The *Saccharomyces cerevisiae CWH8* gene is required for full levels of dolichol-linked oligosaccharides in the endoplasmic reticulum and for efficient *N*-glycosylation

Marianne A.A.van Berkel, Michael Rieger[1], Stephan te Heesen[2], Arthur F.J.Ram, Herman van den Ende, Markus Aebi[2] and Frans M.Klis[3]

Institute for Molecular Cell Biology, University of Amsterdam, BioCentrum Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands, [1]Molekularbiologische und Biotechnologische Forschung GmbH, Angelhofweg 39, D-69259 Wilhelmsfeld, Germany and [2]Mikrobiologisches Institut, ETH Zentrum, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland

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[3]To whom correspondence should be addressed

The *Saccharomyces cerevisiae* mutant *cwh8* was previously found to have an anomalous cell wall. Here we show that the *cwh8* mutant has an *N*-glycosylation defect. We found that *cwh8* cells were resistant to vanadate and sensitive to hygromycin B, and produced glycoforms of invertase and carboxypeptidase Y with a reduced number of *N*-chains. We have cloned the *CWH8* gene. We found that it was nonessential and encoded a putative transmembrane protein of 239 amino acids. Comparison of the *in vitro* oligosaccharyl transferase activities of membrane preparations from wild type or *cwh8*Δ cells revealed no differences in enzyme kinetic properties indicating that the oligosaccharyl transferase complex of mutant cells was not affected. *cwh8*Δ cells also produced normal dolichols and dolichol-linked oligosaccharide intermediates including the full-length form $Glc_3Man_9GlcNAc_2$. The level of dolichol-linked oligosaccharides in *cwh8*Δ cells was, however, reduced to about 20% of the wild type. We propose that inefficient *N*-glycosylation of secretory proteins in *cwh8*Δ cells is caused by an insufficient supply of dolichol-linked oligosaccharide substrate.

Key words: Calcofluor white hypersensitive/carboxypeptidase Y/cell wall mutant/hygromycin hypersensitive/vanadate resistant

Introduction

N-Linked glycosylation is an essential modification of secretory proteins in eukaryotic cells. It starts in the lumen of the rough endoplasmic reticulum (ER), where the oligosaccharyl transferase enzyme complex (OTase) catalyses the transfer of a preassembled oligosaccharide $Glc_3Man_9GlcNAc_2$ from a dolichyl pyrophosphate donor (Dol-PP) onto selected asparagine residues of nascent polypeptide chains. The core oligosaccharide is subsequently trimmed by glycosidases in the ER and extended by glycosyltransferases in the Golgi.

In *Saccharomyces cerevisiae*, the oligosaccharyl transferase was purified as an oligomeric complex of six major subunits (Kelleher and Gilmore, 1994), which are encoded by the essential genes *WBP1* (te Heesen *et al*., 1991, 1992), *SWP1* (te Heesen *et al*., 1993), *OST1* (Pathak *et al*., 1995; Silberstein *et al*., 1995a), and *OST2* (Silberstein *et al*., 1995b) and the nonessential genes *OST3* (Karaoglu *et al*., 1995) and *OST5* (Reiss *et al*., 1997). Genetic screens yielded two additional loci affecting oligosaccharyl transferase activity, *STT3* (Zufferey *et al*., 1995) and *OST4* (Chi *et al*.,1996). Mutations in any of these genes result in transfer of fewer oligosaccharides to proteins *in vivo* as well as defective oligosaccharyl transferase activity *in vitro*.

The oligosaccharide is assembled on the lipid carrier dolichyl phosphate (Dol-P). The assembly is initiated by the Alg7 enzyme, that catalyses the transfer of GlcNAc-P from UDP-GlcNAc to Dol-P (Rine *et al*., 1983; Barnes *et al*., 1984). Additional monosaccharides are added to the GlcNAc-PP-Dol intermediate in an ordered stepwise fashion (reviewed in Herscovics and Orlean, 1993; Orlean, 1997). The reactions leading to the formation of $Man_5GlcNAc_2$-PP-Dol are essential. They occur on the cytoplasmic face of the ER membrane and use cytoplasmic UDP-GlcNAc and GDP-Man as sugar donors. The subsequent nonessential reactions leading to the formation of $Glc_3Man_9GlcNAc_2$-PP-Dol use cytoplasmically synthesized Dol-P-Man and Dol-P-Glc as sugar donors, but occur on the lumenal face of the membrane. Therefore, the $Man_5GlcNAc_2$-PP-Dol intermediate, Dol-P-Man, and Dol-P-Glc are believed to flip over the ER membrane (reviewed in Abeijon and Hirschberg, 1992). The full-length dolichol-linked oligosaccharide is the preferred substrate for the oligosaccharyl transferase, but truncated oligosaccharides can be transferred to protein with reduced efficiency, both *in vitro* (Trimble *et al*., 1980; Sharma *et al*., 1981) and *in vivo* (Huffaker and Robbins, 1983; Stagljar *et al*., 1994; Aebi *et al*., 1996; Burda *et al*., 1996). The *S.cerevisiae alg* mutants are affected in the assembly of the oligosaccharide. These mutants accumulate dolichol-linked oligosaccharide intermediates and transfer fewer oligosaccharide chains to proteins *in vivo*, but display normal oligosaccharyl transferase activity *in vitro* when full-length dolichol-linked oligosaccharides are exogenously supplied (Huffaker and Robbins, 1982; Huffaker and Robbins, 1983; Runge *et al*., 1984; Runge and Robbins, 1986; Albright and Robbins, 1990; Jackson *et al*., 1993; Stagljar *et al*., 1994; Aebi *et al*., 1996; Burda *et al*., 1996; Reiss *et al*., 1996).

Here we describe a new gene, *CWH8*, that is required for efficient addition of *N*-linked oligosaccharides to secretory proteins in the ER. We show that *cwh8*Δ cells are resistant to vanadate and sensitive to hygromycin B, and produce glycoforms of invertase and carboxypeptidase Y with a reduced number of *N*-chains. We found that in *cwh8*Δ cells the level of dolichol-linked oligosaccharides is severely reduced. We therefore propose that in *cwh8*Δ cells part of the *N*-glycosylation sites of secretory proteins pass the oligosaccharyl transferase without being glycosylated due to a limiting amount of dolichol-linked oligosaccharide substrate. The nonessential *CWH8* gene encodes a putative transmembrane protein. Interestingly, the sequence of Cwh8p suggests the presence of a phosphate binding pocket. The possibility is discussed that Cwh8p might act as an accessory protein that interacts with Dol-P, thereby facilitating the initial

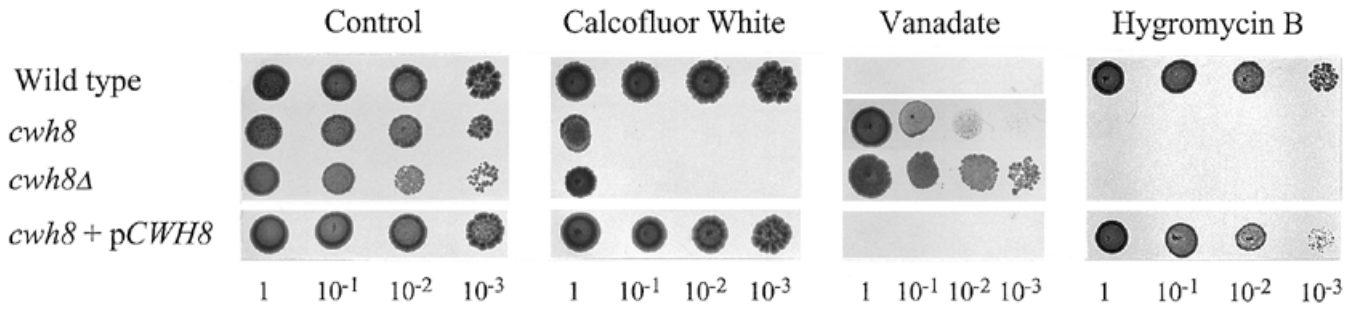


Fig. 1. The *CWH8* locus affects the sensitivity of cells to Calcofluor white, vanadate, and hygromycin B. A 10-fold dilution series of the indicated strains (starting with 5×10^5 cells) was inoculated on selective media containing either no drug (control) or Calcofluor white (5 μg/ml), vanadate (5 mM), or hygromycin B (50 μg/ml).

biosynthetic step in the formation of dolichol-linked oligosaccharides.

Results

The cwh8 mutation affects N-linked glycosylation in vivo

The *cwh8* mutant was isolated in a broad cell wall mutant screen based on examining mutagenized cells for increased sensitivity to the cell wall destabilizing agent Calcofluor white (Ram *et al*., 1994). Earlier studies have shown that *cwh8* cells grow slowly and have an altered wall in which the amount of mannan is reduced to about 50% of the wild type level (Ram *et al*., 1994). As most of the mannan in the wall is *N*-glycosidically linked to wall proteins (Klis, 1994), we tested whether the *cwh8* mutation affects *N*-glycosylation. We therefore grew wild type and *cwh8* cells on media containing vanadate or hygromycin B, as yeast mutants with growth defects due to abnormal *N*-glycosylation often exhibit resistance to vanadate and sensitivity to hygromycin B (Ballou *et al*., 1991; Dean, 1995). Figure 1 clearly shows that *cwh8* cells were resistant to vanadate and hypersensitive to hygromycin B. Subsequent analysis of the secretory proteins invertase and carboxypeptidase Y confirmed that the *cwh8* mutation affects *N*-linked glycosylation. The model glycoprotein invertase contains on average 9–10 *N*-linked glycans, which consist of a core oligosaccharide extended with an outer chain of variable size (Esmon *et al*., 1981; Reddy *et al*., 1988). Outer chain synthesis is blocked in the *mnn9* mutant (Ballou *et al*., 1980). Figure 2A shows that wild type cells produced invertase with an M_r of 130–210 kDa (lane 1). *cwh8* cells, however, synthesized invertase with an M_r of 70–180 kDa (lane 2). The difference in Mr was due to *N*-linked glycans, as removal of these sugar chains with Endo H yielded the same de-*N*-glycosylated species of 62 kDa for both wild type and *cwh8* invertase (lanes 5 and 6). Interestingly, a considerable amount of invertase produced by *cwh8* cells was smaller than the core glycosylated 90 kDa species made by *mnn9* cells (lanes 2 and 4), suggesting that *cwh8* invertase contained fewer *N*-linked glycans. This was further investigated by analyzing another model glycoprotein, carboxypeptidase Y (CPY). Vacuolar CPY contains four *N*-linked oligosaccharides (Hasilik and Tanner, 1978) consisting of a core part that is not extended with an outer chain (Ballou *et al*., 1990). As a result, CPY glycoforms differing in the number of *N*-linked oligosaccharides migrate as distinct, evenly spaced bands on SDS–PAGE. Figure 2B shows that wild type cells produced a predominant glycoform of CPY of 61 kDa (lane 1), corresponding to the mature vacuolar form carrying 4 *N*-linked oligosaccharides. Endo H digestion yielded a deglycosylated 51 kDa protein (lane 5). *cwh8* cells produced multiple CPY glycoforms (lane 2), migrating as a ladder between fully glycosylated CPY and the deglycosylated protein. Based on the number of bands and the stepwise difference in molecular mass of about 2 kDa, we conclude that they contain between 0 and 4 *N*-linked oligosaccharides. The glycoforms containing 1 or 2 *N*-chains were most abundant. As Endo H treatment leaves one GlcNAc residue per *N*-chain on the protein, treated wild type CPY contains four GlcNAc residues, whereas treated *cwh8* CPY contains predominantly 1 or 2 GlcNAc residues. In agreement with this, Endo H digestion yielded a deglycosylated *cwh8* form of CPY that was slightly smaller than the corresponding wild type species (lanes 5 and 6).

Isolation of the CWH8 gene and construction of a cwh8Δ null mutant

The *CWH8* gene was isolated by complementation of the Calcofluor white hypersensitivity of the *cwh8* mutant. The *cwh8* mutant was transformed with a plasmid pool containing partially digested yeast genomic DNA ligated in a centromere-containing plasmid. Approximately 5×10^5 transformants were replica-plated onto medium containing Calcofluor white. Nine transformants grew like wild type and their plasmids were isolated. Restriction mapping showed that all plasmids contained an identical 6.4 kb genomic insert. Subcloning experiments showed that the complementing activity could be delimited to a 1.4 kb fragment (Figure 3A). Searching the SGD sequence database with sequences obtained from both ends of this fragment revealed that it covered one open reading frame. This open reading frame *CWH8* encodes a protein of 239 amino acids (Figure 3B) with several potential membrane-spanning domains (Figure 3C; Kyte and Doolittle, 1982; Nakai, 1991; Nakai and Kanehisa, 1992; Rost *et al*., 1995). *CWH8* not only complemented the Calcofluor white hypersensitivity of the *cwh8* mutant, but also the slow growth rate, the vanadate resistance and the hygromycin B sensitivity (Figure 1) as well as the underglycosylation of CPY (Figure 2B, lane 4).

To further analyze Cwh8p function, we generated haploid cells in which the *CWH8* gene was deleted. These c*wh8*Δ cells displayed similar phenotypes as the *cwh8* mutant. They grew

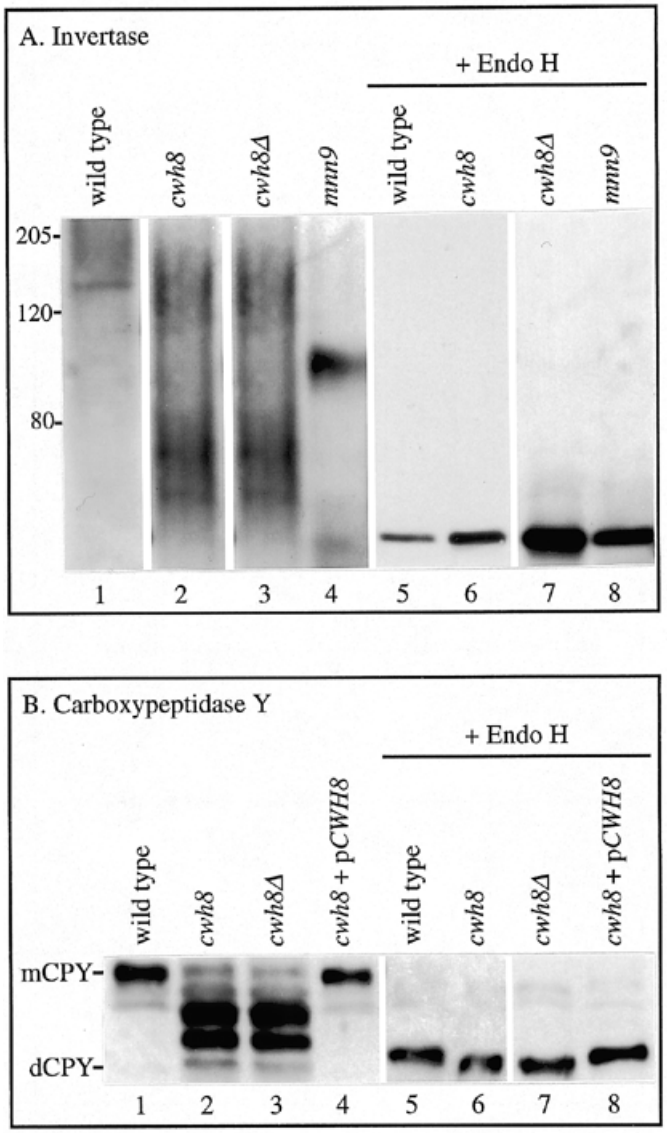


Fig. 2. The CWH8 locus affects the *N*-linked glycosylation of invertase and carboxypeptidase Y. (**A**) Total cell lysates of cells that were grown on low glucose medium to induce invertase synthesis were subjected to Western analysis with invertase antiserum. Marker sizes are indicated in kDa. (**B**) Total cell lysates of cells grown on rich medium were subjected to Western analysis with carboxypeptidase Y antiserum. Mature vacuolar CPY containing 4 *N*-linked oligosaccharides (mCPY, 61 kDa) and vacuolar CPY carrying no *N*-linked oligosaccharides (dCPY, 51 kDa) are indicated. Part of the samples were treated with Endo H.

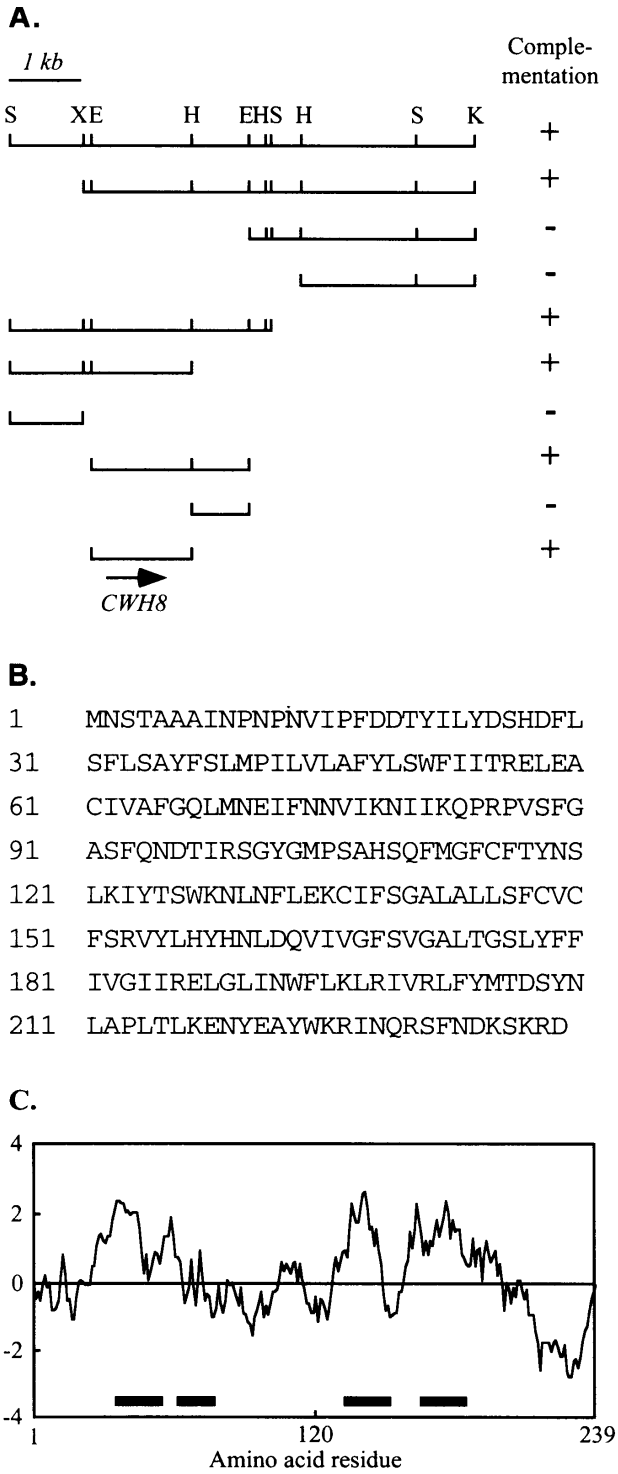


Fig. 3. The *CWH8* gene. (**A**) Isolation of the *CWH8* gene. A 6.4 kb DNA fragment isolated from the yeast genomic library (upper line) was subcloned into various fragments that were tested for their ability (+) or inability (-) to complement the Calcofluor white hypersensitivity of the *cwh8* mutant. Arrow indicates location and orientation of the *CWH8* gene. The *CWH8* gene is identical to ORF YGR036C of the SGD sequence database. Restriction sites are mapped as follows: S, *Sac*I; X, *Xba*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I. The outer *Sac*I and *Kpn*I restriction sites are derived from the multiple cloning site of the vector. (**B**) Deduced amino acid sequence of the *Cwh8* protein. (**C**) Hydrophobicity profile of the Cwh8 protein according to Kyte and Doolittle (1982) using a window of 10 amino acids. Analysis of putative transmembrane domains by the method of Rost *et al*. (1995) revealed four potential transmembrane helices (indicated by bars). Psort analysis (Nakai, 1991; Nakai and Kanehisa, 1992) revealed three transmembrane domains (lacking the second one).

slowly with a doubling time of 250 min, whereas wild type cells doubled in 90 min. They had an altered wall in which the amount of mannan was reduced to about 30% of the wild type level. They were hypersensitive to Calcofluor white, resistant to vanadate and hypersensitive to hygromycin B (Figure 1), and they underglycosylated invertase (Figure 2A, lane 3) and CPY (Figure 2B, lane 3).

The CWH8 locus affects N-linked glycosylation in vitro

The glycoprotein immunoblots of Figure 2 had shown that the *CWH8* locus affects the number of *N*-linked glycans that become

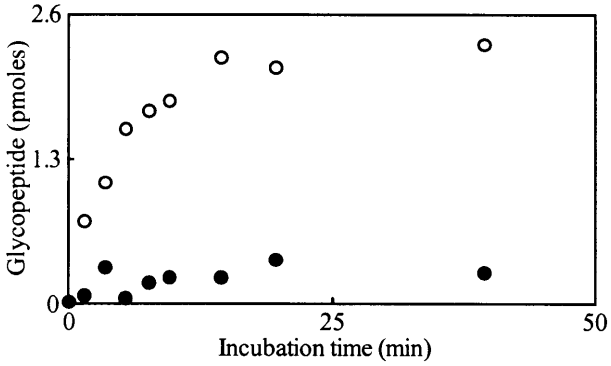


Fig. 4. The *CWH8* locus affects *N*-linked glycosylation *in vitro*. Oligosaccharyl transfer was assayed in membrane preparations isolated from wild type cells (open circles) or *cwh8*Δ cells (solid circles) using synthetic tripeptides as sugar acceptor. Endogenous dolichol-linked oligosaccharides (present in the membrane preparations) functioned as sugar donor (assay two). Membranes containing 100 μg of protein were incubated with 5 μM tripeptide for the indicated times.

attached to secretory proteins *in vivo*. This prompted us to investigate the role of the *CWH8* gene in oligosaccharyl transfer in microsomal membranes *in vitro*. Figure 4 shows that membranes isolated from *cwh8*Δ cells were severely affected in their ability to glycosylate a synthetic tripeptide acceptor *in vitro*, even after prolonged incubation times. In this *in vitro* assay, membranes isolated from either wild type or *cwh8*Δ cells were used as a source for the oligosaccharyl transferase. The dolichol-linked oligosaccharides present in the same membrane preparations functioned as sugar donor and the synthetic tripeptide that we added to the membranes as sugar acceptor. The reduced oligosaccharyl transfer by *cwh8*Δ membranes might therefore be related to the oligosaccharyl transferase or to the dolichol-linked oligosaccharides. The following experiments were done to identify the cause of the reduced oligosaccharyl transfer.

*The oligosaccharyl transferase of cwh8*Δ *cells is not affected*

In the experiments shown in Figure 5, we again used membrane preparations isolated from either wild type or *cwh8*Δ cells as source of the oligosaccharyl transferase, and synthetic tripeptide as oligosaccharide acceptor, but we now also added bovine dolichol-linked oligosaccharides as exogenous oligosaccharide donor. Under these conditions, the time course of oligosaccharyl transfer by *cwh8*Δ membranes was indistinguishable from that by wild type membranes (Figure 5A). The oligosaccharyl transfer was proportional to the incubation time for about 20 min, after which it leveled off and reached a plateau value in about 3 h. When an incubation time of 15 min was used, the oligosaccharyl transfer was proportional to the amount of enzyme (Figure 5B). The plateau value that was reached when an incubation time of 3 h was used was independent of the amount of enzyme and increased linearly with the amount of dolichol-linked oligosaccharides that was added (Figure 5C). When the rate of oligosaccharyl transfer was determined as a function of the concentration of donor oligosaccharide, nearly identical saturation curves were obtained for wild type and *cwh8*Δ membranes (Figure 5D). Similar Km values for the dolichol-linked oligosaccharide were calculated from the data depicted in Figure 5D (0.11 ± 0.01 μM for wild type membranes versus 0.12 ± 0.02 μM for *cwh8*Δ membranes), as well as similar maximal reaction rates (9.0 ± 0.6 pmol min^{-1} mg $protein^{-1}$ for wild type membranes versus 9.4 ± 0.5 pmol min^{-1} mg $protein^{-1}$ for *cwh8*Δ membranes). These maximal reaction rates, however, were limited by the concentration of acceptor peptide that was used (5 μM), as can been seen in Figure 5E, which shows that increasing the acceptor peptide concentration resulted in higher reaction rates. The V^{max} values reached under saturating conditions were calculated to be 54 ± 6 pmol min^{-1} mg $protein^{-1}$ for wild type membranes versus 48 ± 5 pmol min^{-1} mg $protein^{-1}$ for *cwh8*Δ membranes. In addition, the Km value for the acceptor peptide was not significantly changed (69 ± 12 μM for wild type membranes versus 58 ± 9 μM for *cwh8*Δ membranes). Clearly, deletion of the *CWH8* gene does not significantly affect the kinetic properties of the oligosaccharyl transferase.

*Cwh8*Δ *cells produce normal dolichols and full length dolichol-linked oligosaccharides*

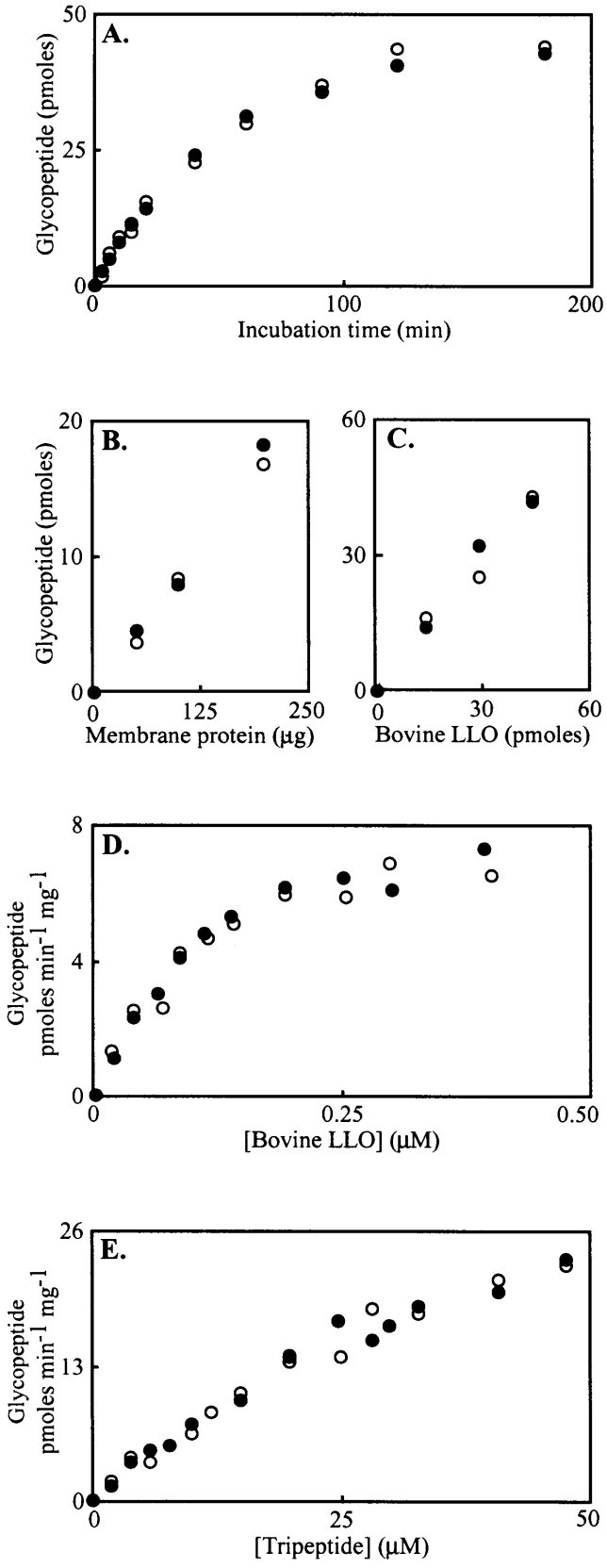
The glycosylation defect of *cwh8*Δ cells might also be caused by a defect in the synthesis of dolichol or by incomplete assembly of the core oligosaccharide, leading to the synthesis of aberrant dolichol-linked oligosaccharides that are suboptimal sugar donors for the oligosaccharyl transferase. It might also be caused by a defect in maintaining optimal levels of the dolichol-linked oligosaccharides, so that less sugar donors are available for the oligosaccharyl transferase. We therefore investigated the synthesis of dolichol-linked oligosaccharides in wild type and *cwh8*Δ cells.

First, we tested whether dolichol-linked oligosaccharides from *cwh8*Δ cells are suitable substrates for oligosaccharyl transfer. We metabolically labeled wild type and *cwh8*Δ cells with D-[2–^{3}H]-mannose and extracted their labeled dolichol-linked oligosaccharides. We then incubated microsomal membranes with equal amounts of labeled dolichol-linked oligosaccharides extracted from either wild type or *cwh8*Δ cells, and assayed oligosaccharyl transfer to endogenous membrane proteins. Table I shows that both wild type and *cwh8*Δ membranes were equally active towards both substrates. This suggests that dolichol-linked oligosaccharides from *cwh8*Δ cells do not have an aberrant structure. To confirm this, we analyzed the synthesis of dolichol and the assembly of the core oligosaccharide in wild type and *cwh8*Δ cells.

Table I. Dolichol-linked oligosaccharides from wild type and *cwh8*Δ cells function equally well in oligosaccharyl transferase assays

Membranes	Oligosaccharyl transferase activity (% of label incorporated)	
	Wild type DLOs	*cwh8*Δ DLOs
Wild type	19 ± 2.6	19 ± 4.7
*cwh8*Δ	25 ± 3.1	22 ± 5.4

DLOs, Dolichol-linked oligosaccharides. Oligosaccharyl transfer was assayed in membrane preparations isolated from either wild type or *cwh8*Δ cells using endogenous membrane proteins as sugar acceptor and equal amounts of 2-[^{3}H]-mannose labeled dolichol-linked oligosaccharides isolated from either wild type or *cwh8*Δ cells as sugar donor (assay three). Membranes containing 100 μg protein were incubated for 30 min with 1×10^5 d.p.m. of 2-[^{3}H]-mannose labeled dolichol-linked oligosaccharides extracted from about 8 mg (wet weight) of radiolabeled wild type cells or about 20 mg (wet weight) of radiolabeled *cwh8*Δ cells. Activity is expressed as the percentage of label incorporated.



To examine the types of dolichol that are present in wild type versus *cwh8*Δ membranes, we incubated wild type and *cwh8*Δ membranes with [γ-^{32}P]CTP using conditions in which the membrane-associated dolichol kinase Sec59p is active (Bernstein *et al*., 1989; Heller *et al*., 1992). ^{32}P-labeled dolichols were then extracted and analyzed by reversed phase chromatography. Four types of ^{32}P-labeled dolichols were detected in wild type extracts, corresponding to chain lengths of 14–17 isoprene units. The elution profiles of mutant extracts were closely similar to that of wild type extracts, and mixing of wild type and *cwh8*Δ extracts did not reveal any differences. These results indicate that deletion of the *CWH8* gene does not affect the structure of the dolichols that are synthesized.

Next, the assembly of the core oligosaccharide was investigated. Therefore, the labeled dolichol-linked oligosaccharides that were isolated from either wild type or *cwh8*Δ cells were released from their dolichol pyrophosphate carriers by acid hydrolysis and analyzed by HPLC. The elution profile of wild type cells (Figure 6A) shows successive biosynthetic intermediates up to the full-length oligosaccharide $Glc^3Man^9GlcNAc^2$, clearly reflecting the sequential addition of monosaccharides by a series of glycosyl transferases. The identity of the full-length oligosaccharide was proven by mixing with reference oligosaccharide $Glc^3Man^9GlcNAc^2$ (results not shown). Figure 6B indicates that in mutant cells the levels of most dolichol-linked oligosaccharides were reduced (see also below). Importantly, the full-length oligosaccharide also seemed present in *cwh8*Δ cells, and this was confirmed by mixing wild type and *cwh8*Δ extracts (Figure 6C). Clearly, deletion of the *CWH8* gene does not lead to a block in oligosaccharide assembly. Wild type and *cwh8*Δ extracts showed differences in the relative amount of biosynthetic intermediates. Compared to wild type cells, very little $Man_5GlcNAc_2$ and less $Man_7GlcNAc_2$ were present in *cwh8*Δ extracts, whereas $Man_6GlcNAc_2$ was abundant. The amount of $Glc_3Man_9GlcNAc_2$ was also reduced. These differences in relative concentrations probably reflect differences in Km values of the various glycosyl transferases. When the supply of dolichol-linked oligosaccharides in the ER decreases and becomes limiting, reactions with a low Km will take preference over those with higher ones.

Fig. 5. The oligosaccharyl transferase of *cwh8*Δ cells is not affected. Oligosaccharyl transfer was assayed in membrane preparations isolated from either wild type cells (open circles) or *cwh8*Δ cells (solid circles) using bovine dolichol-linked oligosaccharides as sugar donor and synthetic tripeptide as sugar acceptor (assay one). (**A**) Time course of oligosaccharyl transfer. Membranes containing 100 μg protein were incubated with 0.45 μM dolichol-linked oligosaccharides and 5 μM tripeptides for the indicated times. (**B**) Oligosaccharyl transfer as a function of membrane concentration. Membranes containing the indicated amounts of protein were incubated with 0.45 μM dolichol-linked oligosaccharides and 5 μM tripeptides for 15 min. (**C**) Linear relationship between substrate and product when reactions were run to completion. Membranes containing 100 μg protein were incubated for 3 h with 5 μM tripeptides and the indicated amounts of dolichol-linked oligosaccharides. (**D**) Rate of oligosaccharyl transfer as a function of the concentration of dolichol-linked oligosaccharides. Membranes containing 100 μg protein were incubated for 15 min with 5 μM peptide and the indicated concentrations of dolichol-linked oligosaccharides. (**E**) Rate of oligosaccharyl transfer as a function of the tripeptide concentration. Membranes containing 50 μg protein were incubated for 15 min with 0.45 μM dolichol-linked oligosaccharides and the indicated tripeptide concentrations.

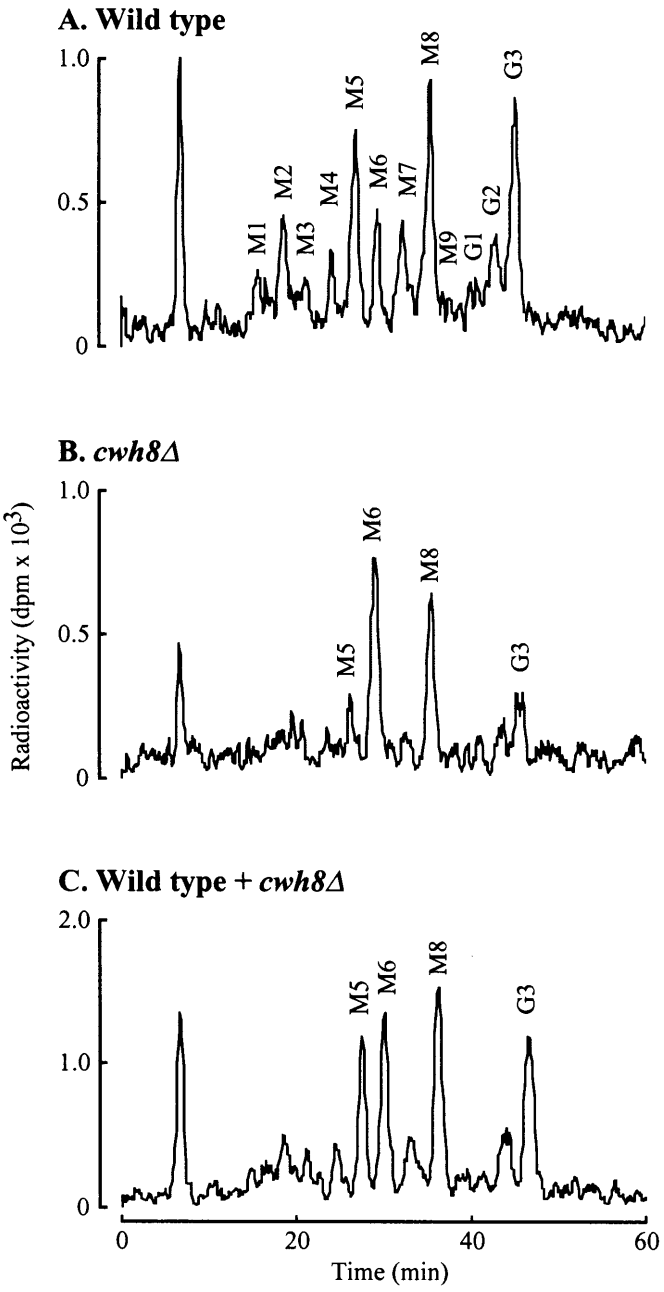


Fig. 6. *cwh8*Δ cells produce full-length dolichol-linked oligosaccharides. Dolichol-linked oligosaccharides were extracted from wild type or *cwh8*Δ cells that were metabolically labeled with D-[2–^{3}H]-mannose. Oligosaccharides were released from their dolicholpyrophosphate carriers by acid hydrolysis and analyzed by HPLC. Equal cell equivalents were loaded, containing 1.5×10^5 d.p.m. of activity for wild type cells and 0.6×10^5 d.p.m. for *cwh8*Δ extracts. (**A**) Wild type oligosaccharides. (**B**) *cwh8*Δ oligosaccharides. (**C**) Mixture of wild type and *cwh8*Δ oligosaccharides. The positions of $Man^{1-9}GlcNAc^2$ (M1–9) and $Glc^{1-3}Man^9GlcNAc^2$ (G1–3) are indicated.

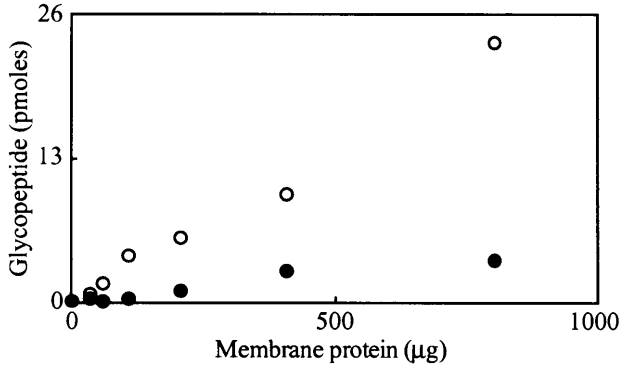


Fig. 7. *cwh8*Δ membranes contain much less dolichol-linked oligosaccharides than wild type membranes. Oligosaccharyl transfer was assayed in membrane preparations isolated from wild type cells (open circles) or *cwh8*Δ cells (solid circles) using synthetic tripeptides as sugar acceptor. Endogenous dolichol-linked oligosaccharides (present in the membrane preparations) functioned as sugar donor (assay two). Reactions were allowed to proceed to completion. Membranes containing the indicated amounts of protein were incubated for 60 min with 5 μM tripeptide.

Cwh8D membranes contain much less dolichol-linked oligosaccharides than wild type membranes

When cells were labeled with D-[2–^{3}H]-mannose and their dolichol-linked oligosaccharides were extracted, *cwh8*Δ extracts contained only 40% of the radioactivity found in wild type extracts ([$7.0 \pm 1.8 \times 10^5$ d.p.m.] and [$17.3 \pm 4.7 \times 10^5$ d.p.m.], respectively). This suggests that *cwh8*Δ cells contained less dolichol-linked oligosaccharides than wild type cells. To test this, we incubated variable amounts of membranes with acceptor tripeptide until the oligosaccharyl transfer was complete. For both wild type and *cwh8*Δ membranes, the plateau values reached were proportional to the amounts of membrane added. However, the *cwh8*Δ values were only ~20% of the wild type values (Figure 7). When *cwh8*Δ cells were transformed with a plasmid containing the *CWH8* gene, wild type values were restored (results not shown). As we have demonstrated that the *cwh8*Δ oligosaccharyltransferase was not affected and as the *cwh8*Δ dolichol-linked oligosaccharides function equally well as sugar donor as those from wild type cells when supplied in equal amounts, we conclude that the level of dolichol-linked oligosaccharides in *cwh8*Δ cells is 20% of the level in wild type cells.

Discussion

CWH8 is required for efficient N*-glycosylation*

We have isolated a new gene, *CWH8*, that is required for the efficient addition of *N*-linked glycans to secretory proteins. It is a nonessential gene encoding a 239 amino acid protein with several potential membrane-spanning domains, that is probably localized in the ER membrane. We have shown that *cwh8*Δ cells grow slowly, are resistant to vanadate and sensitive to hygromycin B, and produce glycoforms of invertase and carboxypeptidase Y with a reduced number of *N*-chains. Furthermore, we have shown that the *CWH8* gene is required for maintaining optimal levels of dolichol-linked oligosaccharides. In *cwh8*Δ cells, the level of dolichol-linked oligosaccharides is reduced to about 20%. The inefficient *N*-glycosylation of secretory proteins in *cwh8*Δ cells is therefore probably caused by an insufficient supply of sugar donors, the dolichol-linked oligosaccharides, for the oligosaccharyl transferase. As a result, some *N*-glycosylation sites may pass the oligosaccharyl transferase complex without being glycosylated.

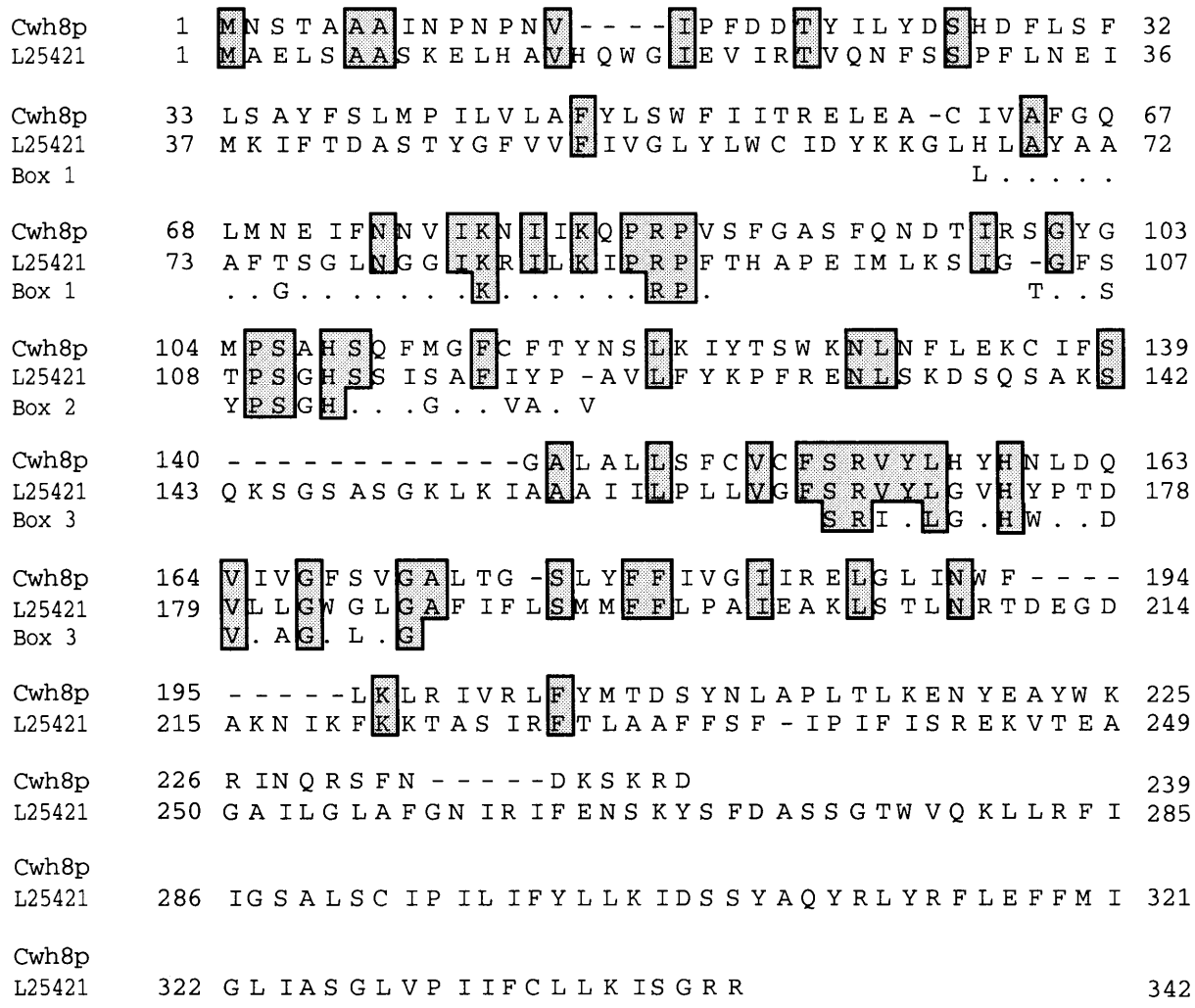


Fig. 8. Cwh8p shows homology with phosphate binding sequences. Alignment of Cwh8p with a *Treponema denticola* phosphatase (Altschul *et al*., 1990; Ishihara and Kuramitsu, 1995; GenBank accession number L25421) and with three short stretches of amino acids that are believed to form a phosphate binding pocket in a wide variety of phosphatases (Hemrika *et al*., 1997, box 1–3). Dashes indicate gaps introduced to improve alignment and dots indicate nonconserved amino acids in the phosphate binding boxes. The homology between Cwh8p and the *T. denticola* phosphatase was disclosed by comparison of the Cwh8p sequence with protein databases using the BLASTP protein sequence comparison algorithm (Altschul *et al*., 1990). Within an overlapping region of 163 amino acids (comprising amino acids 42–193 of Cwh8p) both proteins shared 27% sequence identity.

What is the function of Cwh8p?

A comparison of the Cwh8p sequence with protein sequence databases revealed a homology with phosphate binding sequences (Figure 8; Altschul *et al*., 1990; Ishihara and Kuramitsu, 1995; Hemrika *et al*., 1997). This suggests that Cwh8p might contain a phosphate binding pocket, and raises the question which phosphorylated compound Cwh8p might act upon and how this might affect the levels of dolichol-linked oligosaccharides in the endoplasmic reticulum. Recently, a putative phosphate binding pocket was identified in a lipid phosphatase, namely, the pgpB gene product in *E.coli*, which catalyzes the dephosphorylation of diacylglycerolpyrophosphate and of phosphatidic acid (Icho, 1988; Dillon *et al*., 1996), and in the Wunen protein of *Drosophila melanogaster* which shows strong similarity to the enzyme type II phosphatidic acid phosphatase (Zhang *et al*., 1997). It seems an attractive possibility that Cwh8p functions as phosphatase in the Dol-PP and Dol-P metabolism. This is supported by the recent finding of a multicopy suppressor of *cwh8*Δ encoding a lipid phosphatase (C. Frank and Dr. S. te Heesen, unpublished observations). On the other hand, this is not consistent with recent results (Dr. C. J. Waechter, personal communication) suggesting that the Dol-PP and Dol-P phosphatase activities are increased in *cwh8*Δ cells and that the Dol-PP phosphatase activity is reduced in cells overexpressing the *CWH8* gene. An alternative possibility, therefore, is that Cwh8p plays a role in the initiation of the biosynthesis of dolichol-linked oligosaccharides by binding Dol-P and making it available to the Alg7 enzyme, thereby facilitating the formation of GlcNAc-PP-Dol. According to this hypothesis, Cwh8p is an accessory protein that facilitates the first step of the biosynthesis of dolichol-linked oligosaccharides.

Materials and methods

Yeast strains, growth conditions, and genetic methods

The *S.cerevisiae* strains used were *cwh8* (*MAT*α *cwh8 ura3–52*) and its isogenic wild type AR27 (*MAT*α *ura3–52*) (Ram *et al*., 1994), *cwh8*Δ (*MAT***a** *cwh8*Δ::*HIS3 his3*Δ*300 ura3–52 leu2*Δ*1*

lys2Δ202 trp1Δ63) (this report) and its isogenic wild type FY833 (*MAT***a** *his3Δ300 ura3–52 leu2Δ1 lys2Δ202 trp1Δ63*) (Winston *et al*., 1995), and LB 347–1C (*MATα mnn9*) (Ballou *et al*., 1980). Cells were grown in rich medium (YPD; 1% (w/v) yeast extract, 2% (w/v) Bactopeptone and 2% (w/v) glucose) or selective medium based on SD (0.67% (w/v) yeast nitrogen base and 2% (w/v) glucose) supplemented with the appropriate amino acids and nucleotides (Guthrie and Fink, 1991). For solid media, 2% (w/v) agar was added. Drug-sensitivity was assayed by spotting 5 μl aliquots of a 10-fold dilution series of a cell suspension (containing 1×10^8 cells/ml) on solid media containing 5 μg Calcofluor white/ml, 5 mM vanadate, or 50 μg hygromycin B/ml. Stock solutions of the drugs were appropriately diluted into cooled, autoclaved medium just before pouring the plates. Media containing Calcofluor white were buffered to pH 6.0 with 50 mM MES. Calcofluor white M2RS was obtained from American Cyanamid Co. and hygromycin B was obtained from Boehringer, Mannheim, Germany. Standard procedures were used for genetic crosses, sporulation of diploids, and dissection of tetrads (Rose *et al*., 1990).

Glycoprotein analysis

Total cell lysates from exponentially growing cells were subjected to 7% SDS–PAGE and Western analysis with carboxypeptidase Y antiserum as described previously (Aebi *et al*., 1996). To induce the synthesis of invertase, exponentially growing cells were switched from YPD medium to $YPD_{0.05\%}$ medium (containing 0.05% (w/v) glucose) for 3 h (Ballou, 1990). Total cell lysates were then prepared as described previously (Aebi *et al*., 1996), subjected to 6% SDS–PAGE, and electrophoretically transferred to Immobilon polyvinylidene difluoride membranes for Western analysis with invertase antiserum. Membranes were treated with 50 mM periodic acid, 100 mM sodium acetate (pH 4.5) prior to blocking (Schreuder *et al*., 1993). Invertase antiserum (kindly provided by H. Bussey, McGill University, Montreal, Quebec, Canada) was used in a dilution of 1:1000. Binding of the invertase antiserum was visualized with secondary goat-anti-rabbit-IgG-peroxidase and ECL detection reagents. Recombinant endo-β-N-acetylglucosamidase H (Endo H) was obtained from Boehringer, Mannheim, Germany, and was used according to the manufacturer's instructions. Fractions for Western analysis with carboxypeptidase Y antiserum were equivalent to the total cell lysates of about 0.5 mg cells (wet weight) or to the Endo H–treated total cell lysates of about 0.25 mg cells. Fractions for Western analysis with invertase antiserum were equivalent to cell lysates of about 4 mg cells, or to the Endo H–treated cell lysates of about 2 mg cells.

Isolation of the CWH8 *gene*

Dr. C.Boone (Simon Fraser University, BC, Canada) kindly provided a yeast genomic library containing partially digested chromosomal DNA (size: 5–10 kb) ligated into the centromere based vector pRS316 with the *URA3* selectable marker (Sikorski and Hieter, 1989). The library was transformed into strain *cwh8* by the lithium acetate method of Ito *et al*. (1983), and transformants were selected on medium lacking uracil. About 5×10^5 transformants were replica-plated onto selective medium containing 0.5 mg Calcofluor white/ml. This concentration is lethal to the *cwh8* mutant, but can be overcome by its corresponding wild type strain AR27. Plasmids from transformants that grew like wild type were isolated by the method of Hoffman and Winston (1987), amplified in *Escherichia coli* DH5α, and tested for their ability to restore the Calcofluor white hypersensitivity of the *cwh8* mutant upon retransformation. For subcloning experiments, the vector YCplac33 was used (Gietz and Sugino, 1988). All DNA manipulations were carried out according to standard procedures (Sambrook *et al*., 1989). The *CWH8* gene was mapped to chromosome VII by probing a chromosome blot (Clontech, Palo Alto, CA) with a labeled fragment containing the *CWH8* gene (the 2.1 kb *Eco*RI-*Eco*RI fragment shown in Figure 3A), and to the overlapping λ clones 70129 and 70523 of the λ phage library of mapped yeast genomic DNA (obtained from the ATTC) (Riles *et al*., 1993; results not shown). Partial sequence analysis showed that the *CWH8* gene is identical to ORF YGR036C of the *Saccharomyces* Genome Database (SGD). Northern analysis showed that the *CWH8* gene is expressed in wild type cells (result not shown).

Construction of a cwh8Δ *null mutant*

We used the method of Baudin *et al*. (1993). PCR amplification of the *HIS3* gene from the template plasmid YDp-H (Berben *et al*., 1991) with primers 5′-TCA TTA TAC CAG GCA TTT TAT CTA CAT CAA AAT TTG TTT TGA ATC GGA TCA TAT CGC TAG CTT GGC TGC AGG-3′ and 5′-CTC AAG ATC GCC TCG CTA AAA TAG ATG AAA AAA TAA GAA AAA TGA AAT GTT CAA CGA ATT CCC GGG GAT CCG-3′ generated a 1.2 kb *HIS3* cassette flanked by 55 bp stretches homologous to the DNA immediately upstream and downstream of the *CWH8* coding sequence. The PCR product was transformed to strain FY833. Histidine auxotroph transformants were selected and subjected to Southern analysis to confirm correct integration of the PCR product at the *CWH8* locus. Chromosomal DNA was isolated, digested with *Eco*RI and probed with the labeled 0.8 kb *Hind*III-*Eco*RI fragment located at the 3′ end of the *CWH8* gene (indicated in Figure 3A). A restriction fragment of 1.2 kb characterized a disrupted *CWH8* locus (*cwh8Δ*::*HIS3*), whereas a restriction fragment of 2.2 kb characterized an intact *CWH8* locus (results not shown). The original *cwh8* mutation and the constructed *cwh8Δ* deletion failed to complement each other in a diploid strain *cwh8*/*cwh8Δ*::*HIS3* (results not shown), strongly suggesting that they are alleles of the same locus. Analysis of meiotic tetrads was hindered by the extremely low number of asci that was obtained after induction of sporulation.

Labeling, extraction, and analysis of dolichol-linked oligosaccharides

Metabolic labeling of exponentially growing cells with D-[2–^{3}H]-mannose, extraction of dolichol-linked oligosaccharides, release of oligosaccharides from their dolichol pyrophosphate carriers by acid hydrolysis, and analysis of oligosaccharides by HPLC were performed as described previously (Zufferey *et al*., 1995), except that another gradient was used for the HPLC analysis, namely, acetonitrile/3 mM sodium phosphate buffer, pH 6.0 (NaPi), 70:30 (v/v), to acetonitrile/NaPi, 59:41 (v/v) in 10 min, to acetonitrile/NaPi, 48:52 (v/v) in 40 min, then to NaPi in 5 min, then for 5 min in NaPi, and then back to acetonitrile/NaPi, 70:30 (v/v) in 5 min.

In vitro *oligosaccharyl transfer in microsomal membranes*

Various oligosaccharide donors and acceptors were used to assay the *in vitro* oligosaccharyl transfer in microsomal membranes.

Dolichol-linked oligosaccharides were extracted from bovine pancreas as described by Reiss *et al*. (1997) and were dissolved in chloroform/methanol/water (CMW), 10:10:3 (v/v/v). The concentration of dolichol-linked oligosaccharides in the bovine extract was determined to be 3 pmol/μl based on oligosaccharyl transferase assays (type one, see below) which were run until all oligosaccharide donors were used up. D-[2–^{3}H]-Mannose-labeled dolichol-linked oligosaccharides were isolated from wild type and *cwh8*Δ cells as described by Zufferey *et al*. (1995). The terminally acetylated and amidated tripeptide Nα-AC-NYT-NH_2 (Wieland *et al*., 1987; purchased from Tana Laboratories, Houston) was iodinated with [^{125}I]-NaI (Amersham) according to Reiss *et al*. (1997) and was suspended in H_2O to a concentration of 100 pmol/ml. The initial specific activity was approximately 10,000 c.p.m./pmol. Microsomal membranes were prepared according to Reiss *et al*. (1997) and were suspended in membrane buffer (35% glycerol, 50 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1mM $MnCl_2$,1 mM DTT, 1 mM PMSF, 2 μg/ml each of pepstatin A, leupeptin, chymostatin, antipain, and aprotinin) to a protein concentration of 10 μg/ml (as determined by the Bradford Bio-Rad protein assay using bovine serum albumin as a standard). The membrane preparations were frozen in liquid nitrogen and stored at -80°C. The oligosaccharyl transferase activity of the membrane preparations varied with age. After 1 month of storage at -80°C, a 2-fold reduction in activity was observed. Therefore, in all experiments we used wild type and *cwh8*Δ membranes of the same age. As a control, membranes were inactivated prior to incubation by heating for 10 min at 95°C. All experiments were carried out several times. Representative experiments are shown.

Assay one using bovine dolichol-linked oligosaccharides as sugar donor and [^{125}I]-iodinated synthetic tripeptides as acceptor (Reiss et al*., 1997)*

Unless indicated otherwise, this assay was performed as follows. Fifteen microliters of bovine dolichol-linked oligosaccharides in CMW, 10:10:3 (v/v/v) (45 pmol) were dried in a Savant Speedvac and suspended in 74 μl oligosaccharyl transferase buffer (67 mM Tris–HCl, pH 7.5, 33 mM NaCl, 4 mM $MnCl_2$, 187 mM sucrose, 0.27% Nonidet NP-40). After adding 10 ml membrane buffer, 1 μl 0.1 M DDT, and 5 μl [^{125}I]-iodinated tripeptide (500 pmol), the reaction was started by adding 10 μl membrane preparation (100 μg membrane protein). After a 15-min incubation at 23°C, the reaction was stopped by adding 100 μl ice-cold 2% NP-40 and 1 ml ice-cold wash buffer (50 mM Tris–HCl, pH7.5, 1M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 0.01% NP-40). Glycosylated tripeptide was then recovered by Concanavalin A Sepharose affinity chromatography. The reaction mixture was incubated with 100 μl Concanavalin A Sepharose beads (Pharmacia) in wash buffer (1:1, v/v) for 20 min at 4°C while rotating. After three washes with 1 ml wash buffer, the activity retained on the beads was quantified with a Cobra (Packard) gamma counter.

Assay two using endogenous, membrane-associated dolichol-linked oligosaccharides as sugar donor and [^{125}I]-iodinated synthetic tripeptides as acceptor

This assay was carried out as assay one, except that no bovine dolichol-linked oligosaccharides were added. Yeast dolichol-linked oligosaccharides present in the membrane preparations functioned as oligosaccharide donor. In this assay, the activities were very low. We noticed that the activities increased about 6-fold by omitting NP-40 from the oligosaccharyl transferase buffer. In the experiments presented in Figures 4 and 7, NP-40 was therefore omitted from the oligosaccharyl transferase buffer.

*Assay three using labeled dolichol-linked oligosaccharides isolated from wild type or cwh8*Δ *cells as sugar donor and endogenous proteins as acceptor*

This assay was carried out as assay one, except that 1×10^5 d.p.m. of D-[2–^{3}H]-mannose-labeled dolichol-linked oligosaccharides isolated from wild type or *cwh8*Δ cells were used instead of bovine dolichol-linked oligosaccharides. Also, no [^{125}I]-iodinated tripeptide was added (5 μl of H_2O was used instead). Endogenous membrane proteins functioned as sugar acceptor. In this assay, the reaction was stopped by adding 670 μl of chloroform:methanol (CM), 1:1 (v/v), to yield a ratio of CMW of 10:10:3 (v/v/v). Membranes were pelleted by centrifugation at 10,000 × *g* for 10 min and then washed twice with 1 ml CMW, 10:10:3 (v/v/v), to remove the dolichol-linked oligosaccharides. The activity retained on the membranes was quantified with a Beckman counter.

Labeling, extraction, and analysis of dolichols

Dolichol kinase assays were modified from the procedure of Heller *et al*. (1992) (D. Grassi, personal communication). Microsomal preparations containing 1200 μg membrane protein (120 μl) were incubated with 6 μl [λ-^{32}P]-CTP (10 μCi/μl, 25 Ci/mmol, ICN) in a final reaction volume of 400 μl containing 50 mM Tris–HCl, pH 7.5, 0.4% Nikkol, 30 mM $CaCl_2$, 10 mM UTP, and 9 μM unlabeled CTP. After a 40 min incubation at 24°C, the reaction was stopped by adding 750 μl 1 M KOH in methanol, and alkali-labile lipids were hydrolyzed by incubation for 25 min at 37°C. The lipids were extracted by the Folch-procedure (Folch *et al*., 1957), dried under nitrogen at 37°C, and suspended in 2-propanol/methanol/water (PMW), 40:60:5 (v/v/v), containing 20 mM H_3PO_4. HPLC-analysis of dolichol-phosphates was performed as described by Elmberger *et al*. (1989) except that PMW, 40:60:5 (v/v/v), 20 mM H_3PO_4, was used as eluent for 15 min, followed by a linear gradient of PMW, 40:60:5 (v/v/v), 20 mM H_3PO_4, to acetonitrile over 1 min (D. Grassi, personal communication). After each run, the column was washed for 10 min in acetonitrile, then brought back to PMW, 40:60:5 (v/v/v), 20 mM H_3PO_4, in a linear gradient over 1 min, and eluted for 23 min at PMW, 40:60:5 (v/v/v), 20 mM H_3PO_4. The flow rate was 1 ml/min.

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Abbreviations

CPY, carboxypeptidase Y; Dol-P, dolichyl phosphate; Dol-PP, dolichyl pyrophosphate; Endo H, endo-β-N-acetylglucosamidase H; ER, endoplasmic reticulum; OTase, oligosaccharyl transferase; SGD, *Saccharomyces* Genome Database.

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