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Restrictive Glycosylphosphatidylinositol Anchor Synthesis in *cwh6*/*gpi3* Yeast Cells Causes Aberrant Biogenesis of Cell Wall Proteins

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We previously reported that the defects in the *Saccharomyces cerevisiae cwh6* Calcofluor white-hypersensitive cell wall mutant are caused by a mutation in *SPT14*/*GPI3*, a gene involved in glycosylphosphatidylinositol (GPI) anchor biosynthesis. Here we describe the effect of *cwh6*/*spt14*/*gpi3* on the biogenesis of cell wall proteins. It was found that the release of precursors of cell wall proteins from the endoplasmic reticulum (ER) was retarded. This was accompanied by proliferation of ER structures. The majority of the cell wall protein precursors that eventually left the ER were not covalently incorporated into the cell wall but were secreted into the growth medium. Despite the inefficient incorporation of cell wall proteins, there was no net effect on the protein level in the cell wall. It is postulated that the availability of GPI-dependent cell wall proteins determines the rate of cell wall construction and limits growth rate.

Glycosylphosphatidylinositol (GPI) anchors are commonly used to attach proteins to cell membranes. GPI modification of proteins is found in many eukaryotes, and in prokaryotes, a distant relative of a GPI anchor-synthesizing protein has been identified (47). The core structure of the glycosyl moiety is conserved from yeast cells to humans and consists of a linear sequence of one inositol, one glucosamine, and three mannose residues. Addition of a GPI anchor to protein occurs in the lumen of the endoplasmic reticulum (ER) (46). For import into the ER, the preproprotein uses an N-terminal signal sequence, which is removed upon import. This results in the formation of the proprotein. The signal for GPI anchor addition to the proprotein consists of a C-terminal sequence of hydrophobic amino acids, preceded by suitable amino acids for GPI anchor addition (29, 40). This C-terminal stretch of hydrophobic amino acids presumably serves as a transient membrane anchor until it is cleaved off and replaced by a preformed GPI anchor. The biosynthesis of GPI anchors and their exchange with the hydrophobic C terminus of the protein are complex processes and require multiple enzymes. Several mutant human cell lines which are defective in GPI anchor biosynthesis have been isolated (39). Recently, several yeast mutants and corresponding genes involved in GPI anchor biosynthesis (3, 16–18, 36, 47) and GPI anchor addition (3, 8) have also been isolated. All yeast genes known to be involved in GPI anchor biosynthesis are essential for viability (8, 18, 47). In higher eukaryotes and also in yeasts, a role for GPI anchors in targeting proteins to the plasma membrane is generally accepted (22). But there is also evidence for additional functions of GPI anchors. Attachment of a GPI anchor seems to be required for efficient packaging of Gas1p into COP II vesicles, which are responsible for the transport from the ER to the Golgi compartment (7). Besides this additional role for GPI anchors, there is evidence that they are involved in cross-linking proteins to the cell wall glucan. For example, the mating type α-specific sexual agglutinin, Agα1p, which is known to have a GPI anchor, is released from the plasma membrane and cross-linked to glucan. Simultaneously with the glucan modification, inositol is removed from the protein (20). The GPI anchor apparently plays an important role in this cross-linking step. Deletion of the GPI anchor addition signal prevents the cross-linking of Agα1p to the cell wall and results in secretion of an active molecule into the medium (49). On the other hand, the 30 C-terminal amino acids of Agα1p, which include the GPI anchor addition signal, are sufficient to target a reporter enzyme to the cell wall (42). Once α-agglutinin is linked to glucan, it can be released by treatment with aqueous hydrofluoric acid (HF), which is known to specifically break phosphodiester bonds. These phosphodiester bonds probably originate from the GPI anchor (10). In addition to α-agglutinin, other proteins that contain a GPI anchor addition signal are covalently associated with the cell wall (26, 38, 44, 45). However, as mentioned above, not all proteins containing a GPI anchor are destined for the cell wall. Gas1p is localized in the plasma membrane, and Kre1p is also not covalently linked to the cell wall (38). The molecular basis of this distinction is not known.

In this study, we have investigated further the importance of GPI anchors in cross-linking cell wall proteins to glucan. For this purpose, the temperature-sensitive *cwh6*/*spt14*/*gpi3* mutant in which GPI anchor biosynthesis is restricted was used (32, 36, 47). This resulted in accumulation of ER precursor forms of cell surface proteins and proliferation of the ER. Evidence that the supply of GPI anchors available to cell surface proteins is the limiting factor in cell wall construction and growth rate is presented.

MATERIALS AND METHODS

Yeast strains and media. The yeast strains used in this work are listed in Table 1. All strains used were isogenic to S288C. Strain JV31 was isolated as the temperature-sensitive and leucine auxotrophic haploid offspring from the diploid that arose from crossing AR51 and the *cwh6* strain. Strain JV97 was constructed by transforming FY833 strains to leucine prototrophy with a *LEU2* disruption construct, described by van der Vaart et al. (44). The result of the single-step gene disruption (35) was confirmed by a Southern blot. The *cwp1 cwh6* double mutant strain JV79 was constructed by selecting leucine prototrophic and tem-

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference
AR27	α *ura3-52*	32
cwh6	α *ura3-52 cwh6*^{ts}	32
FY833	**a** *his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63*	48
AR 104	**a** *gas1*::*LEU2 his3Δ200 ura3-52 leu2Δ1* lys2Δ202 trp1 Δ63	31
JV177	**a** *gas1*::*LEU2 cwh6*^{ts} *lys2Δ202 leu2*	This paper
AR51	**a** *leu2*	32
JV31	α *leu2 cwh6*^{ts}	This paper
JV97	**a** *cwp1*::*LEU2 his3Δ200 ura3-52 leu2Δ1* lys2Δ202 trp1Δ63	This paper
JV79	**a** *cwp1*::*LEU2 cwh6*^{ts} *leu2 lys2*	This paper

perature-sensitive offspring from the diploid arising from crossing strains JV31 and JV97. The *gas1 cwh6* double mutant JV177 was constructed by selecting leucine prototrophic and temperature-sensitive offspring from the diploid arising from crossing strains JV51 and AR104.

The strains were grown in synthetic minimal medium (0.17% yeast nitrogen base, 1% MES [morpholinoethanesulfonic acid] [Sigma] buffered at pH 6.0 with NaOH, 0.5% NH_4SO_4, 2% glucose) supplied with necessary amino acids at 28 or 37°C. When cells were labeled with [^{35}S]methionine, low-sulfate medium (100 μM NH_4SO_4) and labeling medium (20 μM NH_4SO_4) were used as described by Rothblatt and Schekman (34). Yeast genetics and sporulation followed established procedures (37). Yeast extract, Bacto Peptone, yeast nitrogen base, and Bacto Agar were from Difco.

[^{35}S]methionine labeling of a-factor-induced cells. Labeling was performed essentially as described by Lu et al. (19). Cells were precultured in low-sulfate medium at 28°C to an optical density at 600 nm of 0.5 (equivalent to 0.5×10^7 cells/ml). The cells were collected, and 2×10^8 cells were transferred to 25 ml of labeling medium and incubated at 28°C. After 5 min, synthetic **a** factor (a gift of Fred Naider, College of Staten Island, Staten Island, N.Y.) was added to 50 ng/ml. Five minutes later, 0.5 mCi of TRAN^{35}S-LABEL (ICN Biochemicals) was added. After a further incubation of 40 min, 0.25 ml of chase mixture (1 M NH_4SO_4, 100 mM DL-cysteine, 100 mM DL-methionine) was added. Before harvesting, NaN_3 was added to 10 mM. Cells were harvested after a 0-min and a 40-min chase, and different protein fractions were isolated as described below.

Protein determination by labeling with ^{35}S. Total protein contents of different fractions were determined by counting radioactivity in these fractions after ^{35}S labeling. For this experiment, the labeling medium as described by Rothblatt and Schekman (34) was slightly modified. Instead of 20 μM NH_4SO_4 as cold sulfate source, we used 50 μM DL-cysteine and 50 μM DL-methionine. The cells were precultured in this medium, and at $t = 0$, cells were diluted in fresh medium to an optical density at 600 nm of 0.25 (equivalent to 0.25×10^7 cells/ml). At $t =$ 5 min, 0.5 mCi of TRAN^{35}S-LABEL was added. Cultures were grown for two doubling times (see Table 2) at 28°C. Cells were harvested and fractionated as described below. Protein contents of the different fractions were calculated as percentages of total label incorporated.

Cell harvesting and storage. Cells were grown on synthetic media complemented with the necessary amino acids as described above. Cells were harvested in the early logarithmic phase by centrifuging at $1,000 \times g$ for 5 min. Harvested cells (2×10^8) were washed twice in wash buffer (30 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF; Sigma]) and stored at −70°C.

Isolation of medium proteins. Remaining cells were removed from the medium by an additional centrifugation step at $4,500 \times g$ for 10 min. Proteins were precipitated from the medium with 0.2 mg of sodium desoxycholate per ml and 6% trichloroacetic acid, during overnight incubation at 4°C (30). To exclude that cell lysis had occurred, these isolated medium proteins, equivalent to 5×10^7 cells, were examined in a Western blot with anti-HDEL antibody. This antibody is directed against the peptide with the sequence HDEL and is known to specifically recognize ER lumen proteins.

Isolation of sodium dodecyl sulfate (SDS) extracts and SDS-extracted cell walls. Frozen cells were resuspended in 250 μl of SDS lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 2% SDS, 1 mM PMSF, 0.7 μg of pepstatin [Boehringer] per ml, 0.5 mg of leupeptin [Sigma] per ml) and were broken with 0.3 g of glass beads. Two hundred microliters of the total lysate was collected, and the SDS extract was separated from the cell walls by centrifugation for 5 min at $14,000 \times g$. The supernatants representing the SDS extracts were stored at −70°C. The cell wall pellets were extracted twice more by being boiled for 5 min in SDS lysis buffer and being washed five times in inhibitor mixture (5 mM EDTA, 2% SDS, 1 mM PMSF, 0.7 μg of pepstatin per ml, 0.5 mg of leupeptin per ml).

Preparation of laminarinase extracts. The SDS-extracted cell walls were washed once in laminarinase buffer (100 mM NaAc [pH 5.5], 1 mM EDTA, 1 mM PMSF, 0.7 μg of pepstatin per ml, 0.5 μg of leupeptin per ml). The pellet was resuspended in 10 μl of laminarinase buffer. Ten microliters of laminarinase solution (0.25 U of mollusc laminarinase/ml [Sigma], 1 mM PMSF, 0.7 μg of pepstatin per ml, 0.5 mg of leupeptin per ml, 50 mM NaAc) was added at $t = 0$ and $t = 2$ h, and the mixture was incubated at 37°C for 4 h in total. The mixture was adjusted to 2% SDS for immunoprecipitations, or 15 μl of 3× sample buffer (6% SDS, 2.25% Tris, 30% glycerol, 15% β-mercaptoethanol, 0.003% bromophenol blue, adjusted to pH 6.8 with HCl) was added for Western analyses. The mixture was boiled for 5 min. Laminarinase-released proteins were in the supernatant after centrifugation for 5 min at $14,000 \times g$.

Preparation of HF extracts. SDS-extracted cell walls were dried in a SpeedVac, and 100 μl of ice-cold 48% HF was added. The mixture was incubated on ice for 48 h. HF was subsequently evaporated by a constant nitrogen flow. The residue was washed three times with ice-cold 90% methanol. The pellet was resuspended in sample buffer and boiled for 5 min. After centrifugation for 5 min at $14,000 \times g$, the HF-extractable cell wall proteins were in the supernatant.

Isolation of membrane-bound and membrane-free fractions. Frozen cells (2×10^8) were resuspended in 250 μl of lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 0.7 μg of pepstatin per ml, 0.5 mg of leupeptin per ml) and were broken with 0.3 g of glass beads. Two hundred microliters of the total lysate was collected and centrifuged for 5 min at $14,000 \times g$. The resulting supernatant was centrifuged again for 5 min at $14,000 \times g$. The supernatant was centrifuged for 30 min at 207 kPa ($100,000 \times g$) with a 18°A-100 rotor in a Beckman Airfuge. The final supernatant, representing the soluble protein fraction, was collected and stored at −70°C. The membrane pellet was incubated in 100 mM Na_2CO_3 for 30 min on ice and recentrifuged for 10 minutes at 207 kPa in a Beckman Airfuge. The pellet was washed twice in membrane wash buffer (50 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1 mM PMSF, 0.7 μg of pepstatin per ml, 0.5 mg of leupeptin per ml). The washed membrane pellet was taken up in a small volume of 2% SDS and stored at −70°C.

Silver staining, immunoblotting, and immunoprecipitation. SDS-polyacrylamide gel electrophoresis (14) was performed on gradient gels from 2 to 20%. For silver staining, the gels were subsequently treated as described by De Nobel et al. (6). Western analysis was performed essentially as described by Montijn et al. (24). Blots were kept for 1 h in periodate buffer (100 mM NaAc, 50 mM H_5IO_6) prior to the blocking step in Western analysis, except when β1-6-glucan antibodies were used. Binding of the peroxidase-labeled secondary antibodies was visualized with the Enhanced Chemo-Luminescence kit from Amersham. The antisera used in this study were anti-Suc2p (27), which was diluted 1:10,000; anti-Gas1p (kindly provided by L. Popolo [41]), which was diluted 1:2,000; anti-β1,6-glucan (kindly provided by R. Montijn [24]), which was diluted 1:5,000; anti-HDEL (kindly provided by N. Dean [9]), which was diluted 1:500; and anti-Cwp1p (described below), which was diluted 1:1,000. α-Agglutinin was immunoprecipitated with the AG3 antibody (49). The immunoprecipitation procedure was as described by Lu et al. (19). Immunoprecipitates were separated on a 6% separating gel with a 4% stacking gel, unless otherwise stated. The gels were fixed, treated with En^{3}Hance (Du Pont), dried, and exposed to Kodak X-Omat AR film.

Raising of antibodies against Cwp1p. The sequence DDGKLKFDDDKYAV from the central part of Cwp1p was used to make a synthetic peptide. This peptide was coupled through an additional C-terminal cysteine to the carrier keyhole limpet hemocyanin. Rabbits were immunized with this neoprotein, and serum was isolated. The above-described procedure was performed by Eurogentec (Seraing, Belgium).

Electron microscopy. Cells were grown overnight in YPD (1% yeast extract, 1% Bacto Peptone, 3% glucose) at 30°C to early logarithmic phase. Cells were washed three times in 0.9% NaCl. The washed cells were fixed chemically in nonbuffered 1.5% potassium permanganate for 20 min at room temperature. Fixed cells were washed three times in phosphate-buffered saline, cryoprotected with 30% dimethyl formamide for 30 min, frozen in liquid propane, and freeze substituted in anhydrous methanol at −90°C for 16 h. The freeze substituted cells were infiltrated with Spurr's resin, and polymerization took place at 65°C. After ultramicrotomy, 80-nm sections were viewed in a transmission electron microscope, EM 420, at 80 kV.

RESULTS

***cwh6* cells contain a temperature-sensitive mutation.** *cwh6*/*gpi3* cells contain a temperature-sensitive allele (47) that allows only limited growth at permissive temperatures (Table 2). The experiments described in this paper were performed at 28°C, unless otherwise stated. At the semipermissive temperature, growth rate and, presumably, also the Cwh6 activity are severely impaired. As *CWH6* is involved in the first biosynthetic step of GPI synthesis, that is, the addition of *N*-acetylglucosamine to phosphatidylinositol, it is expected that in *cwh6* cells grown at the semipermissive temperature only a limited amount of mature GPI anchors will be available for addition to proteins in the ER. As GPI-anchored proteins are destined for

TABLE 2. Definition of growth conditions

Condition	Temp (°C)	Doubling time (h)	
		Wild type	*cwh6*
Permissive	23	2.5	3.5
Semipermissive	28	1.5	5
Restrictive	37	1.5	—[a]

[a] The mutant is nonviable at this temperature.

the cell surface, growth at the semipermissive temperature also allows the study of phenotypic effects with respect to cell surface protein maturation.

Accumulation of ER structures and ER lumen proteins in *cwh6*/*gpi3* cells. At the semipermissive temperature of 28°C, *cwh6* cells accumulated membrane sheets (Fig. 1), suggesting that ER had accumulated. This was supported by the observation that HDEL antigenic proteins accumulated in *cwh6* cells

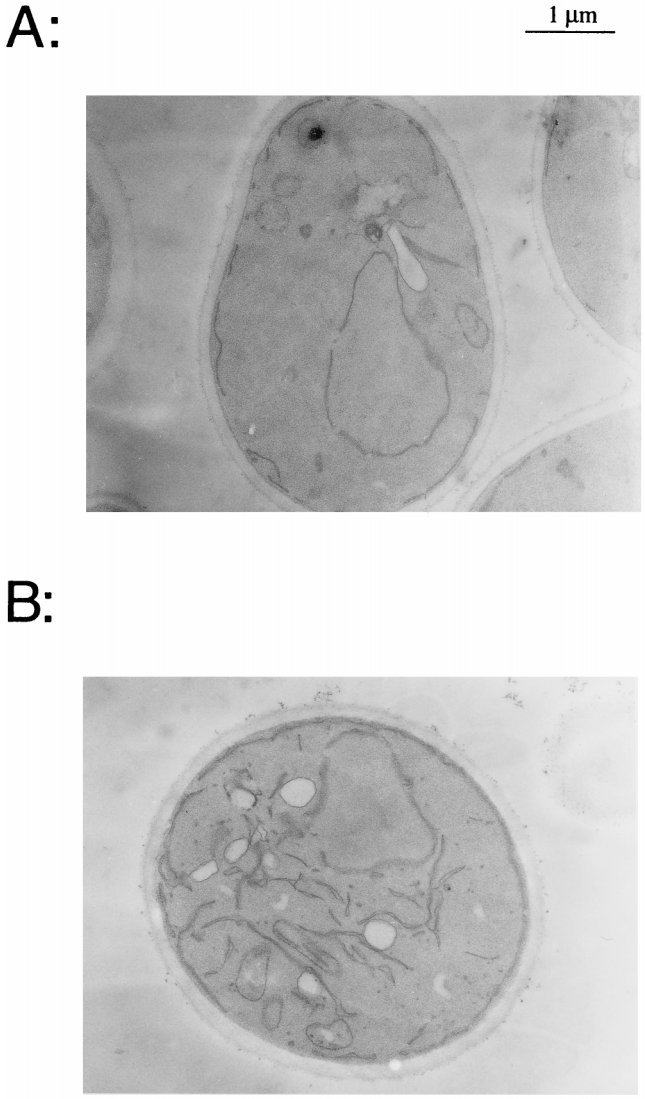

FIG. 1. Transmission electron micrographs of wild-type (A) and mutant *cwh6* (B) cells. ER-like membranous elements accumulate in *cwh6* cells. The cell wall protein layer is manifest as an electron-dense layer at the outside of the cell wall.

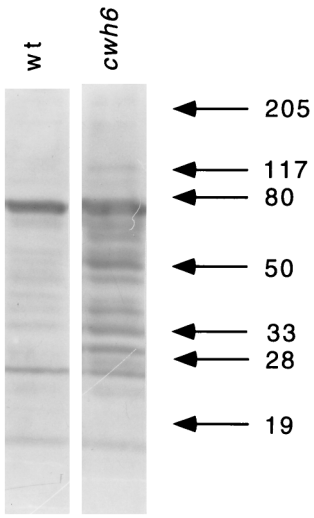

FIG. 2. Accumulation of ER lumen proteins in *cwh6* cells. SDS extracts of cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis in combination with immunoblotting with anti-HDEL antibodies. The arrows indicate molecular mass markers (kilodaltons). In each lane, proteins equivalent to 5×10^7 cells were loaded.

(Fig. 2). The C-terminal HDEL motif is responsible for retention of ER lumen proteins, and antigenic proteins will represent ER residential proteins.

Accumulation of ER forms of GPI-dependent proteins in *cwh6* cells. The observed accumulation of ER indicated that GPI-dependent proteins might accumulate in this compartment. Figure 3A shows that, in *cwh6* cells, the 100-kDa ER form of Gas1p (41) became more prominent than the mature form of about 125 kDa. Similar results have been reported by others (7, 28, 36). In addition, underglycosylated precursors of cell wall proteins were found to accumulate in *cwh6* cells. For example, a 50-kDa form of Cwp1 was found (Fig. 3B). This form is also seen in *sec18* cells grown at the restrictive temperature (23), indicating that the accumulating material is localized in the ER. In addition, Agα1p was found to accumulate in the ER. A 150-kDa form of α-agglutinin, which was also found in *sec18* cells (19), remained present after a 40-min chase period in the mutant cell lysate, whereas in the wild type this form had disappeared (Fig. 3C). In addition, some protein bands with a lower molecular mass and probably representing proteolytic breakdown products of α-agglutinin are more prominent in the mutant cells. In contrast to GPI-dependent proteins, no accumulation of the ER form of invertase was observed in *cwh6* cells (Fig. 3D). This suggests that the limited availability of GPI anchors specifically causes GPI-dependent proteins to accumulate.

The ER form of Agα1p in *cwh6* cells is partially released from the ER membrane. Under normal conditions, a newly synthesized GPI-dependent preproprotein enters the ER through its N-terminal secretion signal. The proprotein is retained in the ER membrane by its hydrophobic C terminus, or GPI anchor addition signal, until a transamidase replaces this region with a GPI anchor. Evidence has been obtained from mammalian cells (21) that cleavage of GPI-dependent proteins at the so-called omega site can take place without the concomitant addition of a GPI anchor, resulting in release of the proteins from the membrane. To determine if the accumulated proteins in yeast cells were associated with the membrane, the partitioning of Agα1p between the membrane fraction (sodi-

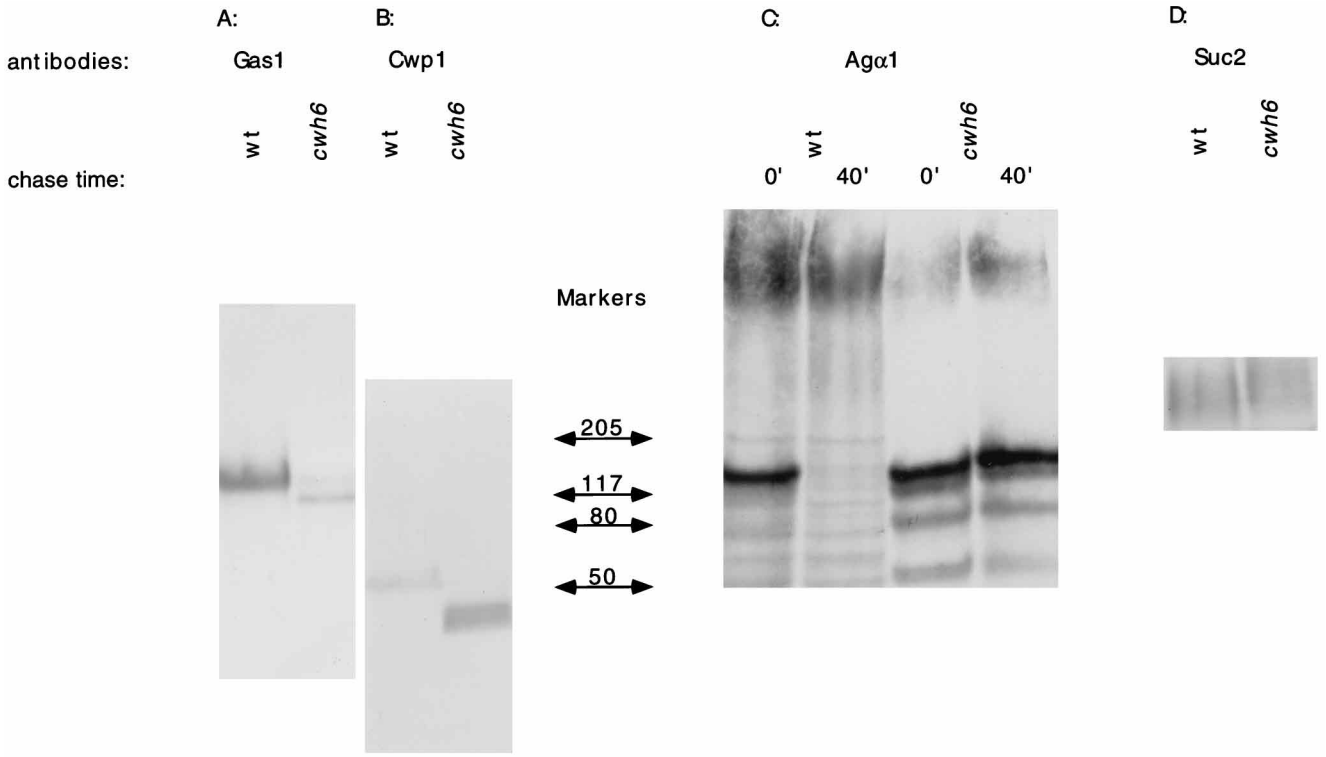

FIG. 3. Intracellular accumulation of precursors of GPI-dependent proteins in *cwh6* cells. (A, B, and D) Western blots of SDS extracts are shown with Gas1p, Cwp1p, and Suc2p antibodies, respectively. (C) α-Agglutinin was immunoprecipitated from SDS extracts of ^{35}S-labeled, **a**-factor-induced cells. The immunoprecipitates were separated on a 6% separating gel with a 4% stacking gel. The autoradiograms are shown. The arrows indicate molecular mass markers (kilodaltons). In each lane, proteins equivalent to 5×10^7 cells were loaded.

um carbonate resistant) and the soluble fraction (100,000 × *g* supernatant) was studied. Figure 4 shows that the low-molecular-weight ER form was present only in the membrane fraction of wild-type cells, whereas in *cwh6* cells the ER form was found also in the soluble fraction together with proteolytic breakdown products. During a 40-min chase period, the ER form of wild-type α-agglutinin was completely converted into a >300-kDa soluble form, which has previously been identified as a soluble periplasmic intermediate (19). In *cwh6* cells, the ER form of α-agglutinin remained present in both the membrane-bound and the soluble fractions. From these results, it can be concluded that Agα1p is partially released from the ER membrane and that transport of both the membrane and the soluble form is strongly retarded in *cwh6* cells.

***cwh6* cells release more proteins into the medium.** Proteins that partition into the soluble or luminal fraction are expected to follow the default pathway and to end up in the vacuole or in the periplasmic region and the growth medium. No HDEL antigenic proteins were found in the medium, indicating that cell lysis was negligible (data not shown). On the other hand, Cwp1p (58 kDa) and Gas1p (125 kDa) were present in the medium of *cwh6* cells (Fig. 5A and B). Also, bands of higher molecular weight were observed. These bands most likely represent protein complexes, since they were absent from the medium of the respective double mutants (JV79 and JV177; data not shown). Cwp1p and Gas1p were not detectable in the medium of wild-type cells. In addition to Cwp1p and Gas1p, *cwh6* cell medium also contained an additional and smaller form of α-agglutinin that was absent in wild-type cell medium (Fig. 5C). This suggests that mistargeting to the medium of GPI-dependent proteins is a general phenomenon in *cwh6* cells. Silver staining of the medium proteins (Fig. 6) showed that the medium of *cwh6* cells contained several proteins that were not seen in the wild-type cell medium. In the medium of the *cwh6 cwp1* double mutant, one band was specifically missing, indicating that the protein migrating at this position in *cwh6* cell medium was Cwp1p. This suggests that the other additional proteins in *cwh6* cell medium are also GPI-depen-

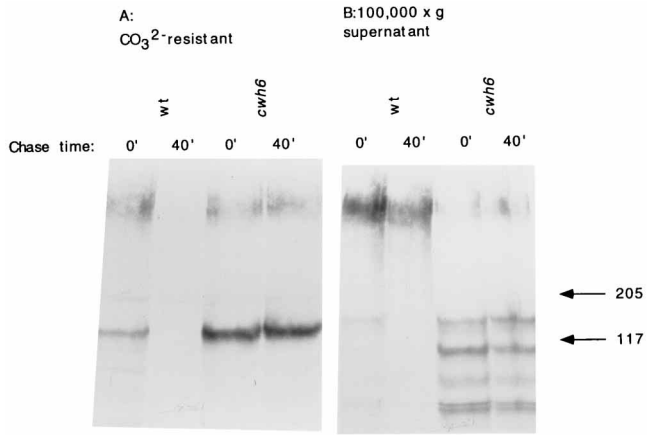

FIG. 4. Membrane association of α-agglutinin precursors. Membrane-bound (A) and membrane-free (B) fractions were prepared as described in Materials and Methods. α-Agglutinin was immunoprecipitated from these fractions, and the precipitates were loaded on 2 to 20% gels. The autoradiograms are shown. The arrows indicate molecular mass markers (kilodaltons). In each lane, proteins equivalent to 5×10^7 cells were loaded.

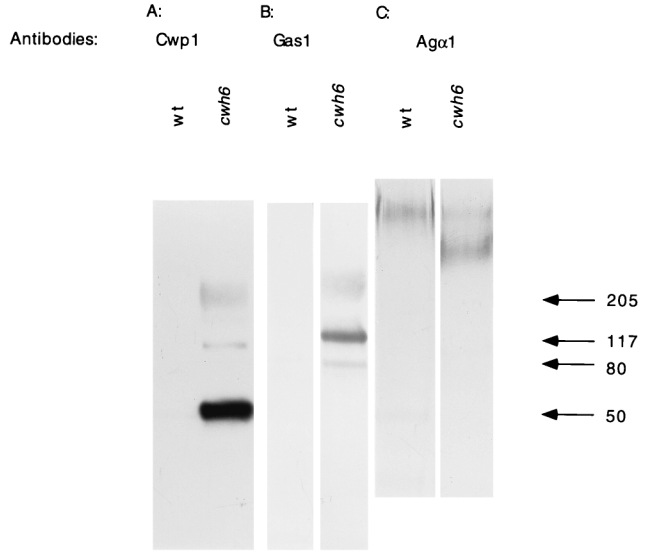

FIG. 5. Secretion of GPI-dependent proteins into the medium of *cwh6* cells. (A and B) Western blots of medium precipitates with Cwp1p and Gas1p antibodies, respectively. (C) α-Agglutinin was immunoprecipitated from medium precipitates of ^{35}S-labeled, **a**-factor-induced cells. The immunoprecipitates were separated on 4% stacking and 6% separating gels. The autoradiograms are shown. The arrows indicate molecular mass markers (kilodaltons). In each lane, proteins equivalent to 5×10^7 cells were loaded.

dent proteins. The total amount of proteins in the medium was measured by [^{35}S]methionine labeling. Compared to wild type, the medium precipitates of *cwh6* cells contained three to four times more label, expressed as a percentage of the total incorporation of radioactive label (Table 3).

The total cell wall protein level in *cwh6* cells is not affected. The increase of proteins in the medium was not accompanied by a decrease in protein levels in the cell wall. When the protein content of the cell wall was measured by ^{35}S labeling, it was found that the protein content of the cell walls—expressed as a percentage of the total label incorporated by the cells—was the same in both the wild type and the mutant (Table 3). From transmission electron microscopy pictures, it was also apparent that the electron-dense outer layer, which corresponds with the cell wall protein layer, was of about equal thickness in mutant and in wild-type cells (Fig. 1). The fact that the limited supply of cell wall proteins in *cwh6* cells did not affect the protein level in the cell wall suggests that cell wall synthesis and doubling time are determined by the supply of cell wall proteins.

TABLE 3. Protein levels in medium, cell walls, and HF-released cell wall material[a]

Strain	Medium	Wall	HF released
Wild type	0.3 ± 0.05	3.7 ± 0.5	1.9 ± 0.3
cwh6	1.1 ± 0.1	3.5 ± 0.2	1.8 ± 0.2

[a] The data are presented as percentages of total incorporated [^{35}S]methionine ± standard errors of the means (n = 3).

A different cell wall protein population is present in *cwh6* cells. The two major polymers of the yeast cell wall, glucan and mannan, are present in about equal monomeric amounts in wild-type yeast cell walls, corresponding to a mannose/glucose ratio of 1.0. Cell walls of *cwh6/gpi3* cells, however, have a mannose/glucose ratio of 0.5 (32). In view of the observation that the total cell wall protein level in *cwh6* cells is not affected, the lowered mannose/glucose ratio indicates that the proteins that are present in *cwh6* cell walls are less extensively mannosylated than in the wild type. Since there was no evidence for a glycosylation defect in α-agglutinin and Cwp1p (Fig. 7A and B), this pointed to a different protein population in the cell wall. Indeed, when proteins in laminarinase extracts from isolated walls were visualized with antibodies against β1,6-glucan (Fig. 7C), three of the four bands seen in wild-type cells were absent or less abundant in the *cwh6* mutant. Cwp1p and α-agglutinin were also less abundant in *cwh6* cell walls (Fig. 7A and

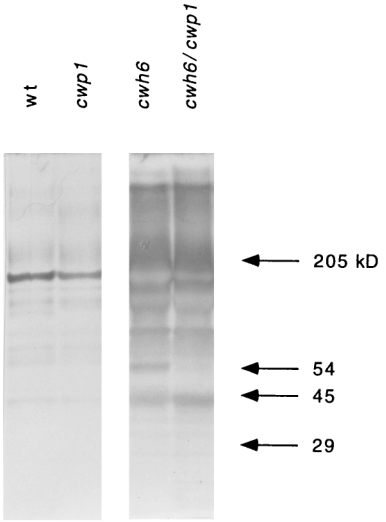

FIG. 6. Protein compositions of the culture medium of wild-type and *cwh6* cells. Concentrated medium proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by silver staining. The medium of *cwh6* cells contains many additional bands. One of the additional bands is missing in the *cwh6 cwp1* double mutant, suggesting that this band represents Cwp1p. The arrows indicate molecular mass markers (kilodaltons). In each lane, proteins equivalent to 5×10^7 cells were loaded.

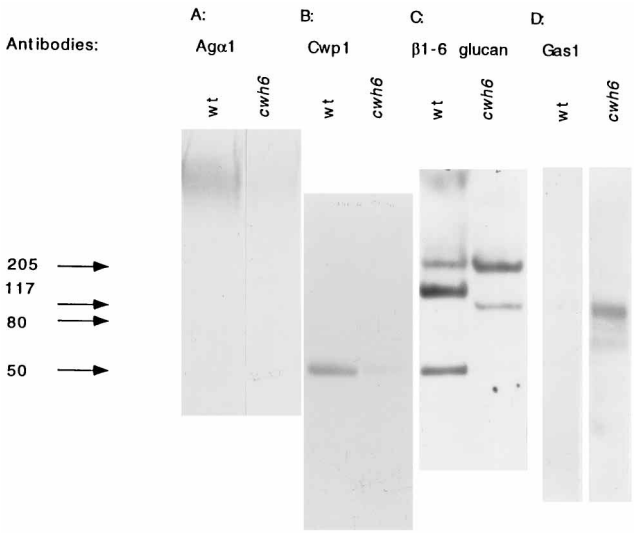

FIG. 7. Cell walls of *cwh6* cells have an altered protein population. Western blots of laminarinase-released proteins with anti-α-agglutinin (A), anti-Cwp1 (B), anti-β-1,6-glucan (C), and anti-Gas1 (D) antibodies are shown. The arrows indicate molecular mass markers (kilodaltons). In each lane, proteins equivalent to 5×10^7 cells were loaded.

B). In contrast, a new β1,6-glucan-containing band appeared and another band became more abundant than in the wild type.

The observed change in the cell wall protein population could be explained by assuming that newly acquired cell wall proteins in *cwh6* cells were not incorporated through a GPI anchor. This idea was tested by making use of the observation by Kapteyn et al. (11) that cell wall proteins can be released with aqueous HF. This treatment specifically cleaves phosphodiester bridges which, among others, are present in the GPI anchor between ethanolamine and mannose. It was, however, observed that HF treatment released equal amounts of label from [^{35}S]methionine-labeled cell walls in both the wild type and the mutant (Table 3), suggesting that equal amounts of proteins in the cell walls of both the wild type and the mutant were linked through a GPI anchor-derived structure.

Mistargeting of Gas1p in *cwh6* cells to the cell wall. In the above paragraph, it was shown that the shift in cell wall protein population was probably not due to a GPI-independent incorporation mechanism of cell wall proteins. This indicates that a different set of GPI-dependent proteins is incorporated into *cwh6* cell walls. As it is known that stress conditions can cause a shift in the expression pattern of β-glucosylated cell wall proteins (13), one might argue that in *cwh6* cells a limited supply of GPI anchors acts as a stress condition, triggering a transcriptional shift in the cell wall protein expression pattern. An alternative or additional explanation for the shift in cell wall protein population might be that proteins that are GPI anchored but that are normally not incorporated into the cell wall, such as Gas1p, are now incorporated in the wall. In wild-type cells, Gas1p localizes—as expected—only to the plasma membrane, but in *cwh6* cells it is also found in the cell wall (Fig. 7D). The absence of any signal in Δ*gas1* and *cwh6*^{ts}Δ*gas1* strains indicated that the antibodies used to detect Gas1p in *cwh6* cell walls were specific (data not shown).

A:

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                        ω
Sed1: VSSSASSHSVVINSN    GANVVVPGALGLAGVAMLFL
Tip1: TSIVETASNAGQRVN    AGAASFGAVVAGAAALLL
Aga1: YQTSSMVTISQYMGG    SGSQIRLPLGKLVFAIMAVACNVIFS
Agα1: QNFTSTSLMISTYFG    KASIFFSAELGSIIFLLLSYLLF
Cwp1: GQIQAPNIVYEQTEN    AGAKAAVGMGAGALAVAAAYLL
Cwp2: VSPSSTETISQQTEN    GAAKAAVGMGAGALAAAAMLL
Flo1: VGYSTASLEISTYAG    SANSLLAGSGLSVFIASLLLAII
Srp1: GQIQATKAVSHQTEN    GAAKAFVGMGAGVVAAAAMLL
```

B:

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Exg2: SSVLSSTTTSRRKSKN    AAISNKLTTSQLLPIKNMSLIWKASVCALAITIAALCASL
Yap3: TTSTASATSTSSKRRN    VGDHIVPSLPLITLISLLFAFI
Mkc7: GMLSPTSSSSPRRKEN    GGHNLNPPFFARFITAIPHHI
Gas1: SSSSSASSSSSSKKRN    AATNVKANLAQVVFTSIISLSTAAGVGFALV
Kre1: VGVIKSAIKKTVSHN     EAQHLGMSSFTSILGGLLTVLIWFL
Sps2: KNGAKSQGSSKKMEN     SAPKNIFIDAFKMSVYAVFTVLFSIIF
Plb1: SASASASGSSTHKKRN    AGNALVNYSNLNINIFIGVLSVISAVFGLI
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FIG. 8. C termini of known GPI-dependent proteins. (A) Cell wall proteins. (B) Plasma membrane-localized GPI-dependent proteins. The potential omega sites are followed by a space. Dibasic motifs are found only in plasma membrane proteins and are in boldface. Plb1 and Sps2 have been tentatively included in the list of plasma membrane proteins because of their dibasic motif close to their potential omega site. References for the sequences are as follows: (A) Sed1, 45; Tip1, 44; Aga1, 5; Agα1, 5, 42; Cwp1, 44; Cwp2, 44; Flo1, 4; Srp1, 43; (B) Exg2, 15; Yap3, 1; Mkc7, 12; Gas1, 41; Kre1, 33.

DISCUSSION

All known covalently linked cell wall proteins contain a putative GPI anchor addition signal, and in some cases, this has been biochemically confirmed (25, 38, 44, 49). Removal of the GPI anchor addition signal results in the release of α-agglutinin into the medium (49). In addition, the C-terminal 30 amino acids of α-agglutinin, which include the GPI anchor addition signal, are sufficient to target a reporter protein to the cell wall (42). These observations indicate that GPI anchors play an important role in the incorporation of proteins in the cell wall. In this study, we wanted to investigate the significance of GPI anchor modification for the biogenesis of cell wall proteins. For this purpose, the temperature-sensitive *cwh6*/*gpi3* cell wall mutant was used. *CWH6*/*GPI3* is involved in the addition of *N*-acetylglucosamine to phosphatidylinositol, which is the first step in the formation of a GPI anchor (36). By growing *cwh6* cells just below the restrictive temperature, the amount of GPI anchor that is available for addition to GPI-dependent proteins is severely reduced but is enough for survival. In this way, the effects of a limited supply of GPI anchors on cell surface growth can be studied. However, formally one cannot exclude the possibility that by growing the cells at a semirestrictive temperature some phenotypic effects may be the result of a more general process of adaptation of the cells.

Abnormal maturation of GPI-dependent proteins in *cwh6* cells. In this study, it is shown that GPI limitation led to the accumulation of GPI-dependent proteins in the ER, with a concomitant increase of ER structures. Under conditions of GPI limitation, export of Gas1p from the ER occurs with reduced efficiency (7). It is shown here that this is also the case for Agα1p and Cwp1p, demonstrating that the GPI modification is a requirement for export from the ER for all GPI-dependent proteins. Since invertase was matured correctly, this is evidence that this was a specific effect on GPI-dependent proteins. This is consistent with the observation that, in cells starved for inositol, which is an essential component of the GPI anchor, secretion of luminal proteins such as invertase and acid phosphatase can continue normally for some time after cell surface growth has stopped (2).

The GPI-dependent proteins that accumulate in the ER are only partially bound to the membrane, suggesting that the accumulated proproteins are gradually converted to a non-membrane-associated form. Maxwell et al. (21) have shown in mammalian cells that, under conditions of impaired processing of GPI-dependent proteins, these proteins can dissociate from the ER membrane. It is likely that a similar reaction also occurs in yeast cells (Fig. 4B) and that, as a result, GPI-dependent proteins are partially mistargeted to the medium (Fig. 6).

Decreased levels of GPI-dependent proteins in the plasma membrane of *cwh6* cells. An additional explanation for the appearance of GPI-dependent cell wall proteins in the medium of *cwh6* cells is based on the observation that in *cwh6* cells the levels of the plasma membrane-localized forms of GPI-dependent proteins are lower. In lysates of *cwh6* cells (Fig. 3), the

125-kDa form of Gas1p, the 54-kDa form of Cwp1p, and the >300-kDa form of Agα1, which represent the plasma membrane-bound forms of these proteins (19, 23, 41), are diminished. This indicates that this might also be the case for other plasma membrane-bound forms of GPI-dependent proteins including Kre1p. As Gas1p and Kre1p are believed to be involved in cross-linking cell wall proteins to the cell wall matrix (31, 33), the appearance of GPI-dependent cell wall proteins in the medium of *cwh6* cells can then be explained by the lack of plasma membrane-localized Gas1p (Fig. 3A) and Kre1p.

Mistargeting of Gas1p to the cell wall of *cwh6* cells. In wild-type cells, Gas1p is directed exclusively to the plasma membrane and not found in the cell wall. In *cwh6*/*gpi3* cells, however, it is detected in the walls even after extraction with hot SDS, indicating that it is tightly associated with the cell wall framework. Mistargeting of Gas1p in *cwh6* cells to the cell wall can be explained in several ways. For example, it is possible that the protein or protein complex at the plasma membrane that cross-links precursors of GPI-containing cell wall proteins to the cell wall uses the suboptimal substrate Gas1p because of a shortage of true cell wall proteins. Alternatively, one might imagine that plasma membrane-bound GPI-dependent proteins, which are expected to be present at a lower level in *cwh6* cells, are themselves involved in sorting or targeting GPI-dependent proteins to the plasma membrane or the cell wall. Interestingly, all putative GPI-dependent plasma membrane proteins including Gas1p possess a dibasic motif, which is located just N terminal from the omega site and is not seen in known cell wall proteins (Fig. 8). This suggests that this motif might be involved in targeting GPI-dependent proteins to the plasma membrane.

Cell wall protein levels in *cwh6* cells are not affected. Despite intracellular accumulation and mistargeting of GPI-dependent proteins to the medium and in contrast to the lower levels of GPI-dependent proteins in the plasma membrane of *cwh6* cells, the cell wall of *cwh6* cells contains similar amounts of proteins as the wild type. As at the semipermissive temperature, the doubling time of *cwh6* cells has increased more than threefold; this indicates that the growth rate of *cwh6* cells is determined by the supply of cell wall proteins. This suggests that GPI-dependent cell wall proteins are essential for cell wall construction. It is also consistent with the notion that the cell wall complex GPI-dependent cell wall protein–β1,6-glucan–β1,3-glucan fraction as described by Kapteyn et al. (11), in which GPI-dependent cell wall proteins function as receptors for the β-glucan fraction, represents a major building block of the yeast cell wall.

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