass beads in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1.5 μg of leupeptin per ml, and 3.0 μg of pepstatin A per ml), the cell wall fraction was collected by centrifugation at 1,000 × g for 5 min at 4°C. Cell walls were extracted twice with hot sodium dodecyl sulfate (SDS) by boiling in lysis buffer containing 2% SDS for 5 min each time, washed three times with cold 1 M NaCl-M-1 mM PMSF, and washed three times with 10 mM Tris-HCl (pH 7.5)-1 mM PMSF.

**Cell wall analysis.** Alkali-insoluble glucans were extracted from isolated cell walls of stationary-phase cultures. After β1,3-glucanase (Zymolyase; IC Pharmaceuticals, Inc., Irvine, Calif.) digestion and dialysis, the β1,6-glucan was collected and quantified as described by Boone et al. (1). The total alkali-insoluble glucan (β1,3- plus β1,6-glucan) was determined as the hexose content before dialysis (43), and the β1,3-glucan level was calculated by subtracting the β1,6-glucan content from the total glucan level. The isolated cell wall preparations were used directly to quantify the cell wall total hexose level.

**Epitope tagging.** Epitope tagging of Cwh41p was performed by inserting a 123-bp BglII fragment, which codes for three tandem copies of the influenza virus hemagglutinin (HA) epitope (24), into the unique BamHI site of the CWH41

![FIG. 1. Restriction map and cloning of the CWH41 gene. DNA fragments isolated from the yeast genomic DNA library are represented as thin lines. The open bars indicate various subclones derived from the original genomic DNA fragment. The ability (+) or inability (−) of each fragment to complement the cwh41 mutation is shown on the right. The arrows represent open reading frames. Abbreviations for restriction sites: B, BamHI; E, EcoRI; H, HindIII; S, SpeI; X, XbaI.](image-url)
coding region. Subclones carrying the inserted BglII fragment in the correct orientation were confirmed by DNA sequencing.

To epitope tag the Cwp1p protein, two complementary oligonucleotides, oligonucleotide 1 (AATTCATGTACCCATACGACGTCGCTA) and oligonucleotide 2 (AATTCCATAGCGTAGTCTGGGACGTCGTA), were designed. In addition to containing the sequence encoding the HA epitope, these oligonucleotides also contain complementary EcoRI termini at their ends (underlined). The two oligonucleotides were phosphorylated, annealed, and ligated into the unique EcoRI site located between codons 24 and 25 of CWP1. Constructs containing the epitope insertion were screened by restriction mapping the AatII site (shown in boldface type) present in each of the oligonucleotides. After sequencing the DNA, we identified positive subclones carrying one, two, or four copies of the inserted fragment in the correct orientation. Thus, we obtained single-, double-, and quadruple-HA epitope-tagged Cwp1p. All these tagged Cwp1p proteins are correctly targeted and anchored to the cell wall matrix, and we chose to use the quadruple-HA epitope-tagged Cwp1p (Cwp1p-HA) in this study.

Preparation of total cell lysates and extraction of membrane proteins. Exponentially growing cells expressing Cwh41p-HA were harvested and broken with glass beads in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1.5 μg of leupeptin per ml, and 3.0 μg of peptatin A per ml). After spinning at 1,000 ×g for 5 min at 4°C to remove the cell walls and unbroken cells, the supernatant was collected as the total cell lysate. To determine the nature of Cwh41p-HA’s membrane association, 80 μl of total cell lysate was mixed with 20 μl of 0.5 M Na2CO3 (pH 11), 3 M NaCl, 8 M urea, 5% Triton X-100, or 2.5% SDS. These mixtures were incubated at 4°C for 15 min and then subjected to a high-speed centrifugation of 150,000 ×g for 15 min at 4°C. The resulting membrane pellets were resuspended in 100 μl of the appropriate extraction buffer, and supernatant and pellet fractions were diluted with SDS-polyacrylamide gel electrophoresis (PAGE) sample loading buffer, heated at 95°C, and analyzed by Western blotting (immunoblotting).

Western blotting analysis. Western blots were performed with a 1:2,500 dilution of anti-HA antibody (12CA5) and a 1:2,500 dilution of horseradish peroxidase-conjugated goat anti-mouse secondary antibody. The blots were developed with the ECL chemiluminescence detection kit (Amersham).

Immunofluorescence. Log-phase (optical density at 600 nm’ ≈ 0.5; approximately stationary phase) cells were harvested and stained as described for Western blotting.

FIG. 2. DNA and predicted amino acid sequences of CWH41. The nucleotide sequence of a 3.3-kb BclI-BglII fragment is shown. The predicted 833-amino-acid protein product is shown in the one-letter code below the nucleotide sequence. The stop codon is shown as an asterisk. The potential transmembrane domain is underlined. The predicted N-glycosylation sites are boxed.
mately 2.8 x 10^6 cells per ml) homogenous cwh41Δ diploid cells expressing either Cwh41p-HA or native Cwh41p protein were fixed with 3.7% formaldehyde. Immunofluorescence microscopy was performed as described by Pringle et al. (40). For cells containing 2µm-based plasmids, anti-HA antibody (12CAS) was used at a dilution of 1:2,000 and Texas red-conjugated goat anti-mouse secondary antibody was diluted 1:1,000. For strains carrying centromere-based plasmids, one additional layer of secondary antibody was used to amplify the immunofluorescence signal. Antibody dilutions were 1:2,000 for anti-HA antibody (12CAS), 1:1,000 for Texas red-conjugated goat anti-mouse secondary antibody, and 1:500 for Texas red-conjugated donkey anti-goat secondary antibody. Images were recorded on Kodak T-Max 400 black-and-white film with an epifluorescence microscope (Zeiss Axiophot).

**Cell labeling and immunoprecipitation.** Cells expressing Cwh41p-HA were grown in 10 ml of YNB(50) medium to log phase (optical density at 600 nm = 0.5; approximately 2.8 x 10^6 cells per ml). Labeling was initiated by adding 100 µCi of Trans-35Slabel (ICN Biochemicals) to the cell culture, continued for 20 min, and terminated by adding NaCl to 10 mM and chilling the cells on ice. Labeled cells were lysed with glass beads and immunoprecipitated with anti-HA antibody (12CAS) as described by Roemer et al. (45). N-terminal Tail 1,6-glucanase digestion and protein precipitation from growth media. Washed cell walls were digested with a recombinant B1.3-glucanase, Quantzyme ygl (Quantum Technologies Inc., Montreal, Canada), at a concentration of 2.5 µg/mg of cell wall (wet weight) in 100 µl of a solution containing 50 mM Tris-HCl (pH 7.5), 100 mM dithiothreitol, 1 mM PMSF, 1.5 µg of leupeptin per ml, and 3.0 µg of pepstatin A per ml at 37°C for 18 h. After the digestion, the remaining insoluble material was removed by centrifugation at 15,000 x g for 5 min and the supernatant was analyzed by Western blotting. Proteins secreted into the growth media were recovered by deoxycholate precipitation as described by Ozols (36).

**Nucleotide sequence accession number.** The DNA sequence of the CWH41 gene has been entered in the GenBank database and assigned accession no. U235609.

## RESULTS

**Cloning and sequencing of the CWH41 gene.** The cwh41 Δ mutant was originally isolated from a broad cell wall mutant screen based on the calcofluor white-hypersensitive phenotype. Initial studies indicated that cwh41 displayed a higher cell wall mannose-to-glucose ratio and was resistant to K1 killer toxin, suggesting that this mutant had defects in β1,6-glucan assembly (41). To further characterize the gene identified by this mutant allele, we cloned the CWH41 gene by functional complementation. Two overlapping genomic DNA fragments complementing the cwh41−1 calcofluor white-hypersensitive phenotype were isolated from a yeast genomic DNA library. Restriction mapping and subcloning analyses located the cwh41−1 gene, and the cwh41−1 complementing activity to a 4.4-kb HindIII fragment (Fig. 1). To determine if the cloned DNA fragments contained the CWH41 gene, we crossed the cwh41Δ::HIS3 deletion mutant (see below) with the original cwh41−1 allele. The resulting diploid strain was sporulated and analyzed by tetrad dissection. Of the 10 tetrads examined, all four spores from each tetrad were calcofluor white hypersensitive, with the HIS3 marker segregating 2:2:2:2. The diploid strain showed hypersensitivity to calcofluor white as well. These results demonstrate that the cwh41Δ::HIS3 deletion not only failed to complement but also was tightly linked to the original cwh41−1 locus, indicating that the cloned DNA fragments contained the CWH41 gene.

DNA sequence analysis of the 4.4-kb HindIII fragment revealed a single, 2.5-kb open reading frame encoding a protein of 833 amino acid residues (Fig. 2). The predicted Cwh41p protein sequence contains features characteristic of a type II integral membrane protein (16, 38); it has a positively charged N-terminal tail of 10 amino acid residues followed by a stretch of 16 hydrophobic residues that could form a potential membrane-spanning domain and a large 807-amino-acid C-terminal domain containing four potential N-linked glycosylation sites (Asn-X-Ser/Thr). Comparison of the Cwh41p sequence with those from GenBank, EMBL, PIR, and SwissProt sequence databases has not revealed any proteins with significant similarities to Cwh41p.

A sequence search of the S. cerevisiae GenBank database revealed that the DNA sequence 5′ to the CWH41 coding region was identical to the DNA sequence 3′ to the TRP5 gene (57), thus demonstrating that the CWH41 gene is physically adjacent to TRP5 on the left arm of chromosome VII.

**Phenotypes of the cwh41Δ::HIS3-null mutant.** To study its in vivo function, a deletion-null mutant of the CWH41 gene was constructed and the resulting phenotypes were examined by tetrad analysis. Disruption of the CWH41 gene did not give rise to any detectable growth defects under standard growth conditions, indicating that CWH41 is a nonessential gene. However, as found for the original cwh41−1 allele, the cwh41Δ::HIS3-null mutant displayed cell wall-related defects: the mutant was hypersensitive to calcofluor white, more resistant to K1 killer toxin, and showed an approximately 50% reduction in cell wall β1,6-glucan levels (Table 2). The levels of the cell wall total hexose (glucans plus mannans) and β1,3-glucan were not affected by the cwh41Δ::HIS3 mutation. These phenotypes indicated that the CWH41 gene was involved in β1,6-glucan assembly.

**Immunodetection of Cwh41p.** To facilitate detection and further characterization of the CWH41 gene product, we tagged the N terminus of CWH41 with a quadruple-HA epitope, which is recognized by the monoclonal antibody 12CAS (see Materials and Methods). The epitope-tagged gene (CWH41-HA) remained fully functional, as judged by its ability to complement both the killer-resistant phenotype and the β1,6-glucan defect in the cwh41Δ strain (Table 2).

Western blot analysis with 12CAS antibody detected a single, 107-kDa polypeptide from a strain expressing a centromere-based CWH41-HA plasmid (Fig. 3). The 107-kDa protein was overproduced in cells containing a 2µm-based CWH41-HA plasmid but was absent from strains lacking the tagged CWH41 gene. These results showed that the 107-kDa protein is the product of the tagged CWH41 gene. Cwh41p appears to localize to the ER. Immunofluorescence microscopy was performed to determine the intracellular lo-

### TABLE 2. Phenotypes of CWH41 gene disruption

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Killer toxin zone size (mean ± SD [mm])</th>
<th>Cell wall polymer level* (mean ± SD [µg (dry wt)/ml])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td>16.9 ± 0.5</td>
<td>118 ± 3.4 228 ± 37 592 ± 92</td>
</tr>
<tr>
<td>cwh41Δ</td>
<td>HA-CWH41 in pRS316</td>
<td>11.8 ± 0.4</td>
<td>56.7 ± 6.0 208 ± 3.0 568 ± 46</td>
</tr>
<tr>
<td>cwh41Δ</td>
<td></td>
<td>17.0 ± 0.6</td>
<td>105 ± 10 210 ± 37 537 ± 22</td>
</tr>
</tbody>
</table>

*Total cell walls were first isolated from stationary-phase cells, and then the major cell wall polymers were fractionated and measured as described by Boone et al. (1).
FIG. 3. Immunodetection of the Cwh41-HA protein. Total cell lysates from strains expressing native or HA epitope-tagged CWH41 gene were analyzed by Western blot with the 12CA5 anti-HA monoclonal antibody. CEN, centromere-based low-copy-number plasmids; 2μ, 2μm-based high-copy-number plasmids. Molecular masses (in kilodaltons) are indicated on the left.

Calization of Cwh41p-HA. Cells containing a 2μm-based CWH41-HA plasmid, but not those with the untagged CWH41 plasmid, showed clear perinuclear-rim staining, with some staining at the periphery of the cell (Fig. 4). This staining pattern resembles that observed for several known ER proteins, including Dpm1p, Kar2p, Sec62p, and Sec63p (9, 11, 39, 46). Since overproduction of a protein might lead to mislocalization in the ER, we also examined the subcellular location of Cwh41p-HA with a centromere-based plasmid. By standard experimental procedures we could not observe any detectable signals for Cwh41p-HA, probably because of its low abundance. However, by using an antibody sandwiching method (40) we were able to detect a weak but highly reproducible perinuclear-staining pattern very similar to that seen from cells containing a 2μm-based CWH41-HA plasmid (data not shown). This showed that overexpression of Cwh41p-HA did not result in its mislocalization. Under the experimental conditions we used, approximately 47% of the cells counted (total counted ~ 1,000) gave detectable fluorescence signals: 13% showed strong perinuclear-rim staining, and 34% displayed a weak perinuclear signal. No cells showed Golgi, vacuolar, or plasma membrane staining. These observations suggest that Cwh41p-HA is an ER protein.

Cwh41p is an integral membrane N-glycoprotein. Examination of the predicted Cwh41p amino acid sequence revealed the presence of a putative transmembrane domain near the N terminus and four potential N-linked glycosylation sites (Asn-X-Ser/Thr) in the C-terminal region. To test whether Cwh41p is an integral membrane protein, we extracted total cell lysates with various reagents and then fractionated the samples into membrane pellet and soluble supernatant by centrifugation at 100,000 × g. Figure 5A shows that Cwh41p-HA fractionated exclusively to the membrane pellet after treatment with 0.1 M Na2CO3 (pH 11), 0.6 M NaCl, or 1.6 M urea, conditions commonly used to strip nonintegral proteins from membranes (14, 45). In contrast, 1% Triton X-100 released a fraction of the protein into the soluble fraction and treatment with 0.5% SDS almost completely solubilized the protein. Thus, Cwh41p behaved as an integral membrane protein.

To determine whether the protein is N glycosylated, we immunoprecipitated 35S-labeled Cwh41p-HA proteins from tunicamycin-treated and nontreated cell cultures and examined their electrophoretic mobilities by SDS-PAGE (Fig. 5B). In the presence of tunicamycin, which specifically inhibits N
The four potential N-glycosylation sites are indicated by asterisks.

Figure 5. Membrane association and N-glycosylation of Cwh41p-HA. (A) Cell lysates from a strain expressing the 2μm-based CWH41-HA plasmid were treated with H2O, 0.1 M Na2CO3 (pH 11), 0.6 M NaCl, 1.6 M urea, 1% Triton X-100, or 0.5% SDS and separated into supernatant (S) and pellet (P) fractions as described in Materials and Methods. These fractions were then analyzed by Western blot, with the 12CA5 anti-HA monoclonal antibody. (B) Cells expressing a 2μm-based native CWH41 plasmid or a 2μm-based CWH41-HA plasmid were labeled with Trans-35S label in the presence (+) or absence (−) of tunicamycin. The radiolabeled Cwh41p-HA protein was immunoprecipitated and analyzed by SDS-PAGE as described in Materials and Methods. Molecular masses (in kilodaltons) are indicated on both sides of the panel. (C) A schematic diagram of Cwh41p protein. The shaded box indicates the transmembrane domain. The arrow points to the insertion site of the HA epitope. The four potential N-glycosylation sites are indicated by asterisks.

Genetic interactions between CWH41 and KRE genes. Previous studies suggested that β1,6-glucan is synthesized in a stepwise manner within the yeast secretory pathway (3, 44). At least three distinct steps were identified: a Kre5p-dependent ER step involved in the initiation of the polymer synthesis (32), a Kre6p- and Skn1p-dependent Golgi step required for further modification of the polymer (45), and a Kre1p-dependent cell surface step for β1,6-linked side chain addition or elongation (1). Having shown that Cwh41p is an ER membrane protein involved in cell wall β1,6-glucan assembly, we searched for possible genetic interactions between CWH41 and KRE1, KRE6, SKN1, and KRE5. Cwh41p was an N-glycoprotein. All N-glycoproteins are initially modified by the attachment of a core oligosaccharide (GlcNAc2Man9Glc3) in the ER (17, 26, 53). As each core oligosaccharide contributes about 2 kDa to the molecular mass (35), the tunicamycin-induced 2-kDa shift suggested that the Cwh41p-HA was modified by a core oligosaccharide without outer-chain extensions.

The cwh41Δ kre1Δ double mutant displayed defects in cell wall anchorage of Cwp1p. It has recently been shown that some cell wall mannanproteins are covalently cross-linked to β1,6-glucans, and these β1,6-glucan side chains have been suggested to play functional roles in the anchorage of mannanproteins to the cell wall matrix (28, 34, 56). To obtain additional insights about its in vivo function, we examined the effects of CWH41 deletion on the cell wall anchorage of Cwp1p, a known β1,6-glucan-modified cell wall protein (55). In wild-type cells, as well as in the cwh41Δ and kre1Δ single mutants, the epitope-tagged Cwp1p-HA protein was correctly anchored into the cell wall matrix (Fig. 8). In the cwh41Δ kre1Δ double mutant, however, little if any Cwp1p-HA protein could be detected in the cell wall fraction. Instead, the protein was secreted into the growth medium by the double mutant. To test whether the secretion of Cwp1p-HA is specific to the cwh41Δ kre1Δ mutant, we examined the kre5Δ mutant and found that the Cwp1p-HA protein was secreted by the kre5Δ mutant as well. These results showed that severe β1,6-glucan defects, caused either by the cwh41Δ kre1Δ double mutation or by a kre5Δ single mutation, resulted in the failure of Cwp1p-HA cell wall
anchorage, indicating that β1,6-glucan plays a functional role in anchoring cell wall proteins within the extracellular matrix.

**DISCUSSION**

In this study, we report the cloning and characterization of the CWH41 gene from *S. cerevisiae*. Our gene deletion analysis revealed that the *cwh41Δ* null mutant displayed phenotypes characteristic of cell wall defects: hypersensitivity to calcofluor white and resistance to K1 killer toxin. In addition, we showed that disruption of the CWH41 gene resulted in a 50% reduction of cell wall β1,6-glucan levels. The effects of CWH41 gene deletion appeared to be specific for β1,6-glucan, since the β1,3-glucan level and the total cell wall hexose content were not altered by loss of the CWH41 gene. Furthermore, we demonstrated that a null mutation in the CWH41 gene displayed severe synergistic defects with null mutations in KRE1 and KRE6, two genes known to be involved in the β1,6-glucan biosynthetic pathway (1, 43). The *cwh41Δ kre1Δ* double mutant showed an extremely slow-growth phenotype and a 75% reduction in cell wall β1,6-glucan level. Cells carrying the *cwh41Δ kre6Δ* double mutation were not viable. Together, these results provide strong genetic evidence indicating that Cwh41p plays a functional role in the assembly of cell wall β1,6-glucan. The exact biochemical function of Cwh41p is not clear. It could function as a new component of the β1,6-glucan synthetic machinery. Alternatively, it could play a regulatory role, for example, as an activator of synthase components.

DNA sequencing revealed that the CWH41 gene encodes a novel, 833-amino-acid-residue polypeptide. Using a functional epitope-tagged protein, we showed that Cwh41p-HA is an integral membrane N-glycoprotein, consistent with the structure predicted from the DNA sequence. Because all four potential N-glycosylation sites are located in the C-terminal region of Cwh41p, the presence of N-linked oligosaccharide on the protein also showed that the C-terminal domain was situated within the lumen of the secretory pathway. Assuming that the 16-amino-acid-residue transmembrane domain spans the membrane once, this would indicate that Cwh41p has a type II membrane protein topology.

Immunofluorescent analysis of Cwh41p-HA revealed a perinuclear-rim staining pattern indicative of ER localization in yeast cells. Since it has been documented that some misfolded or overproduced proteins accumulate within the ER (15, 39), one has to interpret the ER localization of an epitope-tagged protein cautiously. We think the observed ER staining reflects the authentic subcellular location of Cwh41p for the following reasons. Firstly, a centromere-based CWH41-HA plasmid is

**FIG. 6.** Germination and growth of tetrads from *cwh41Δ/cwh41Δ* and *cwh41Δ/kre1Δ*kre1Δ/kre1Δ heterozygous diploids. Diploid strains were sporulated and dissected onto yeast extract-peptone-dextrose plates and incubated at 30°C. Tetrad types shown are parental ditype (PD), nonparental ditype (NPD), and tettype (TT). The four spore progeny derived from each tetrad are indicated by the letters A, B, C, and D to the left of each panel. (A) Tetrads from the *cwh41Δ/cwh41Δ* and *kre1Δ/kre1Δ* heterozygous diploid. The spore progeny containing the *cwh41Δ**kre1Δ* double mutation displayed a slow-growth phenotype. The tetrads were photographed after 2 days of incubation. (B) Tetrads from the *cwh41Δ/cwh41Δ* and *kre6Δ/kre6Δ* heterozygous diploid. Cells carrying the *cwh41Δ kre6Δ* double mutation were not viable. The tetrads were photographed after 4 days of incubation.

**FIG. 7.** Quantification of cell wall β1,6-glucan levels. Alkali-insoluble β1,6-glucan was extracted from the cell wall preparations of various strains and quantified (in micrograms per milligram [dry weight]) of cell wall) as described in Materials and Methods. The data shown represent the results of at least three independent experiments. Error bars represent standard deviations. WT, wild type.
able to completely complement the cwh41Δ-null mutation. This shows that the epitope-tagged protein is fully functional, thus making the possibility of misfolding very unlikely. Secondly, the centromere-based CWH41-HA plasmid and the 2μm-based CWH41-HA plasmid both showed similar perinuclear-rim staining patterns. These results indicate that overexpression of the Cwh41p-HA did not lead to mislocalization. Thirdly, the perinuclear-rim staining was the only staining pattern detected: no Golgi, vacuolar, or plasma membrane staining was seen. Collectively, these data indicate that the Cwh41p is an ER protein.

Previous studies of the Kre5p, the two ER proteins involved in β1,6-glucan assembly? Several lines of evidence suggest that Cwh41p and Kre5p have distinct functions. Firstly, the phenotypes displayed by the two null mutants are very different. Disruption of the Kre5 gene gave rise to an extremely severe β1,6-glucan defect, with no detectable amount of polymer made (32). In comparison, the β1,6-glucan defect caused by CWH41 gene disruption was quite modest. Secondly, the cwh41Δ kre5Δ double mutant displayed the same phenotype as the kre5Δ single mutant, indicating that Kre5 is epistatic to CWH41. Thirdly, we showed that overexpression of either gene cannot compensate for the defect caused by the loss of the other, suggesting that their functions are not interchangeable. On the basis of all these results, Cwh41p appears to play a distinct functional role either downstream of Kre5p or as an auxiliary component perhaps in a complex with Kre5p. Further biochemical characterizations will be needed to distinguish these possibilities.

Our results also reveal a correlation between defects in β1,6-glucan assembly and the anchorage of a glucanmannoprotein in the cell wall matrix. Recently, a number of glucanase-extractable cell wall proteins have been identified (55). These proteins, Cwp1p, Cwp2p, Tip1p, and Srp1p, have been shown to be rich in serine and threonine residues and all contain putative glycosylphosphatidylinositol attachment sites. Cwp1p and Tip1p (25) proteins have been demonstrated to carry glycosylphosphatidylinositol attachment sites. Cwp1p and Tip1p (25) proteins have been demonstrated to carry glycosylphosphatidylinositol attachment sites. Cwp1p and Srp1p (25) proteins have been demonstrated to carry glycosylphosphatidylinositol attachment sites. Cwp1p and Tip1p (25) proteins have been demonstrated to carry glycosylphosphatidylinosositol attachment sites.
results provide experimental evidence for the proposal that β1,6-glucan plays a functional role in anchoring glucosamino-protein in the cell wall, probably by covalently cross-linking the protein to the extracellular β1,3-glucan matrix (22, 34). How and where the β1,6-glucan side chain is attached onto the protein is not clear, although several possibilities, including that the β1,6-glucan is linked to the glycosylphosphatidylinositol anchor (8, 21, 28, 54), have been suggested. Detailed analyses of the glycosylation of Cwp1p in various kars mutants should provide insights on the in vivo functions of these KRE genes and on the molecular mechanisms underlying the processes of protein glycosylation and cell wall anchorage.

ACKNOWLEDGMENTS

We thank members of the Bussey laboratory for advice and discussions, Charlie Boone for his great yeast library, and Diane Oki for manuscript preparation. This work was supported by Operating and Strategic grants from the Natural Sciences and Engineering Research Council of Canada.

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