Identification and Characterization of a cDNA Encoding a Dolichyl Pyrophosphate Phosphatase Located in the Endoplasmic Reticulum of Mammalian Cells*

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Jeffrey S. Rush†‡, Steve K. Cho†, Songmin Jiang‡, Sandra L. Hofmann§, and Charles J. Waechter¶¶

From the †Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, Lexington, Kentucky 40536 and the ‡Department of Internal Medicine and the Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, Texas 75390

The CWH8 gene in Saccharomyces cerevisiae has been shown recently (Fernandez, F., Rush, J. S., Toke, D. A., Han, G., Quinn, J. E., Carman, G. M., Choi, J.-Y., Voelker, D. R., Aebl, M., and Waechter, C. J. (2001) J. Biol. Chem. 276, 41455–41464) to encode a dolichyl pyrophosphatase (Dol-P-P) phosphatase associated with crude microsomal fractions. Mutations in CWH8 result in the accumulation of Dol-P-P, deficiency in lipid intermediate synthesis, defective protein N-glycosylation, and a reduced growth rate. A cDNA (DOLPP1, GenBank™ accession number AB030189) from mouse brain encoding a homologue of the yeast CWH8 gene is now shown to complement the defects in growth and protein N-glycosylation, and to correct the accumulation of Dol-P-P in the cwh8Δ yeast mutant. Northern blot analyses demonstrate a wide distribution of the DOLPP1 mRNA in mouse tissues. Overexpression of Dolpp1p in yeast, COS, and SF9 cells produces substantial increases in Dol-P-P phosphatase activity but not in dolichyl monophosphate or phosphatidic acid phosphatase activities in microsomal fractions. Subcellular fractionation and immunofluorescence studies localize the enzyme encoded by DOLPP1 to the endoplasmic reticulum of COS cells. The results of protease sensitivity studies with microsomal vesicles from the lpp1Δ/dpp1Δ yeast mutant expressing DOLPP1 are consistent with Dolpp1p having a luminal oriented active site. The sequence of the DOLPP1 cDNA predicts a polypeptide with 238 amino acids, and a new polypeptide corresponding to 27 kDa is observed when DOLPP1 is expressed in yeast, COS, and SF9 cells. This study is the first identification and characterization of a cDNA clone encoding an essential component of a mammalian lipid pyrophosphate phosphatase that is highly specific for Dol-P-P. The specificity, subcellular location, and topological orientation of the active site described in the current study strongly support a role for Dolpp1p in the recycling of Dol-P-P discharged during protein N-glycosylation reactions on the luminal leaflet of the endoplasmic reticulum in mammalian cells.

The multisubunit complex, oligosaccharyltransferase (1), catalyzes the transfer of Glc3Man9GlcNAc2 from dolichyl pyrophosphate (Dol-P-P) to appropriate asparagine residues during the co-translational N-glycosylation of nascent polypeptides in yeast and mammalian cells (2–4). During the primary N-glycosylation reaction, Dol-P-P is released on the luminal surface of the endoplasmic reticulum (ER). In order for Dol-P-P to be re-utilized as a glycosyl carrier lipid for additional rounds of lipid intermediate biosynthesis, it must be converted to dolichyl phosphate (Dol-P) and translocated to the cytoplasmic leaflet of the ER (4). Although it cannot yet be excluded that Dol-P-P, or perhaps Dol-P, diffuses transversely from the luminal leaflet to the cytoplasmic face by a protein-mediated mechanism, it is more likely that it is dephosphorylated on the luminal surface to form free dolichol that could more readily diffuse back to the cytoplasmic leaflet. Cytoplasmically oriented dolichol would then be re-phosphorylated by dolichyl phosphate kinase (5, 6). Recent studies (7, 8) have shown that the CWH8 gene in Saccharomyces cerevisiae encodes a Dol-P-P phosphatase that actively converts Dol-P-P to Dol-P and is also capable of dephosphorylating Dol-P at a slower rate. Moreover, the yeast Dol-P-P phosphatase is recovered in crude microsomal fractions, but its subcellular location has not been established. Although there have been numerous reports (4, 7–13) that crude microsomal fractions from a variety of mammalian cells contain enzymes that can hydrolyze exogenous Dol-P-P, the identity, specificity, number, and exact subcellular location of the enzymes catalyzing the hydrolysis of Dol-P-P have not been established. Recently, Inoue et al. (14) have described a mouse brain cDNA, referred to as gene 2-23, with a high degree of homology to yeast CWH8. The current study presents several lines of evidence that mouse 2-23 encodes a mammalian homologue of the CWH8 gene product from yeast. In this report, this gene is referred to as Dol-P-P phosphatase 1 (DOLPP1). The identification of the mammalian homologue as a Dol-P-P pyrophosphate phosphatase is based on its ability to complement defects in growth and protein N-glycosylation in the cwh8Δ mutant (8, 15) and to prevent the accumulation of Dol-P-P in the mutant cells. In addition, expression of DOLPP1 modified with either a carboxyl-terminal His6 tag in the yeast mutants or an amino-terminal extension encoding the T7 bacteriophage epitope in SF9 cells results in the appearance of a new polypeptide with an apparent molecular mass of 27 kDa and a substantial increase in the level of Dol-P-P phosphatase activity.

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†To whom correspondence should be addressed.

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1 The abbreviations used are: Dol-P-P, dolichyl pyrophosphate; Dol-P, dolichyl monophosphate; ER, endoplasmic reticulum; LPP, lipid phosphate phosphohydrolase; OG, n-octyl glucoside; PA, phosphatidic acid; TMD, transmembrane domain; RACE, rapid amplification of cDNA ends; CPY, carboxypeptidase Y; CDG, congenital disorders of glycosylation.
The mouse enzyme exhibits a marked preference for Dol-P-P over Dol-P and phosphatidic acid (PA), as reported for the yeast enzyme.

The results described in this paper provide solid evidence that DOLPP1 encodes the mammalian homologue of the Dol-P-P pyrophosphate phosphatase encoded by the CHW8 gene in *S. cerevisiae*, and represent the first experimental proof that this enzyme is located in the ER. The possible function of this novel lipid pyrophosphate phosphatase in the recycling of the glycosyl carrier lipid in the ER of brain and other mammalian cells is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Plasmid YEp352 containing the yeast gene CHW8 coding sequence and 240 bp of 5′-flanking sequence was a gift from Drs. Fabiana Fernandez and Markus Aebi (ETH, Zurich, Switzerland). The cDNA designated as m2-23 was a gift from Dr. S. Inoue (Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Ibaraki Prefecture, Japan). Yeast strains W303-1A, cwh8Δ, and lpp1Δ/lpp1Δ were the generous gifts from Dr. George Carman, Department of Food Science, Rutgers University, New Brunswick, NJ. α-Octyl-β-D-glucopyranoside was purchased from Calbiochem-Novabiochem. Triton X-100 was from Pierce. Trichloroacetonitrile, tetrabutylammonium hydroxide, and anhydrous acetonitrile were obtained from Aldrich. Nycodenz, dioleoylphosphatidic acid (lot number 129F8359), and dиеolin (D:8884) were obtained from Sigma. Dolichol (C95) was a generous gift from Dr. M. Mizuno, Kuraray Chemical Co. (Okayama, Japan). Carrier-free [32P]phosphoric acid was purchased from American Radiolabeled Chemicals (St. Louis, MO). Econo-safe™ biodegradable scintillation counting mixture was a gift from Research Products International (Mount Prospect, IL). Yeast nitrogen base, yeast extract, and Bacto-Peptone are products of BD Biosciences. Casamino acids is a product of Fisher. Antibodies were obtained from the following commercial sources and used according to instructions from the supplier: rabbit anti-calnexin polyclonal antibody and mouse anti-KDEL monoclonal antibody (StressGen Biotechnologies, Victoria, British Columbia, Canada); mouse anti-KDEL receptor monoclonal antibody, mouse anti-β-COP monoclonal antibody, horseradish peroxidase-conjugated sheep anti-rabbit IgG, horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Biosciences); and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and Texas Red-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). Anti-CPY antiserum was generously provided by Dr. Neta Dean, State University of New York, Stony Brook, NY. All other chemicals and reagents were purchased from standard commercial sources.

**Affinity-purified Rabbit Anti-Dolpp1 Antibodies and Immunoblotting**—Three New Zealand White rabbits were each immunized with 300 μg of a synthetic peptide corresponding to amino acid residues 86–105 of mouse Dolpp1 coupled to keyhole limpet hemocyanin as described previously (16). The antigen was injected intradermally in Freund’s complete adjuvant, and rabbits were boosted three times at 3-week intervals with 300 μg of peptide in Freund’s incomplete adjuvant. An IgG fraction was prepared from preimmune and immune serum by specific binding to protein A-Sepharose CL-4B (Amersham Biosciences). Goat anti-rabbit IgG was affinity-purified by specific binding to a column consisting of the Dolpp1 peptide cross-linked to SulfoLink coupling gel (Pierce). For analysis of Dolpp1 expression by immunoblotting, samples were analyzed by electrophoresis in 15% SDS-PAGE gels and immunoblotting essentially as described (17). Filters were blocked for 1 h with Sea Block (East Coast Biologicals, North Berwick, ME) and washed in PBS-T (0.25% (v/v) Tween 20 in phosphate-buffered saline) followed by incubation for 2 h with rabbit anti-Dolpp1 polyclonal antibody (1 μg/ml). The filters were washed with PBS-T and incubated for 45 min in a solution containing horseradish peroxidase-conjugated secondary antibody (sheep anti-rabbit IgG, Amersham Biosciences) diluted 1:2500 in PBS-T. The filters were washed and developed using ECL chemiluminescence reagent (Amersham Biosciences).

**Preparation of Radiolabeled Phosphorylated Lipid Substrates**—[32P]P-Dol-P, [α-32P]-Dol-P-P, and [β-32P]-Dol-P-P were chemically synthesized in anhydrous acetonitrile from the appropriate lipid using [32P]tetrabutyrammonium phosphate and trichloroacetonitrile as described by Danilov et al. (18). Unlabeled polyisoprenyl phosphates were synthesized using tetrabutyrammonium phosphate. Synthetic substrates were purified by preparative thin layer chromatography on Baker Si250 thin layer plates developed in CHCl3/CH3OH/H2O/NH4OH (65:35:6.1, v/v).

**Recombinant DNA Methods**—Plasmid and genomic DNA preparation, restriction enzyme digestion, and DNA ligations were performed by standard methods (19). Transformation of yeast (20) and *Escherichia coli* (19) were performed as described. The annealing temperature for the PCR was 55 °C, and extension times were typically 2.0 min at 72 °C. PCRs were routinely run for a total of 30 cycles. DNA sequencing reactions were performed by Davis Sequencing, LLC (Davis, CA). For blot hybridization of RNA, total RNA was isolated from mouse tissues as described (21). Blots were pre-hybridized for 30 min at 65 °C in Rapid-Hyb buffer (Amersham Biosciences), followed by hybridization with a random hexamer-primed 32P-labeled mouse cDNA fragment corresponding to the entire 717 bp coding region of Dolpp1 for 3 h at 65 °C. The blots were washed in 2× SSC (1× SSC = 150 mM NaCl and 15 mM sodium citrate (pH 7)), 0.1% SDS at ambient temperature for 30 min, followed by stringent washes in 0.5× SSC, 0.1% SDS at 65 °C for 15 min and in 0.1× SSC, 0.1% SDS at 65 °C for 15 min.

**Yeast Culture**—Yeast cultures were grown at 30 °C in either 1% yeast extract, 2% BactoPeptone, and 2% dextrose or in yeast nitrogen base (6.7 g/liter), 50 mM sodium succinate (pH 5.0), 2% dextrose, amino acids (25 mg/ml), and purine and pyrimidine bases (25 mg/ml) as required to meet auxotrophic requirements for selective growth. Yeast transformants were screened on 2% agar plates containing 6.7 g/liter yeast nitrogen base, 0.5% casamino acids, 50 mM sodium succinate (pH 5.0), 2% dextrose, and 25 mg/ml adenine or leucine as required. The yeast strains used in this study and their respective genotypes are contained in Table 1.

**Expression of Mus musculus DOLPP1 and DOLPP1-His6 in Yeast**—A DNA fragment containing 240 bp upstream of the coding sequence of the yeast CHW8 gene was amplified by PCR using the appropriate oligonucleotide primers (forward primer, 5′-ACGTATATCGGTTTCTTGGGAAAAC-3′; reverse primer, 5′-CCCGTGACGATGATGATGATGCCGTCACAAA-3′) and plasmid YEp352 containing the CHW8 gene (8, 15) as template. The forward and reverse primers were designed to contain altered bases to insert flanking EcoRI and KpnI restriction sites to facilitate cloning (underlined above). The resulting PCR product was digested with EcoRI and KpnI and ligated into similarly prepared YEp352 (22) to generate a plasmid containing the putative yeast CHW8 promoter sequence, YEp352/pro. A DNA fragment containing the entire murine 2-23 coding sequence flanked by KpnI and BamH I restriction sites (underlined in sequences below) was amplified by PCR using the appropriate oligonucleotide primers (forward, 5′-GGTCTCCGCGGCACACAGAGAGAG-3′; reverse, 5′-AGGGAATCGCGTGCGCGAGTTGGTTTT-3′) and a cDNA containing the mouse 2-23 gene obtained from Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., (Tsukuba, Ibaraki, Japan), as template. The PCR product was digested with KpnI and BamHI to yield a 0.74-kb DNA fragment. This fragment was then ligated into the KpnI/BamH I sites of plasmid YEp352/pro to form the plasmid YEp352/pro-Dolpp1. The correct DNA sequence of YEp352/pro-Dolpp1 was confirmed by direct sequencing using the M13 primer sites in YEp352. This construct was then transformed into the pertinent yeast strains for the expression of the mouse DOLPP1 gene according to Schiestl and Gietz (20).

DOLPP1 carrying a carboxyl-terminal His6 tag was prepared by PCR
using appropriate oligonucleotide primers (forward, 5'-ACTGAACT- TGGCGCCAGGAAAC-3', reverse, 5'-TTCTGCATTTGTCG- GGTGGTGTGTGTGCAGTTTGTGTT-3') and pBSPro-DOLP1 cDNA as template. The PCR product was digested with EcoRI/HindIII (underlined in sequences above) and ligated into similarly prepared YEp352. The construct was transformed into the yeast strains for the expression of DOLPP1-αHis according to Schiestl and Gietz (20). Yeast were cultured in a 250 ml baffled Erlenmeyer flask containing 50 ml of YPD medium (2%) and aeration at 37 °C. After harvesting, cells were resuspended in distilled water, sedimented again, resuspended in distilled water to 200 A260/ml, and incubated for 30 min at 30 °C in 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2 containing 1 mg/ml lyticase (Sigma). The yeast spheroplasts were sedimented at 1,000 × g for 20 min; the supernatant was decanted, and the pellet was mixed vigorously with 40 volumes of CHCl3/CH3OH (2:1, v/v). Dol-PP was separated by centrifugation at 100,000 × g for 10 min, resuspended in 20 ml of ice-cold 10 mM HEPES (pH 7.0), 0.25 M sucrose and aliquoted into several 500-μl samples. Each sample was added to an equal volume of ice-cold 0.25 M sucrose, 1 mM EDTA, and 1 mM DTT in a 0.2 ml PCR tube. The samples were then centrifuged at 500 × g for 5 min at 4 °C and the resulting pellets were collected by centrifugation. The pellets were then resuspended in 0.2 ml of CHCl3/CH3OH/H20 (10:10:3, v/v), organic extracts were separated by centrifugation and the aqueous layer was discarded. The organic phase was then dried under nitrogen to a small volume and redissolved in 0.2 ml of CHCl3/CH3OH (10:10:3, v/v). Dol-P and Dol-PP in the organic extracts were separated by ion exchange chromatography on a 20-ml column of DEAE-650M (Toyopearl, Supelco, Bellefonte, PA) equilibrated with CHCl3/CH3OH/H2O (10:10:3, v/v) and eluted with a linear gradient of 0 to 2 M ammonium acetate (60 ml). The fractions containing Dol-P and Dol-PP were pooled separately and supplemented with CHCl3 and water to give a final composition of CHCl3/CH3OH/H2O (5:2:1, v/v). The phases were separated by a brief centrifugation, and the aqueous layer was discarded. The organic layer was then partitioned once with 1/5 volume of CHCl3/CH3OH (2:1, v/v) and twice with 5 ml of CHCl3/CH3OH (10:10:3, v/v). The organic extracts were combined, taken to dryness under nitrogen vaporization under 50 °C in a water bath that was then deacetylated in 1 ml of toluene/CH3OH (1:1, v/v) containing 0.1 M KOH on ice for 30 min. Following deacetylation, the reaction was neutralized with acetic acid, diluted with 5 ml of CHCl3/CH3OH (2:1, v/v), and partitioned with 1 ml of water. The aqueous layer was discarded, and the organic phase was further partitioned twice with 1 ml of CHCl3/CH3OH (2:1, v/v) to remove the last traces of acetic acid. The organic phase was dried under nitrogen to a small volume of N2 gas and redissolved in 0.2 ml of CHCl3/CH3OH/H2O (10:10:3, v/v). Dol-P and Dol-PP in the organic extracts were separated by a final ion exchange chromatography on a 20-ml column of DEAE-650M (Toyopearl, Supelco, Bellefonte, PA) equilibrated with CHCl3/CH3OH/H2O (10:10:3, v/v) followed by elution with a linear gradient of 0 to 2 M ammonium acetate (60 ml). The fractions containing Dol-P and Dol-PP were pooled separately and supplemented with CHCl3 and water to give a final composition of CHCl3/CH3OH/H2O (5:2:1, v/v). The phases were separated by a brief centrifugation, and the aqueous layer was discarded. The organic layer was then partitioned once with 1/5 volume of CHCl3/CH3OH/H2O (2:1, v/v) and twice with 5 ml of CHCl3/CH3OH (2:1, v/v) to remove the last traces of acetic acid. The organic phase was further partitioned twice with 1 ml of CHCl3/CH3OH (2:1, v/v) to remove the last traces of acetic acid. 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The organic phase was dried under nitrogen to a small volume of N2 gas and redissolved in a small volume of CHCl3/CH3OH/H2O (10:10:3, v/v). Aliquots representing equivalent portions of the initial extracts were spotted on 10 × 20-cm Baker Si250 Silica Gel thin layer plates and developed in CHCl3/CH3OH/H2O (3:4:8:7, v/v), taken to dryness under a stream of N2 gas, and redissolved in 50 mM Tris-HCl (pH 7.0), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were plated on Day 0 in 100-mm dishes and were transiently transfected on Day 1 using 6 μg of DNA (pCHA7/DOLP1) and 18 μl of FuGene 6 reagent (Boehringer Molecular Biochemicals) per 100-mm dish and cultured at 37 °C in 5% CO2 for 48 h. Cells were harvested with a rubber policeman in 800 μl of buffer containing 10 μg/ml each of chymostatin, leupeptin, and pepstatin and homogenized by 15 passages through a 25-gauge needle on a 1-ml syringe. Nuclei and intact cells were removed by microcentrifugation at 1,200 × g for 5 min at 4 °C. The postnuclear supernatant was further centrifuged at 100,000 × g for 1 h at 4 °C in a Beckman Optima TLX ultracentrifuge. The resulting supernatant (S100) was reserved, and the membrane pellet (P100) was washed once and resuspended in homogenization buffer for immunoblotting or in 10 mM HEPES (pH 7.0), 0.25 μg/ml streptomycin, 5 mM EDTA, and 1 mM dithiothreitol. Cells were harvested with a rubber policeman in 800 μl of buffer containing 10 μg/ml each of chymostatin, leupeptin, and pepstatin and homogenized by 15 passages through a 25-gauge needle on a 1-ml syringe. Nuclei and intact cells were removed by microcentrifugation at 1,200 × g for 5 min at 4 °C. The postnuclear supernatant was loaded on preformed Nyodenz gradients prepared exactly as described (27). Briefly, linear Nyodenz gradients were prepared for the Beckman SW 41 Ti rotor from initial discontinuous gradients (24, 19, 15, and 10% Nyodenz in 10 mM Tris-HCl, pH 7.5) by extending the resulting gradients prepared exactly as described (27). Briefly, linear Nyodenz gradients were prepared for the Beckman SW 41 Ti rotor from initial discontinuous gradients (24, 19, 15, and 10% Nyodenz in 10 mM Tris-HCl, pH 7.5) by extending the resulting gradients prepared exactly as described (27). Briefly, linear Nyodenz gradients were prepared for the Beckman SW 41 Ti rotor from initial discontinuous gradients (24, 19, 15, and 10% Nyodenz in 10 mM Tris-HCl, pH 7.5) by extending the resulting gradients prepared exactly as described (27). Briefly, linear Nyodenz gradients were prepared for the Beckman SW 41 Ti rotor from initial discontinuous gradients (24, 19, 15, and 10% Nyodenz in 10 mM Tris-HCl, pH 7.5) by extending the resulting gradients prepared exactly as described (27).
antibody or anti-KDEL receptor monoclonal antibody. The secondary antibodies used were fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and Texas Red-conjugated goat anti-mouse IgG. Immunofluorescence was visualized under a Zeiss Axiophot immunofluorescence microscope.

**Preparation of Sealed Microsomal Vesicles and Estimation of Vesicle Integrity—**Sealed microsomal vesicles were prepared from the pertinent yeast strains and evaluated for integrity as described previously (8, 28).

**General Analytical Methods—**Protein was determined by the method of Rodriguez-Vico et al. (29) using a commercial protein assay reagent (BCA, Pierce) and bovine serum albumin as standard. Lipid-phosphate analysis was according to Bartlett (30). Radioactivity was measured by liquid scintillation spectrometry in a Packard Tri-Carb TR-2100 Liquid Scintillation Analyzer (Packard Instrument Co., Meriden, CT) in the presence of Econo-Safe Biodegradable Counting Mixture (Research Products International, Mount Prospect, IL).

**RESULTS**

**Mammalian Cells Express a Homologue of the Yeast CWH8 Gene—**Recently, Inoue and co-workers (14) identified and sequenced 64 cDNA clones from a mouse brain cDNA library that suppress bacterial growth when expressed in *E. coli*. One of these clones (GenBank™ accession number AB030189) has a high degree of homology to the yeast CWH8 gene (GenBank™ accession number NP_011550), an ER Dol-P-P phosphatase with a luminally oriented active site (7, 8). Fig. 1 compares the deduced amino acid sequences of yeast Cwsh5p and the predicted amino acid sequences from the mouse brain cDNA (designated Dolpp1p). The sequences (Fig. 1, panel A) are 29.8% identical and 49.4% strongly similar. In addition, Dolpp1p contains the consensus lipid-phosphate phosphatase motif (Fig. 1, panel B) defined by Stukey and Carman (31), suggesting that Dolpp1p is the mammalian homologue of yeast CWH8.

**The DOLPP1 mRNA was found to be widely expressed in various mouse tissues as assessed by RNA blotting (Fig. 2). A single transcript migrating at 2.0 kb was observed in all tissues examined, with highest expression in brain, kidney, lung, and intestine and low but detectable levels in liver, spleen, and uterus. The broad tissue distribution is consistent with the ER Dol-P-P phosphatase having an essential function in the lipid intermediate pathway for protein N-glycosylation in all mammalian cells.**

**Overexpression of Dolpp1p Corrects Defects in Growth Rate and N-Glycosylation of CPY in the cwh8Δ Mutant—**The mutant yeast strain, cwh8Δ, grows at an abnormally slow rate and exhibits a defect in protein N-glycosylation (8, 15). To determine whether expression of mammalian DOLPP1 corrects the growth deficiency of the cwh8Δ yeast strain, the DOLPP1 gene was subcloned into YEp352 under the control of the putative yeast CWH8 promoter and transformed into cwh8Δ. The effect on rate of growth in complete media was then assessed. As shown in Table II, the cell density of wild type yeast cultures typically doubles approximately every 2 h, whereas the doubling time ($T_2$) for cwh8Δ was $2.5$ h. Overexpression of DOLPP1 in cwh8Δ reduces the doubling time to 3 h, similar to the effect of transformation with YEp352 containing the yeast CWH8 gene ($T_2 = 2.5$ h).

**As reported previously (8, 15), mutation of the CWH8 gene in yeast results in defects in protein N-glycosylation, as assessed by N-glycosylation of carboxypeptidase Y (CPY) (Fig. 3). Although SDS-PAGE of wild type yeast cultures typically doubles approximately every 2 h, whereas the doubling time ($T_2$) for cwh8Δ was $4$ h. Overexpression of DOLPP1 in cwh8Δ reduces the doubling time to 3 h, similar to the effect of transformation with YEp352 containing the yeast CWH8 gene ($T_2X = 2.5$ h).**
A

B

FIG. 3. Overexpression of DOLPP1 cDNA corrects hypoglycosylation of CPY in the yeast cwh8Δ mutant. Total cell extracts from the various yeast strains were separated by SDS-PAGE and analyzed by Western blotting using α-CPY serum as described previously (56). Lane 1, wild type; lane 2, cwh8Δ; lane 3, cwh8Δ/DOLPP1; and lane 4, cwh8Δ/CWH8. The positions of mature CPY and underglycosylated isoforms are indicated.

FIG. 4. Overexpression of Dolpp1p corrects Dol-P-P accumulation in the yeast cwh8Δ mutant. Dol-P-P was extracted from either wild type (lane 1), cwh8Δ (lane 2), cwh8Δ/DOLPP1 (lane 3), or cwh8Δ/CWH8 yeast strains and chromatographed on Baker Si250 Silica Gel thin layer plates as described under “Experimental Procedures.” Dol-P-P was visualized by anisaldehyde staining (24).

FIG. 5. Time course of Dol-P-P hydrolysis (panel A) and comparison of Dol-P-P, Dol-P, and PA as substrates (panel B) for phosphatase activity in microsomal fractions from lpp1Δ/dpp1Δ/DOLPP1 yeast. For the time course shown in panel A, reaction mixtures for the determination of Dol-P-P phosphatase contained yeast microsomes (10 μg of protein) from either lpp1Δ/dpp1Δ/DOLPP1 (○) or lpp1Δ/dpp1Δ (○) strain, 10 mM EDTA, 50 mM sodium citrate/sodium phosphate (pH 7.0), 0.6% OG, and the indicated concentration of either [β-32P]Dol-P-P (●), [32P]Dol-P (▲), or [32P]PA (▲) in a total volume of 0.02 ml. Following incubation at 30 °C for the indicated periods, the amount of [32P] released was determined as described previously (9, 23). For the comparison of the different phospholipid substrates (panel B), reaction mixtures contained yeast microsomes from lpp1Δ/dpp1Δ/DOLPP1 (10 μg of protein), 10 mM EDTA, 50 mM sodium citrate/sodium phosphate (pH 7.0), 0.6% OG, and the indicated concentration of either [β-32P]Dol-P-P (●), [32P]Dol-P (▲), or [32P]PA (▲) in a total volume of 0.02 ml. After 2 min at 30 °C, the amount of [32P] released was determined as described previously (9, 23).

These results indicate that Dol-P-P accumulation in cwh8Δ yeast cells, due to a lack of the Dol-P-P phosphatase activity responsible for the hydrolysis of the Dol-P-P released during the transfer of the precursor oligosaccharide from Glc3Man9GlcNAc2-P-P-Dol to nascent polypeptides in the lumen of the ER (8). To determine whether overexpression of Dolpp1p in cwh8Δ will restore normal Dol-P-P levels, total lipid extracts were prepared from either wild type, cwh8Δ plus YEp352, cwh8Δ plus YEp352/yPro-DOLPP1, or cwh8Δ plus YEp352/CWH8 yeast cells and analyzed for Dol-P-P (Fig. 4, panel A, lanes 1–4). As reported earlier (8), the lipid extracts from cwh8Δ cells (Fig. 4, panel A, lane 2), contained a prominent anisaldehyde-positive compound with the chromatographic mobility of standard Dol15-P-P. In contrast to this result, virtually no Dol-P-P was detected in extracts from wild type cells (Fig. 4, panel A, lane 1) or from cwh8Δ yeast cells expressing either the DOLPP1 gene (Fig. 4, panel A, lane 3) or the CWH8 gene (Fig. 4, panel A, lane 4). To confirm the identity of the anisaldehyde-positive compound as Dol-P-P, this lipid was quantitatively converted to a product chromatographically identical to Dol17-P by mild acid hydrolysis (2 h at 80 °C in CHCl3, CH3OH, 2 N HCl, 10:10:3, v/v). It should be noted that the levels of Dol-P-P in the various yeast strains were not significantly different (Fig. 4, panel B, lanes 1–4).

Increase in Dol-P-P Phosphatase Activity Upon Expression of Dolpp1p in lpp1Δ/dpp1Δ Yeast or Sf9 Insect Cells—The results described above indicate that the DOLPP1 gene encodes a functional homologue of the yeast Dol-P-P phosphatase. To provide more direct biochemical evidence that Dolpp1p functions as a Dol-P-P phosphatase, microsomal fractions were prepared from the lpp1Δ/dpp1Δ yeast mutant strain after transformation with YEp352/yPro-DOLPP1, and the rate of hydrolysis of Dol-P-P was assayed. The lpp1Δ/dpp1Δ strain was chosen as the parental strain for this study in order to minimize the nonspecific phosphatase activities encoded by LPP1 and DPP1 (7, 8, 13). Microsomes from lpp1Δ/dpp1Δ/DOLPP1 were found to enzymatically dephosphorylate Dol-P-P at a markedly higher rate than microsomes from the lpp1Δ/dpp1Δ (Fig. 5, panel A) parental strain. Preliminary studies determined that the Dol-P-P phosphatase activity encoded by DOLPP1 was active in the presence of n-octyl glucoside and slightly less sensitive to inactivation by Triton X-100 than Cwh8p. Divalent cations were not required for activity for the mammalian enzyme (data not shown).

To examine the specificity of Dolpp1p, yeast microsomes from various yeast strains were assayed for Dol-P-P, Dol-P, or PA phosphatase activity. As seen in Table III, the overexpression of either Dolpp1p or Cwh8p in the lpp1Δ/dpp1Δ yeast
Fig. 6. Expression of T7 and His-tagged constructs of Dolpp1p phosphatase in Sf9 and yeast cells. (A) When COS cells overexpressing T7-DOLPP1 (panel A, lane 2) or after transfection with pFASBac/DOLPP1-T7 (panel A, lanes 2 and 3, two separate isolates) were probed with α-T7 antiserum. Extracts from cwh8Δ yeast cells following transformation with either Yepl352 (panel B, lane 1), Yepl352/HIS-DOLPP1 (panel B, lane 2), or lpp1Δdpp1Δ yeast following transformation with Yepl352/HIS-DOLPP1 (panel B, lane 3) were probed with α-His antiserum.

Table III

| Yeast strain | Phospholipid substrate | Dol-P | Dol-P-P | PA |
|--------------|------------------------|-------|---------|----|
| Sf9          | 0.1                    |       |         |    |
| Sf9(DOLPP1)  | 5.0                    |       |         |    |
| COS cells    | 1                      |       |         |    |
| COS cells    | 0.8                    |       |         |    |
| COS(DOLPP1)  | 16.2                   |       |         |    |
| COS(DOLPP1)  | 12.2                   |       |         |    |

The minor immunoreactivity in Golgi shown in Fig. 8 was not confirmed by subcellular fractionation. Cells—Although mutations in the CWH8 gene affect the rate of lipid intermediate synthesis and protein N-glycosylation in the ER, the precise subcellular location of the yeast enzyme has not been firmly established. To explore the properties of the DOLPP1 expressed in mammalian cells, the full-length mouse brain DOLPP1 cDNA was inserted into the eukaryotic expression vector pCHAT to yield the plasmid pCHAT/DOLPP1, and expression in COS cells was detected by enzyme assay and immunoblotting. When microsomes from transfected cells were assayed, a large (12–16-fold) increase in Dol-P-P phosphatase activity was seen compared with identical microsomal fractions from cells transfected with the empty vector alone (Table IV). It is noteworthy that no concomitant increase in Dol-P or PA dephosphorylation was observed, corroborating the specificity for Dol-P-P.

SOLuble (S100) and particulate (P100) fractions from DOLPP1-transfected COS cells were also analyzed by SDS-PAGE and immunoblotting using a rabbit polyclonal antibody raised against a Dolpp1p peptide (Fig. 7). This analysis revealed a single band in the P100 fraction (Fig. 7, lane 4) at an Mr of 27 in accord with the molecular mass of Dolpp1p predicted from the DOLPP1 cDNA sequence (27.1 kDa). No bands were visible in the S100 fraction or the fractions from the vector-transfected cells (Fig. 7, lanes 1–3).

Expression and Subcellular Localization of Dolpp1p in COS cells—Unlike mutations in the CWH8 gene affect the rate of lipid intermediate synthesis and protein N-glycosylation in the ER, the precise subcellular location of the yeast enzyme has not been firmly established. To explore the properties of the DOLPP1 expressed in mammalian cells, the full-length mouse brain DOLPP1 cDNA was inserted into the eukaryotic expression vector pCHAT to yield the plasmid pCHAT/DOLPP1, and expression in COS cells was detected by enzyme assay and immunoblotting. When microsomes from transfected cells were assayed, a large (12–16-fold) increase in Dol-P-P phosphatase activity was seen compared with identical microsomal fractions from cells transfected with the empty vector alone (Table IV). It is noteworthy that no concomitant increase in Dol-P or PA dephosphorylation was observed, corroborating the specificity for Dol-P-P.

To determine further the subcellular location of Dolpp1p, transfected COS cells were fractionated on nonlinear Nycodenz gradients, using a method previously developed to provide effective separation of ER and Golgi complex proteins (27). As shown in Fig. 8, >95% of the immunoreactivity sedimented to the bottom of the gradient (upper panel), co-sedimenting with calnexin, a well established ER marker. A much smaller amount was found at the top of the gradient co-fractionating with the Golgi markers, KDEL receptor and β-COP (Fig. 8). The minor immunoreactivity in Golgi shown in Fig. 8 was not observed in every experiment and seemed to correlate with higher levels of Dolpp1p expression.

When COS cells overexpressing DOLPP1 were examined by immunofluorescence using a specific Dolpp1p antibody, a reticular and nuclear envelope pattern characteristic of ER localization was observed (Fig. 9). This pattern essentially completely overlapped with the ER marker (KDEL, upper panel) and showed no overlap with a Golgi marker (KDEL receptor, lower panel).
that was proposed to function in a model for the recycling of the carrier lipid (7, 8). This paper describes the identification and characterization of a mammalian homologue of the yeast CWH8 gene, DOLPP1, from a mouse brain cDNA library. Although there have been numerous reports (7–10, 13, 32–41) describing Dol-P-Dol-P phosphatase activities in crude microsomal fractions from various mammalian tissues, the exact number, location, and specificity have not been rigorously defined. Previous studies have been complicated by an inability to study the enzymatic properties of these proteins independent of the activities of other contaminating phosphatases. Expression of Dolpp1p in the lpp1Δ/dpp1Δ yeast strain has allowed us to characterize the brain Dol-P-P phosphatase in the absence of other endogenous lipid phosphatase activities hydrolyzing Dol-P and Dol-P. The comparisons reported in Tables III and IV show that expression of Dolpp1p in either yeast, insect Sf9 cells, or COS cells results in a substantial and highly specific increase in Dol-P-P phosphatase activity. The experiment described in Fig. 5 reveals that Dolpp1p exhibits a marked preference for Dol-P-P (Km ≤ 10 μM), similar to Cwh8p (~25 μM) when expressed in the lpp1Δ/dpp1Δ yeast double

**TABLE V**

| Vesicle treatment | Time of exposure to trypsin | Dol-P-P phosphatase activity |
|-------------------|-----------------------------|-----------------------------|
| None              | 0 min                       | 3.3 nmol/min/mg % control   |
| Unsealed with 0.2% Triton X-100 | 60 min | 2.9 87.5 |
| Unsealed with 0.2% Triton X-100 | 0 min | 0 100 |

**DISCUSSION**

When Glc3Man9GlcNAc2 is transferred from the glycosyl carrier lipid to appropriate asparagine residues in nascent proteins, Dol-P-P is formed on the luminal monolayer of the ER. This luminally oriented Dol-P-P must be converted to Dol-P and return to the cytoplasmic leaflet of the ER in order to participate in subsequent rounds of lipid intermediate biosynthesis (4). The CWH8 gene in *S. cerevisiae* has recently been shown to encode a Dol-P-P-specific pyrophosphate phosphatase
Fig. 10. Predicted topological arrangement of mammalian Dolpp1p and yeast Cwh8p in the ER. The topology of potential transmembrane domains of Dolpp1p was predicted using the TMpred transmembrane helix prediction algorithm (43). Identical and highly conserved residues in Dolpp1p and Cwh8p are shaded black and grey, respectively.

This enzyme to the Dol-P-P phosphatase activity previously reported (9, 10) to be enriched in the Golgi compartment in brain is not clear. However, it seems likely that unrelated lipid-phosphate phosphatases with broad substrate specificity and overlapping subcellular distribution may have obscured the proper localization of this activity. In this regard, other researchers have reported that Dol-P-P phosphatase activity is found in the plasma membrane in rat liver (32) or throughout the plasma membrane, rough ER, smooth ER, Golgi, and lysosomal fractions in mouse L-1210 cells (33). A small and variable proportion (<5%) of Dolpp1p was seen in the Golgi upon overexpression in COS cells. This result raises the possibility that the enzyme might be partially localized in a Golgi compartment under some conditions. However, it is clear from these studies that the DOLPP1 gene product is predominantly localized in the ER, the site of lipid intermediate biosynthesis, and protein N-glycosylation.

The specificity, location in the ER, and luminally oriented active site of Dolpp1p meet all the criteria for a role in the recycling of Dol-P-P released during primary protein N-glycosylation reactions on the luminal surface. There is substantial evidence that cellular levels of Dol-P are a rate-controlling factor in the regulation of lipid intermediate biosynthesis (44–50). The induction of cis-isoprenyltransferase activity correlates closely with the onset of developmental increases in Dol-P and Glc3Man9GlcNAc2-P-P-Dol biosynthesis (51, 52), suggesting that de novo synthesis is an important source of Dol-P for lipid intermediate synthesis. However, considering the impressive effect (~80% reduction in Glc3Man9GlcNAc2-P-P-Dol synthesis) produced by mutations in CWHS (8, 15), it seems likely that recycling of Dol-P plays an important role in maintaining Dol-P supplies for Glc3Man9GlcNAc2-P-P-Dol biosynthesis. An important future goal will be to determine whether Dol-P was formed via the recycling of Dol-P-P exists in a separate pool to initiate new rounds of lipid intermediate synthesis or intermixes with the pool acquired by a de novo pathway.

The hypothetical models for Dolpp1p and Cwh8p, based on the TMpred program (43), indicate that the proteins contain four transmembrane helices and are topologically identical (Ref. 8, Fig. 10). The transmembrane domains (TMD) and the large luminal domains between TMD 1 and 2 are highly conserved in the two proteins. The high degrees of conservation in the TMDs suggest that these domains may be critical to the function of Dolpp1p phosphatase and could be involved in the interaction with the polyisoprenyl moiety of Dol-P-P. The two putative luminal domains in Dolpp1p and Cwh8p have a significantly higher degree of homology than the cytoplasmic domains and may form part of the reactive site. Studies are in progress to determine the precise domains forming the active site of the enzyme.

The identification of the DOLPP1 gene is an important development, with implications for the inherited metabolic diseases in humans that occur due to defects in the synthesis of the dolichol-linked oligosaccharide donor required for protein N-glycosylation. These disorders are collectively referred to as congenital disorders of glycosylation (CDG) (53–55). Considering the wide variety of enzymes that have already been described as causing CDG, and the severity of the protein N-glycosylation deficiency in the yeast CWHS mutants, it seems inevitable that a human CDG related to human DOLPP1 deficiency will be discovered. The accumulation of Dol-P-P in the luminal leaflet resulting from defects in Dolpp1p could block protein N-glycosylation by end product inhibition and interfere with other ER functions by causing biophysical alterations due to anionic microheterogeneities. The description of this man-
Mammalian cDNA should facilitate the clinical diagnosis of the underlying metabolic disorder in patients of this type.

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Jeffrey S. Rush, Steve K. Cho, Songmin Jiang, Sandra L. Hofmann and Charles J. Waechter

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