The CWH8 Gene Encodes a Dolichyl Pyrophosphate Phosphatase with a Luminally Oriented Active Site in the Endoplasmic Reticulum of Saccharomyces cerevisiae*

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Mutations in the CWH8 gene, which encodes an ER transmembrane protein with a phosphate binding pocket in Saccharomyces cerevisiae, result in a deficiency in dolichyl pyrophosphate (Dol-P-P)-linked oligosaccharide intermediate synthesis and protein N-glycosylation (van Berkel, M. A., Rieger, M., te Heesen, S., Ram, A. F., van den Ende, H., Aebi, M., and Klis, F. M. (1999) Glycobiology 9, 243–253). Genetic, enzymological, and topological approaches were taken to investigate the potential role of Cwh8p in Dol-P-P/Dol-P metabolism. Overexpression of Cwh8p in the yeast double mutant strain, lacking LPP1/DPP1, resulted in an impressive increase in Dol-P-P phosphatase activity, a relatively small increase in Dol-P phosphatase activity, but no change in phosphatidate (PA) phosphatase activity in microsomal fractions. The Dol-P phosphatase encoded by CWH8 is optimally active in the presence of 0.5% octyl glucoside and relatively unstable in Triton X-100, distinguishing this activity from the lipid phosphatases encoded by LPP1 and DPP1. Stoichiometric amounts of P, and Dol-P are formed during the enzymatic reaction indicating that Cwh8p cleaves the anhydride linkage in Dol-P-P. Membrane fractions from Sf-9 cells expressing Cwh8p contained a 30-fold higher level of Dol-P phosphatase activity, a slight increase in Dol-P phosphatase activity, but no increase in PA phosphatase relative to controls. This is the first report of a lipid phosphatase that hydrolyzes Dol-P-P/Dol-P but not PA. In accord with this enzymatic function, Dol-P-P accumulated in cells lacking the Dol-P phosphatase. Topological studies using different approaches indicate that Cwh8p is a transmembrane protein with a luminally oriented active site. The specificity, subcellular location, and topological orientation of this novel enzyme are consistent with a role in the re-utilization of the glyceryl carrier lipid for additional rounds of lipid intermediate biosynthesis after its release during protein N-glycosylation reactions.

Although the biosynthesis of dolichol-linked saccharide intermediates is initiated on the cytoplasmic side of the endoplasmic reticulum (ER), dolichyl pyrophosphate (Dol-P-P) and several dolichyl monophosphate (Dol-P) molecules are released on the luminal surface during protein N-glycosylation reactions, glycosylphosphatidylinositol anchor synthesis, C- and O-mannosylation of proteins (see reviews in Refs. 1–3). For the dolichyl moiety of Dol-P-P to be re-utilized for additional rounds of lipid intermediate biosynthesis, it must be converted to Dol-P before or after returning to the cytoplasmic leaflet of the ER.

There have been many reports of Dol-P phosphatase activities in microsomal fractions from mammalian tissues (see reports cited in Ref. 3). However, the subcellular location and topological arrangement of the active sites of these enzymes have not been definitively established. A Dol-P phosphatase activity reported in brain (4) is curiously enriched in Golgi fractions and is an unlikely candidate to be involved in the recycling of Dol-P-P. The LPP1 and DPP1 genes in Saccharomyces cerevisiae, which have homology to a Mgp††-independent phosphatidate (PA) phosphatase, have been shown to encode enzymes capable of converting Dol-P-P to Dol-P and dephosphorylating Dol-P, as well as PA in vitro (5–7). However, because there are no growth or protein N- or O-glycosylation defects associated with mutations in these enzymes, it is doubtful that Dol-P/P and/or Dol-P are the primary substrates in vivo.

Recently, van Berkel et al. (8) isolated a cwh8 mutant from S. cerevisiae using a broad cell wall mutant screen and demonstrated that it is deficient in the synthesis of dolichol-linked oligosaccharide intermediates and protein N-glycosylation. CWH8 encodes a putative ER transmembrane protein of 239 amino acid residues with a lipid phosphate binding pocket, raising the possibility that it could plausibly be involved in Dol-P-P/Dol-P metabolism. To explore this potential function, we have conducted enzymological and topological studies with the yeast double mutant, Δlpp1Δdpp1, and Sf-9 cells expressing CWH8.

By expressing Cwh8p in the yeast double mutant and Sf-9 cells, it was possible to distinguish the properties and specificity of this novel lipid phosphatase from the enzymes encoded by LPP1/DPP1. The enzymatic studies presented here reveal that

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1 The abbreviations used are: ER, endoplasmic reticulum; Dol-P, dolichyl monophosphate; Dol-P-P, dolichyl pyrophosphate; PA, phosphatidate; OG, octyl glucoside; PCR, polymerase chain reaction; TNM-FH, Trichoplusia ni medium with yeastolate and lactalbumin hydrolysate; PMSF, phenylmethylsulfonyl fluoride; TM, transmembrane region.
CWH8 encodes a Dol-P-P phosphatase that was previously overlooked because of its characteristic lability in the presence of Triton X-100 under conditions that are optimal for the lipid phosphatases encoded by LPP1/DPP1. These results provide solid evidence that CWH8 encodes an enzyme, which can be readily assayed in vitro in the presence of 0.5% octyl glucoside (OG), capable of converting Dol-P-P to Dol-P and P_i on the luminal surface of the ER in yeast. The support for these conclusions and the possible role of the enzyme in the recycling of the glycosyl carrier lipid for additional rounds of lipid intermedi-ate synthesis are discussed. Some aspects of this study have been presented in preliminary form (9).

**EXPERIMENTAL PROCEDURES**

**Materials**—n-Octyl-β-D-glucopyranoside was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). Triton X-100 was from Pierce Chemical Co. (Rockford, IL). Trichloroacetonitrile, tetrabutylammonium pyrophosphate was synthesized enzymatically using Catharanthus ro- seus (PharMingen) into a monolayer of Autographa californica DNA (PharMingen) into a monolayer of Sf-9 cells grown in 75-cm² tissue culture flasks. The Sf-9 cells were infected at a multiplicity of 10 and grown in TMN-FH medium under 10% heat-inactivated fetal bovine serum for 48 h. The infected cells were collected by gentle trituration with medium, harvested by centrifugation, and washed twice with phosphate-buffered saline. The final cell pellet was snap-frozen over dry ice and stored at −80 °C.

**Preparation of Radiolabeled Phosphorylated Lipid Substrates**—[32P]Dol-P-P, [32P]Dol-P, and [32P]Poly-P-P were chemically synthesized in anhydrous acetone from the appropriate lipid using [32P]Tetrabutylammonium phosphate and trichloroacetonitrile as described by Danilov et al. (18). Nonradio- active polisoprenyl phosphates were synthesized employing tetrabutylammonium phosphate. Dioleyl-PA was obtained from Sigma-Aldrich (St. Louis, MO). Synthetic substrates were purified by prepara-tive thin-layer chromatography on Baker Si250 thin-layer plates de-veloped in CHCl₃/CH₃OH/H₂O/NH₄OH (65:35:6.1 v/v, and quantitated by lipid-phosphorus analysis according to Bartlett (19).

**Preparation of Microsomal Fractions and Assay of Dol-P-P, Dol-P, and PA Phosphatase Activity in Vitro**—Yeast strains were routinely grown in liquid culture containing Yeast Nitrogen Base (Difco), 50 mg/ml L-arginine, 40 mg/ml L-glutamine, 0.5% glucose, 2 mM 5′-adenosine pyrophosphate/5′-guanosine pyrophosphate bases (25 mg/liter) as required. Yeast cultures were grown to an A₆₀₀ of ~1.0 and sedimented by centrifugation (1000 × g, 10 min). The cell pellets were washed by resuspension in phosphate-buffered saline and re-sedimented. The cell pellets were washed in ice-cold 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 5 mM EDTA, and 5 mM 2-mercaptoethanol and resuspended in this buffer to ~100 A₆₀₀/ml. Cells were then lysed at 0−4 °C by shaking (five 1-min cycles) in a Braun homogenizer with glass beads (5 mm). Following removal of unbroken cells and dense organelles by sedimentation at 10,000 × g, 20 min. Yeast microsomes were resuspended to a membrane protein concentration of 10−20 mg/ml in 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 5 mM EDTA, and 5 mM 2-mercaptoethanol and stored at −20 °C until used for enzyme assays. The lipid phosphatase activities were assayed essentially by the procedures described previously (4, 7).

**Preparation of Cell Extracts and Assay Procedure for Dicacylglycerol Pyrophosphate Phosphatase**—All steps were performed at 5 °C. Yeast cells were disrupted with glass beads with a Mini-Bead-Buster (Biosped Products) in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM disodium EDTA, 1% Triton X-100, 50 mM 2-mercaptoethanol (20). Cell debris, broken yeast cells, and cell debris were removed by centrifugation at 1500 × g, 5 min. The supernatant was used as the cell extract.

**Analysis of Dol-P and Dol-P-P—Wild type (SS238) and Δcwh8 (YG 1001) strains were grown in YPD to an A₆₀₀ of ~1.5. Cells were collected by centrifugation. The cell pellets were resuspended in water, and total lipids were extracted twice with 5 volumes of CHCl₃/CH₂OH (2:1, v/v, the organic solvent was evaporated, and the sample was redissolved in 2 ml of CH₃OH-toluene (1:1) and 2 ml of 0.2 M KOH in CH₃OH. Samples were incubated at 0 °C for 60 min and were neutralized with 0.4 M of NaOH acetic acid. CHCl₃ (4 ml) and 1.3 M of 0.9% NaCl were added and incubated on ice for 10 min. The lower phase (organic) was washed twice with CHCl₃/CH₂OH/0.9% NaCl (3:4:87). The washed lower phase was dried under an atmosphere of N₂. The lipid sample was dissolved in CHCl₃/CH₂OH/H₂O (10:10:3) and loaded onto a DEAE-Toyopearl column (0.5 × 5 cm). The column was washed sequentially with 10 volumes of CHCl₃/CH₂OH/H₂O (10:10:3), 5 volumes of CHCl₃/CH₂OH/H₂O (10:10:3) with 2 and 20 mM ammonium acetate. Then 3 ml of CHCl₃/CH₂OH/H₂O (10:10:3) with 200 mM ammonium acetate was loaded. Dol-P-P was eluted with CHCl₃/CH₂OH/H₂O (10:10:3) and dried. Lipid phosphatase activities were determined with Dol-P-P as the substrate. For Dol-P, the pertinent fractions were subjected to strong alkaline hydrolysis (3 × KOH in 40% methanol, 100 °C, 60 min). Dol-P was extracted after the addition of 12 ml of methanol and 48 ml of dichloromethane to the hydrolysate, and the mixture was incubated for 1 h at 40 °C. The organic (lower) phase was removed and washed four times with equal volumes of dichloromethane/methanol/water (3:48:47). The washed or-
Dolichyl Pyrophosphate Phosphatase

**TABLE I**

<table>
<thead>
<tr>
<th>Yeast strains used in these studies and their genotypes</th>
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<tbody>
<tr>
<td><strong>Strain</strong></td>
</tr>
<tr>
<td>W303-1A</td>
</tr>
<tr>
<td>Δcwh8</td>
</tr>
<tr>
<td>Δlpp1 Δdpp1 (TRY1)</td>
</tr>
<tr>
<td>Δlpp1 Δdpp1/CWH8</td>
</tr>
<tr>
<td>Δcwh8/LPP1 (YG 099)</td>
</tr>
<tr>
<td>Δcwh8/RER2 (YG 099)</td>
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<tr>
<td>Δcwh8/SEC59 (YG 099)</td>
</tr>
<tr>
<td>Δcwh8/YEp352 (YG 1001)</td>
</tr>
<tr>
<td>SS328</td>
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<td>FC2a</td>
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</table>

Expression of the *S. cerevisiae* CWH8 Gene in a Δlpp1Δdpp1 Mutant and in Insect Cells—The CWH8 gene was isolated by PCR amplification using genomic DNA from strain W303-1A as the template. The gene was then used to construct a multicopy plasmid for the overexpression of the CWH8 gene product in wild type and Δlpp1Δdpp1 mutant cells. CWH8 was expressed in the Δlpp1Δdpp1 mutant so that its properties could be readily distinguished from the lipid phosphate phosphatase activities encoded by the DPP1 and LPP1 genes. In *vitro*, the DPP1 and LPP1 gene products account for most of the Mg^{2+}-independent PA phosphatase, lysophosphatidate phosphatase, and isoprenoid phosphatase activities, and all of the diacglycerol pyrophosphate phosphatase activity of *S. cerevisiae* (5–7). All of the yeast strains used in this study and their corresponding genotypes are listed in Table I. To further examine the activity encoded by the CWH8 gene product, we used heterologous expression of the CWH8 gene in SF-9 insect cells. The CWH8 gene was placed within the genome of baculovirus under control of the polyhedrin promoter and expressed by viral infection of SF-9 cells.

Dol-P-P Phosphatase Encoded by CWH8 Is Labile in Triton X-100 in Contrast to the Lipid Phosphatases Encoded by LPP1/DPP1 in *S. cerevisiae*—A recent study has indicated that the lipid phosphatases encoded by LPP1 and DPP1 are responsible for the labile performance of Dol-P-P phosphatase activity.
Dolichyl Pyrophosphate Phosphatase

At present it is established that the lipid phosphatase encoded by the CWH8 gene, when expressed in Sf-9 cells, has the same activity and specificity as the phosphatase encoded by the LPP1 gene. The presence of Triton X-100 in the assay medium, however, was quite stable (8) (CWH8/CWH8) strain contained a lipid phosphatase gene which encodes a microsomal lipid phosphatase activity encoded by CWH8 in vitro assays were conducted with microsomal fractions from the Δlpp1Δdpp1 double mutant expressing CWH8. These results were compared with the lipid phosphatase activity encoded by LPP1/DPP1 using microsomes from a strain in which CWH8 was deleted. From Table II it can be seen, as previously reported, that the lipid phosphatases related to LPP1/DPP1 (5–7) are capable of actively hydrolyzing Dol-P-P, Dol-P, and PA when assayed in the presence of 1% Triton X-100. The activity seen in the presence of Triton X-100 is greatly diminished by deleting the LPP1/DPP1 genes. However, the results in Table II reveal that the novel phosphatase encoded by CWH8, which is active in the presence of OG, hydrolyzes Dol-P-P at a very high rate, and Dol-P at a much lesser rate. It is important to note that no dephosphorylation of PA was observed under these in vitro conditions in contrast to the lipid phosphatase encoded by LPP1/DPP1 (5–7). This novel lipid phosphatase also actively hydrolyzed the fully unsaturated, long-chain (C95)polyprenyl pyrophosphate, a substrate closely related structurally to Dol-P-P (data not shown).

When complete saturation curves were generated for Dol-P-P, Dol-P, and PA, Dol-P-P (Fig. 2A) was actively hydrolyzed in a concentration-dependent manner whereas Dol-P was dephosphorylated at a much lower rate (Fig. 2B). PA dephosphorylation was barely detectable. When the initial rates for Dol-P-P and Dol-P were analyzed by double-reciprocal plots, the catalytic efficiency for Dol-P-P cleavage was 164 times greater than that for Dol-P. The rates of hydrolysis of PA were not significantly higher enough to calculate meaningful kinetic constants.

This is the first report of a lipid phosphatase that hydrolyzes Dol-P-P/Dol-P but not PA (5–7, 27–29). Similarly, the level of diacylglycerol pyrophosphate phosphatase activity was not changed compared with wild type strains, in microsomes from the yeast strains overexpressing CWH8 or the cwh8 null allele mutant (data not included).

The reaction used to determine if Cwh8p cleaved the anhydride bond of Dol-P-P or released pyrophosphate ions and dolichol, Dol-32P-32P, labeled in both the α- and β-phosphorus atoms, was incubated with microsomes from the Δlpp1Δdpp1/CWH8 strain, and the water-soluble and lipid products were analyzed. As seen in Table III, there were virtually stoichiometric amounts of Dol-32P and 32Pi formed during the enzymatic reaction. The slightly higher proportion of 32P, observed is probably due to the subsequent, but relatively slow, dephosphorylation of Dol-32P catalyzed by Cwh8p in vitro. Based on all of these results we conclude that the CWH8 gene encodes a microsomal lipid phosphatase that specifically converts Dol-P-P to Dol-P and P,

Expression of CWH8 in Sf-9 Cells—To obtain additional proof that CWH8 encodes a Dol-P-P phosphatase, the yeast gene was expressed in a baculovirus system. As compared with control membrane fractions, membranes isolated from Sf-9 cells expressing CWH8 contained at least a 30-fold higher level of related Dol-P-P/Dol-P phosphatase activity was overlooked in previous in vitro studies (5–7).

When the concentration of OG was varied, a fairly sharp optimum was seen at 0.3–0.5% (w/v). The Dol-P-P phosphatase apparently does not have a strict divalent cation requirement, because the reaction was not affected by an excess of EDTA. A very modest stimulation was observed variably with 2 mM MgCl2. When assayed in the presence of OG, The CWH8 Dol-P-P phosphatase exhibited a broad pH optimum between 6 and 7.

Lipid Phosphatase Encoded by CWH8 Actively Converts Dol-P-P to Dol-P but Does Not Hydrolyze PA or Diacylglycerol Pyrophosphate—To examine the specificity of the lipid phosphatase encoded by CWH8, in vitro assays were conducted with microsomal fractions from the Δlpp1Δdpp1 double mutant expressing CWH8. These results were compared with the lipid phosphatase activity encoded by LPP1/DPP1 using microsomes from a strain in which CWH8 was deleted.

For virtually all of the isoprenyl mono- and pyrophosphatase activities in yeast microsomes when assayed in the presence of Triton X-100 (7). However, the observation that the Δlpp1Δdpp1 double mutant had no growth or protein N- or O-glycosylation defect suggested that yeast contained other lipid phosphatases capable of dephosphorylating Dol-P/Dol-P-P in vivo that were not detected when assayed in vitro in the presence of Triton X-100.

In an attempt to evaluate the potential role of Cwh8p in Dol-P/Dol-P-P metabolism, microsomal fractions were prepared from the Δlpp1Δdpp1 double mutant overexpressing CWH8 to explore the possibility that the CWH8 gene encoded a lipid phosphatase with different properties and substrate specificity than the enzymes encoded by LPP1/DPP1. A preliminary screen of various detergent indicated that microsomes from the Δlpp1Δdpp1/CWH8 strain contained a lipid phosphatase that was unstable in the presence of Triton X-100 but active in the presence of OG with optimal activity seen at 0.5%. The initial experiments also revealed that Dol-P-P was an excellent substrate, and it was used for further enzymatic analyses. As seen in Fig. 1, the Dol-P-P phosphatase encoded by CWH8 was extremely labile when preincubated in the presence of Triton X-100 (panel A, open circles), but the activity encoded by LPP1/DPP1 was quite stable (panel A, closed circles), as reported previously (7). The Dol-P-phosphatase encoded by LPP1/DPP1 was also active and stable in the presence of Triton X-100 (panel B, closed circles) whereas the Dol-P-phosphatase activity in the microsomes from the Δlpp1Δdpp1 double mutant expressing CWH8 was barely detectable when the lipophilic substrate was dispersed in Triton X-100 (panel B, open circles). These results provide a tenable explanation of why the CWH8-
Phosphatase activities were assayed in the presence of Triton X-100 or octyl glucoside in microsomes from various yeast strains.

Phosphatase activities were assayed in the presence of Triton X-100 by incubating microsomes (6 μg of protein) from the indicated yeast strain, 10 mM EDTA, 50 mM Na-HEPES (pH 7.4), 0.5% Triton X-100, and 100 μM \([\text{32P}]\text{Dol-32P}\) or \([\beta-\text{32P}]\text{Dol-P-P}\) in a total volume of 0.02 ml. Phosphatase activities were assayed in the presence of OG by incubating microsomes (6 μg of protein) from the indicated yeast strain, 1 mM EDTA, 5 mM MgCl\(_2\), 0.5% OG, 50 mM sodium HEPES (pH 7.7), and 100 μM \([\text{32P}]\text{Dol-P-P}\) in a total volume of 0.02 ml. Following incubation for 5 min (1 min for Dow-P phosphatase) at 30 °C, the amount of \(^{32}\)P released from each substrate was assayed essentially as described elsewhere (7, 32, 55).

<table>
<thead>
<tr>
<th>Microsomes from</th>
<th>Detergent</th>
<th>Substrate for lipid phosphatase</th>
<th>Dow-P-P</th>
<th>Dow-P</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Triton X-100</td>
<td>5.3</td>
<td>7.6</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>octyl glucoside</td>
<td>2.0</td>
<td>5.7</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>(\Delta)cwh8</td>
<td>Triton X-100</td>
<td>5.3</td>
<td>11.0</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>(\Delta)cwh8</td>
<td>octyl glucoside</td>
<td>0.7</td>
<td>5.4</td>
<td>1.1</td>
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</tr>
<tr>
<td>(\Delta)lpp1Δdpp1</td>
<td>Triton X-100</td>
<td>0.8</td>
<td>0.01</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>(\Delta)lpp1Δdpp1</td>
<td>octyl glucoside</td>
<td>0.6</td>
<td>&lt;0.01</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>(\Delta)lpp1Δdpp1(CWH8)</td>
<td>Triton X-100</td>
<td>2.2</td>
<td>0.3</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>(\Delta)lpp1Δdpp1(CWH8)</td>
<td>octyl glucoside</td>
<td>26.2</td>
<td>0.5</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

The activity of Dow-P phosphatase when assayed in the presence of 0.3% OG. Under the same in vitro conditions, expression of CWH8 also produced an increase in Dow-P phosphatase, although considerably lower than the increase seen for Dow-P phosphatase (Table IV). Very significantly, the lipid phosphatase encoded by CWH8 does not hydrolyze PA in contrast to virtually all other Mg\(^{2+}\)-independent lipid phosphatases reported previously (5–7, 27–33). Membrane fractions from the insect cells overexpressing CWH8 also did not exhibit diacylglycerol pyrophosphatase (data not included).

As seen for the enzyme activity encoded by CWH8 in yeast, the enzyme expressed in Sf-9 cells was quite labile when incubated in the presence of Triton X-100 (Fig. 3). Thus, the lability of the Dow-P phosphatase is apparently not due to the membrane environment in yeast but rather is an intrinsic property of the enzyme. When the Dow-P and Dow-P phosphatase activities in membranes from Sf-9 cells expressing CWH8 were analyzed by double-reciprocal plots (Fig. 4), similar apparent \(K_m\) values were calculated for both substrates, but the catalytic efficiency of the Dow-P phosphatase reaction was substantially higher (\(V_{\text{max}}/\text{app.} K_m = 796\)) compared with Dow-P dephosphorylation (\(V_{\text{max}}/\text{app.} K_m = 51\)).

Deletion of Cwh8p Causes an Elevation in Dow-P Levels in Vivo—Because the enzymological experiments suggested that CWH8 encoded a Dow-P phosphatase, the physiological significance of this activity was assessed further by comparing Dow-P levels in vivo in the wild type and mutant strains. Although virtually no Dow-P was detected in wild type cells (Fig. 5A, left trace), increased levels of Dow-P were observed in \(\Delta\)cwh8 cells (Fig. 5A, right trace). The identity of the lipid that accumulates in the mutant cells was confirmed by showing that the lipid with the chromatographic properties of Dow-P was converted to Dow-P by strong base hydrolysis (Fig. 5B, right trace). This in vitro analysis provides additional evidence for a direct role of the Cwh8p phosphatase in Dow-P metabolism. These in vivo and in vitro results are consistent with a role for this novel enzyme in the conversion of Dow-P, formed during protein N-glycosylation reactions, to Dow-P.

Cwh8p Is a Transmembrane Protein with the Putative Lipid Phosphate-binding Site Located in the Lumen of the ER—To learn more about the function of Cwh8p in Dow-P metabolism, topological studies were conducted to investigate the localization and orientation of the lipid phosphate-binding site in the ER.

Cwh8p contains a phosphate-binding domain conserved among many lipid phosphatases (34) (Fig. 6A), and a C-term-
The indicated lipid phosphatase activities in membrane fractions from control and SF-9(CWH8) cells were assayed in the presence of either Triton X-100 or OG under the same conditions described for Table II.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Detergent</th>
<th>SF-9 control</th>
<th>SF-9(CWH8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dol-P</td>
<td>Triton X-100</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Dol-P</td>
<td>Octyl glucoside</td>
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<td>2.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dol-P-P</td>
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<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Dol-P-P</td>
<td>Octyl glucoside</td>
<td>0.8</td>
<td>24.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PA</td>
<td>Triton X-100</td>
<td>0.12</td>
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<tr>
<td>PA</td>
<td>Octyl glucoside</td>
<td>0.43</td>
<td>0.11</td>
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</table>

<sup>a</sup> ND, not detected.

<sup>b</sup> Numbers in parentheses are ratios of Dol-P and Dol-P-P phosphatase activity in SF-9(CWH8)/SF-9 control cells.

These results provide good evidence that essential residues of the lipid phosphate-binding motif of Cwh8p are oriented toward the ER lumen and the C-terminal domain is exposed on the cytoplasmic side of the ER. Based on these results the topological model for Cwh8p depicted in Fig. 6C is proposed.

**Active Site of Dol-P-P Phosphatase Is Protected from Protease Inactivation in Sealed Microsomal Vesicles**—Because the lipid phosphate-binding site appeared to be exposed in the lumen, and Dol-P-P phosphatase activity is not affected. However, when the vesicles were unsealed by the addition of 0.5% OG exposing the luminal surface, the Dol-P-P phosphatase activity was reduced by at least 90% in the presence of chymotrypsin (Fig. 7). Consistent with this result, the sensitivity of the phosphatase activity to chymotrypsin increased in parallel to the loss of latency of the luminal processing glucosidases I/II (Fig. 8).

These experiments provide good evidence for a luminal orientation of the active site of this novel Dol-P-P phosphatase.

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**TABLE IV**

Dol-P, Dol-P-P, and PA phosphatase activities assayed in the presence of Triton X-100 or octyl glucoside in membranes from control cells and SF-9 cells expressing Cwh8p.

**FIG. 3.** The Dol-P-P phosphatase encoded by CWH8 is also labile to Triton X-100 when expressed in SF-9 cells. Microsomes (18 

**FIG. 4.** Dol-P-P and Dol-P phosphatase activities in microsomal fractions from SF-9 cells expressing CWH8 as a function of the concentration of Dol-P and Dol-P-P. Reaction mixtures for the assay of Dol-P-P phosphatase (upper panel) contained microsomes from the Sf-9(CWH8) strain (7.7 ng of protein), 1 mM EDTA, 50 mM sodium HEPES (pH 7), 2 mM MgCl<sub>2</sub>, 0.3% OG, and the indicated concentration of [32P]Dol-P-P in a total volume of 0.025 ml. Assay mixtures for the determination of Dol-P phosphatase (lower panel) contained microsomes from the SF-9(CWH8) strain (15 ng of protein), 1 mM EDTA, 50 mM sodium HEPES (pH 7), 2 mM MgCl<sub>2</sub>, 0.3% OG, and the indicated concentration of [32P]Dol-P in a total of 0.025 ml. Following incubation for either 2 min (Dol-P-P phosphatase) or 10 min (Dol-P phosphatase) at 30 °C the amount of [32P]<sub>2</sub> released was determined (7, 32, 55).

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To address the topological arrangement of Cwh8p experimentally, two different N-terminal portions of the protein were fused to the carboxyl-domain of the His4Cp containing histidinol dehydrogenase activity (22). A cytoplasmic location of His4C protein allowed us to verify the luminal orientation of the histidinol dehydrogenase of the TM III fusion, visualized by a mobility shift of the fusion protein on SDS-PAGE (−10 kDa) after treatment with endoglycosidase H (data not shown).
RER2 and SEC59 Act as High Copy Number Suppressors of the Growth Phenotype of \(\Delta cwh8\) Cells—

RER2 encodes the long chain cis-isoprenyltransferase, a key enzyme catalyzing a rate-controlling step in the \textit{de novo} synthesis of Dol-P (reviewed in Ref. 3). Overexpression of \textit{RER2} has been found to produce an increase in the levels of dolichol (36) and Dol-P.\(^2\) To determine if increasing the rate of dolichol/Dol-P synthesis \textit{de novo} might suppress the growth deficiency of these cells, \textit{RER2} was overexpressed in \(\Delta cwh8\) cells. Overexpression of Rer2p was, indeed, capable of partially restoring the growth of \(\Delta cwh8\) cells (Fig. 9). The restoration of growth by both \textit{RER2} and \textit{SEC59} is at least as robust as for \textit{CWH8}. These results indicate that limited supplies of Dol-P contribute to the growth phenotype of \(\Delta cwh8\) cells and suggest that Cwh8p plays a role in maintaining sufficient Dol-P levels that are required for the efficient synthesis of lipid intermediates and protein N-glycosylation \textit{in vivo}.

Protein N-Glycosylation Is Partially Restored by Overexpression of \textit{LPP1}—In contrast to a disruption of \textit{DPP1} (5) and \textit{LPP1} (6), the deletion of \textit{CWH8} yielded a severe growth deficiency on all media tested (8) (Fig. 10A). Because the \textit{in vitro} and \textit{in vivo} results presented above suggested that the growth phenotype in the mutant was due to the absence of Dol-P phosphatase, the possibility that overexpression of \textit{LPP1}, which encodes a

\(^2\) B. Schenk and M. Aebi, unpublished results.
Dolichyl Pyrophosphate Phosphatase

Fig. 7. Yeast Dol-P-P phosphatase activity is inactivated by chymotrypsin in unsealed (●) but not intact microsomal vesicles (○). Sealed yeast microsomal vesicles from the Δlpp1Δlpp1/CWH8 strain (17 μg of protein) were preincubated with (●) or without (○) 0.5% OG at 21 °C in a reaction mixture containing 2 mM EDTA, 10 mM MgCl2, 50 mM sodium HEPES (pH 7), 0.25 M sucrose, and the indicated concentration of chymotrypsin in a total volume of 0.01 ml. After 20 min proteolysis was stopped by the addition of 2 mM PMSF, and Dol-P-P phosphatase activity was determined (7, 55).

Fig. 8. Dol-P-P phosphatase activity is inactivated (●) in parallel to loss of glucosidase I/II latency (○) during unsealing of yeast microsomes. Sealed yeast microsomal vesicles from the Δlpp1Δlpp1/CWH8 strain (17 μg of protein) were preincubated in the indicated concentration of octyl glucoside with (●, △) and without (○) 0.2 mg/ml chymotrypsin. After 20 min at 21 °C, proteolysis was stopped by the addition of 2 mM PMSF, and Dol-P-P phosphatase (55) and glucosidase I/II activity (24) were assayed.

Fig. 9. RER2 and SEC59 are high copy number suppressors of growth phenotype in Δcwh8 cells. Δcwh8 cells were transformed with plasmid YEpC59 (empty vector, YG 1001) or YEpR2 harboring either CWH8 (YG 1000), RER2 (RER2, YG 0998) or SEC59 (SEC59, YG 0997) loci. Cells were plated on YPD and grown for 3 days at 30 °C.

Fig. 10. Overexpression of LPP1 restores growth and partially compensates for hypoglycosylation of CPY in Δcwh8 cells. A, serial dilutions of wild type and different mutants were plated on YPD media and incubated for 3 days at 30 °C. The relevant genotypes of the strains are indicated. B, total cell extracts from strains indicated were analyzed by SDS-PAGE and immunoblotting using anti-CPY serum (8). The positions of mature CPY (mCPY) and different glycoforms lacking one to four N-linked oligosaccharides (−1 to −4) are indicated.

In addition, overexpression of LPP1 partially rescued the hypoglycosylation observed in Δcwh8 cells. Deletion of CWH8 resulted in the appearance of CPY molecules lacking up to four N-linked glycans, with the majority of CPY molecules containing one or two oligosaccharides chains (Fig. 10B, lane 2). Full glycosylation was restored by expressing CWH8 (Fig. 10B, lane 3) and improved by LPP1 overexpression in Δcwh8 cells (Fig. 10B, lane 4). Importantly, no hypoglycosylation was observed in Δlpp1Δlpp1 cells (Fig. 10B, lanes 5 and 6). Thus, the lipid phosphatase encoded by LPP1 appears to be capable of at least partially compensating for the loss of a functional Cwh8p.

DISCUSSION

During protein N-glycosylation reactions the glucosylated precursor oligosaccharide is transferred from Glc3Man9GlcNAc2-P-Dol to appropriate asparagine residues in the polypeptide acceptors, and the glycosyl carrier lipid is released as Dol-P-P in the luminal leaflet of the ER (3). For Dol-P-P to be re-utilized for additional rounds of lipid intermediate synthesis, it must initially be converted to the phosphomonoester form. Although this pathway has been studied for over 30 years, the enzyme required for this reaction has not been identified. In this paper we present evidence that the CWH8 gene encodes a Dol-P-P phosphatase that could perform this function in S. cerevisiae.

The ability of the lipid phosphatases encoded by the LPP1/DPP1 genes to hydrolyze Dol-P and Dol-P-P suggested that they might be involved in Dol-P metabolism. However, because the Δlpp1Δlpp1 double mutant has no obvious growth or protein N-glycosylation defects, it is unlikely that Dol-P/Dol-P-P are the primary substrates for those lipid phosphatases in vivo.
phatase encoded by CWH8 on the luminal surface of the ER. A role for the Dol-P-P phosphatase, the enzyme could convert Dol-P-P to Dol-P, which is essential for phosphatase activity in other lipid-mediated reactions (3). The enzymological characterization described here have shown that Dol-P-P accumulates in cwh8 mutants.

The CWH8 phosphatase was also shown to convert a fully unsaturated, long-chain polyisoprenyl pyrophosphate, an intermediate in Dol-P biosynthesis (3), to the monophosphate form. Mechanistically, this activity would allow it to play a role in the de novo pathway, because the polyisoprenyl pyrophosphate intermediate is proposed to undergo complete dephosphorylation prior to the reduction of the α-isoprene unit (40). Because the cis-isoprenyltransferase system producing this intermediate has an active site exposed to the cytoplasmic face of the ER (41, 42), the CWH8 phosphatase appears to have the wrong topological orientation for this function.

Two experimental approaches were taken to determine if the active site of the novel Dol-P-P phosphatase was oriented toward the luminal surface where the substrate is discharged during the primary protein N-glycosylation reactions (3). First, topological studies based on histidinol dehydrogenase constructs indicate that the domain implicated as the lipid-phosphate-binding site is luminally oriented. This study establishes that, indeed, Cw8p has at least one transmembrane region (domain IV) and the C terminus is located in the cytosol. Based on the proposal for the topological arrangement of motif 3 in Cw8p, which is essential for phosphatase activity in other lipid phosphatases, the enzyme could convert Dol-P-P to Dol-P on the luminal surface of the ER. A role for the Dol-P-P phosphatase encoded by CWH8 in recycling, but not in the de novo biosynthetic pathway, is also supported by the observation that a chymotrypsin-sensitive site was protected in sealed microsomes but exposed to the protease when the vesicles were unsealed. A luminal orientation of the active site would allow this ER enzyme to convert Dol-P-P discharged during protein N-glycosylation reactions to Dol-P in the luminal leaflet.

In view of the evidence that the CWH8 gene encodes a Dol-P-P phosphatase with a luminally oriented active site, it is possible to speculate about the underlying metabolic defect responsible for the deficiency in lipid intermediate synthesis and protein N-glycosylation documented by van Berkel et al. (8). First, it is very likely that the inability to convert Dol-P-P to Dol-P in the luminal leaflet blocks the recycling of this form of the carrier lipid. The Dol-P formed from Dol-P-P and the other lipid-mediated reactions could be dephosphorylated by another phosphatase that has yet to be identified or directly "flip-flop" to the cytoplasmic monolayer by a protein-mediated process. In this scheme free dolichol would be expected to more readily diffuse back to the cytoplasmic face of the ER after the carrier lipid is completely dephosphorylated on the luminal surface. The transverse diffusion of the free polyisoprenyl could then be driven by the subsequent phosphorylation of dolichol by the CTP-mediated kinase on the cytoplasmic leaflet in the rough ER (41, 42). It should be emphasized, however, that the existence of a Dol-P "flipase" that could mediate the direct transverse diffusion of the luminal pool of Dol-P to the cytoplasmic leaflet or a Dol-P phosphatase with a luminally oriented active site has not yet been established.

The rate of protein N-glycosylation could be affected in the cwh8 mutants due to the accumulation of Dol-P-P on the luminal leaflet reducing the rate of transfer of Glc₃Man₉GlcNAc₂ from the carrier lipid to polypeptide acceptors by "end-product inhibition" of the oligosaccharyltransferase. Another potential problem is that the accumulation of Dol-P-P produced by protein N-glycosylation reactions and possibly the degradation of Dol-P-P-linked oligosaccharides (43) might result in an anionic microheterogeneity in the ER, resulting in a biophysical alteration in the luminal monolayer. Such an alteration could affect protein N-glycosylation and other functions of the organelle. If these biophysical alterations do occur, it is clear that they do not prevent cell growth when Dol-P synthesis is increased by overexpression of RER2 and SEC59.

If Cw8p does not actively dephosphorylate Dol-P in vivo, the Dol-P discharged during luminal Man-P-Dol- and Glc-P-Dol-mediated reactions (see papers cited in Ref. 3) could be recycled normally during early stages of growth in cwh8 mutants. However, during each succeeding new round of lipid intermediate synthesis, one Dol-P would be converted to Glc₃Man₉GlcNAc₂-P-Dol and, subsequently, to Glc₃Man₉GlcNAc₂-P-Dol. The Dol-P molecules used in this sequence of reactions would be "arrested" as Dol-P during the transfer of the precursor oligosaccharide to protein. If the rate of de novo biosynthesis could not compensate for the gradual accumulation of the carrier lipid as the pyrophosphate form, it is reasonable to expect that the rate of lipid intermediate biosynthesis would be impaired. Although these explanations are plausible, additional work will certainly be required to confirm the speculation regarding the biochemical basis for the growth and protein N-glycosylation phenotype of the cwh8 mutation.

The role of Dol-P-P phosphatase is clearly pertinent to the regulation of lipid intermediate biosynthesis. There is an abundance of experimental support that the level of Dol-P in the ER is one rate-controlling factor for lipid intermediate synthesis in this pathway (44–50). Dol-P levels can be primarily determined by the rate of de novo biosynthesis and recycling of Dol-P/Dol-P from the luminal monolayer. Several reports indicate that the level of the cis-isoprenyltransferase system involved in the chain-elongation stage is a key to the regulation of de novo biosynthesis (50–52).

One factor that could control the rate of recycling is the rate of conversion of Dol-P-P to Dol-P emphasizing the importance of this new Dol-P-P phosphatase. Experiments described here demonstrate that the lack of an adequate amount of cytoplasmically oriented Dol-P to sustain normal rates of lipid inter-
mediate biosynthesis in Δcwh8 cells can be partially compensated by overexpression of RER2 or SEC59. Moreover, the finding that LPP1 acts as a high copy number suppressor suggests that this enzyme can act as a Dol-P(P) phosphatase in vivo, although it is apparently not its principal function. Indeed, if LPP1 is at the plasma membrane, where some mammalian phosphatases reside (53, 54), it might convert Dol-P-P to Dol-P.

The ability of RER2, SEC59, and LPP1 to suppress the growth and N-glycosylation phenotype of the CWH8 mutation suggests that the recycling of the carrier lipid may contribute significantly to the pool of Dol-P available for lipid intermediate synthesis. It is also plausible that the dephosphorylation of the Dol-P formed by this phosphatase and the other lipid-mediated reactions on the luminal surface could influence the rate of movement of the carrier lipid back to the cytoplasmic leaflet if it occurs by simple diffusion. The marked preference of the CWH8 phosphatase for Dol-P-P over Dol-P suggests that dephosphorylation of Dol-P by this enzyme may not be quantitatively significant in vivo. The possibility that Dol-P formed on the luminal leaflet directly diffuses transversely to the cytoplasmic site of lipid intermediate synthesis via a protein-mediated mechanism cannot yet be excluded.

Determining if the Dol-P, formed by Cwh8p and the other lipid-mediated glycosylation reactions on the luminal surface, diffuses directly to the cytoplasmic leaflet via a protein-mediated mechanism or by simple diffusion after it is dephosphorylated will be an important goal for future studies.

REFERENCES