A Homologue of Saccharomyces cerevisiae Dpm1p Is Not Sufficient for Synthesis of Dolichol-Phosphate-Mannose in Mammalian Cells*

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Dolichol-phosphate-mannose (Dol-P-Man) serves as a donor of mannosyl residues in major eukaryotic glyco-conjugates. It donates four mannol residues in the N-linked oligosaccharide precursor and all three mannol residues in the core of the glycosylphosphatidylinsitol anchor. In yeasts it also donates one mannose to the O-linked oligosaccharide. The yeast DPM1 gene encodes a Dol-P-Man synthase that is a transmembrane protein expressed in the endoplasmic reticulum. We cloned human and mouse homologues of DPM1, termed hDPM1 and mDPM1, respectively, both of which encode proteins of 260 amino acids, having 30% amino acid identity with yeast Dpm1 protein but lacking a hydrophobic transmembrane domain, which exists in the yeast synthase. Human and mouse DPM1 cDNA restored Dol-P-Man synthesis in mouse Thy-1-deficient mutant class E cells. Mouse class E mutant cells had an inactivating mutation in the mDPM1 gene, indicating that mDPM1 is the gene for class E mutant. In contrast, hDPM1 and mDPM1 cDNA did not complement another Dol-P-Man synthesis mutant, hamster Lec15 cells, whereas yeast DPM1 restored both mutants. Therefore, in contrast to yeast, mammalian cells require hDPM1/mDPM1 protein and a product of another gene that is defective in Lec15 mutant cells for synthesis of Dol-P-Man.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) D86198 for hDPM1 cDNA, D86199, D86200, D86201, and D86202 for hDPM1 genomic sequences, and AB004789 for mDPM1 cDNA.

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‡ The abbreviations used are: Dol, dolichol; GPL, glycosylphosphatidylinositol; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; CHO, Chinese hamster ovary; GST, glutathione S-transferase.
reverse primer, cloned into the EcoRV site of pBSII (Strategene, La Jolla, CA) and sequenced. An insert with the correct sequence was excised with XhoI and NotI and cloned into the mammalian expression vector pMEneo (16).

Cell Lines and Culture—The mouse thymoma BW5147 cell line and its Thy-1-negative mutant of complementation class E were gifts from Dr. R. Hyman (Salk Institute, San Diego, CA). Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Lec15 mutant (Lec15.2) of CHO cells (a gift from Dr. M. A. Lehrman, University of Texas Southwestern Medical Center, Dallas, TX (17) and CHO-K1 cells were cultured in Ham’s F-12 medium containing 10% fetal bovine serum. To inhibit α-mannosidase II, cells were cultured in the presence of 5 μg/ml swainsonine (Wako Chemicals, Osaka, Japan) for 4 days. For transfection, cells (5 × 10⁴) were suspended in 0.8 ml of HEPES-buffered saline (18), mixed with 20 μg of plasmids, and electroporated with a Gene Pulser (Bio-Rad) at 350 V/25 μF for class E cells and 350 V/960 μF for Lec15 and CHO-K1 cells.

Fluorescence Staining of Cell Surface Thy-1 and Complex Oligosaccharides—Thy-1 cells were stained for Thy-1 with biotinylated anti-Thy-1 monoclonal antibody G7 (a gift from Dr. T. Tadakuma, National Defense Medical College, Japan) and phycoerythrin-conjugated streptavidin (Biomeda, Foster City, CA). Cell surface complex oligosaccharides were stained with 25 μg/ml fluorescein isothiocyanate-conjugated phytohemagglutinin-M (Seikagaku Co., Tokyo, Japan). Stained cells were analyzed in a FACScan cytometer (Becton Dickinson, Mountain View, CA).

Assay of Dol-P-Man Synthase Activity—Cells were destroyed by a Teflon homogenizer. After removal of cell debris and nuclei by centrifugation at 15,000 × g for 10 min, membranes were collected by centrifugation at 100,000 × g for 60 min and suspended in a buffer consisting of 50 mM Hepes/NaOH (pH 7.4), 25 mM KCl, 5 mM MgCl₂, and 5 mM MnCl₂. Dolichol phosphate (4 μg, Sigma) dried under a stream of N₂ was suspended in 10 μl of the same buffer containing 0.2% Triton X-100 by vigorous agitation and sonication, and then GDP-[3H]mannose (0.4 Ci, American Radiolabeled Chemicals, St. Louis, MO) and the membranes (60 μg of protein) were added to a final volume of 100 μl. The mixture was incubated for 15 min at 37 °C. The Dol-P-[3H]Man generated was assayed by measuring radioactivity extracted into the organic phase (19).

Analysis of Mannolipids—Cells (4 × 10⁶) were metabolically labeled with 100 μCi of [2-3H]mannose (American Radiolabeled Chemicals) for 45 min, then the lipid fraction was extracted and separated with chloroform/methanol/water (10:10:3) on DC-Alufolien Kieselgel 60 (Merck, Germany) (20). The plate was analyzed by fluorography.

Cloning and Analysis of Genomic hDPM1 Clone and Ribonuclease Protection Assay—A human genomic library (Strategene) was screened with an hDPM1 cDNA probe (1-kilobase pair XhoI-NotI fragment). The ribonuclease protection assay was done with a HybSpeed RPA kit (Ambion, Austin, TX) and total RNA of HeLa cells.

RESULTS

Cloning of a Human Homologue of S. cerevisiae DPM1

A search of the TIGR EST data base for a homologue of a nucleotide sequence of yeast DPM1 identified a cluster of ESTs. This cluster contained a contiguous sequence of 451 nucleotides (Fig. 1, nucleotides 5–455) that has 41% identity to the corresponding sequence of yeast DPM1. We amplified both the 5’ and 3’ ends of this cDNA sequence by RACE methods from a cDNA library derived from human placenta and ligated and cloned them in a mammalian expression vector, then transfected the vector into mouse class E mutant cells. The cloned cDNA, 1043 base pairs (Fig. 1, nucleotides 5–1047), partially restored the surface expression of Thy-1 (data not shown), indicating that Dol-P-Man synthesis was partially restored leading to a synthesis of the GPI anchor and the surface expression of GPI-anchored proteins. Since there was no methionine codon near the 5′ end and a reading frame was open to the 5′ end, we tried to amplify the 5′ end by RACE methods again, but only three more nucleotides (Fig. 1, nucleotides 2–4) were obtained. It is possible that some structural constraint hampered reverse-transcription of the 5′ end.

To obtain the 5′ end of the coding region, we isolated a genomic clone of human homologue of DPM1. We identified four exons in the genomic clone of 17 kilobase pairs by comparing its sequence with the cDNA sequence. The exon sequences

FIG. 1. Nucleotide sequence of hDPM1. Nucleotide numbers are above the sequence with A of the start codon as 1. Nucleotides shown in uppercase letters were derived from a genomic clone. Those in lowercase letters were derived from cDNA. Arrows indicate exon boundaries. The poly(A) additional signal is boxed. The stop codon corresponds to nucleotides 781–783.
were exactly the same as the corresponding cDNA sequences, indicating that this clone represented the human gene homologous to DPM1. The four exon boundaries corresponded to cDNA nucleotide numbers 161, 261, and 295 (nucleotide number 1 at A of the initiation codon ATG; see Fig. 1).

The genomic clone contained an exon that included a methionine codon at two residues amino-terminal to the end of the partial cDNA (Fig. 1). This methionine codon was within a Kozak consensus sequence (21). We prepared an expression construct including these two residues (spanning nucleotides −54 to 1047) and transfected it into a class E mutant. The surface expression of Thy-1 was completely restored (Fig. 2), indicating that we cloned the full-length coding region. Based on this functional activity, we termed the cloned gene hDPM1.

Since the reading frame was still open to the 5' end, we analyzed a start site of transcription with the ribonuclease protection assay using RNA of HeLa cells. Only one protected fragment of the hDPM1 RNA probe was seen and from its size the start site of transcription was determined to be at around nucleotide −10 (data not shown). This is consistent with the idea that the methionine codon at position 1–3 is the initiation codon.

**Structural Properties of hDPM1**

Human DPM1 encodes a protein of 260 amino acids. Fig. 3 compares amino acid sequences of yeast Dpm1p (267 amino acids) and hDPM1 protein. The amino acid identity between homologous regions is about 30%. There are two significant differences. Human DPM1 has 22 extra residues at the amino terminus. This sequence is hydrophilic. Human DPM1 is shorter than Dpm1p at the carboxyl terminus by 29 residues. The hydropathy profile of hDPM1 protein (not shown) indicated that it lacks a transmembrane domain and an amino-terminal hydrophobic signal sequence. So, hDPM1 has characteristics of a cytoplasmic protein or a peripheral membrane protein. In contrast, yeast Dpm1p has a carboxyl-terminal hydrophobic segment that may be a transmembrane domain. Human DPM1 had a sequence RKKIS (Fig. 3, amino acids 137–141) (6).

**Complementation of Class E Mutant by hDPM1**

**Dol-P-Man Synthase—**Restoration of Dol-P-Man synthase activity in class E mutants was tested after stable transfection of hDPM1 (Fig. 4). Class E cells had almost no activity. hDPM1 and yeast DPM1 restored Dol-P-Man synthase in class E cells to levels about 3 times and 2.5 times as high as the wild-type level, respectively.

**GPI Anchor Synthesis—**To confirm that restoration of Dol-P-Man synthase activity in class E cells by hDPM1 led to restoration of synthesis of GPI anchor precursors, we labeled the class E cells and transfectants with [3H]mannose, and analyzed mannolipids. As shown in Fig. 5, the synthesis of Dol-P-Man and mannose-containing GPI anchor precursors was restored. These results indicate that hDPM1 restored the defective synthesis of Dol-P-Man in class E mutant cells.

**FIG. 2.** Restoration of the surface Thy-1 expression on class E mutant cells with stable transfection of hDPM1. Solid lines, anti-Thy-1; broken lines, second reagent only. A, parental wild-type BW5147 cells. B, class E cells transfected with vector. C, class E cells transfected with yeast DPM1. D, class E cells transfected with hDPM1. After transfection, cells were cultured for 10 days in G418-containing medium. A shift of staining profile after transfection of the vector alone (B) may be due to nonspecific association of anti-Thy-1 antibody.

**FIG. 3.** Comparison of amino acid sequences of hDPM1 and yeast Dpm1p. Identical and similar amino acids are indicated by asterisks and dots, respectively. Amino acid numbers are on the right. Putative consensus sequences for phosphorylation by the cAMP-dependent kinase are boxed.
A Lack of Complementation of Lec15 Mutant by hDPM1

We next tested whether hDPM1 complements Lec15 mutant. It was reported previously that yeast DPM1 fully complemented defective Dol-P-Man synthesis of Lec15 (8) as well as that of class E. On the other hand, there is a report that Lec15 and class E are different mutants (5). We confirmed the latter by a cell hybridization experiment; heterokaryons expressed Thy-1 on the cell surface 2 days after fusion, indicating complementation (data not shown).

We stably transfected Lec15 mutant cells with hDPM1 and DPM1, and assessed restoration of Dol-P-Man synthase activity. Consistent with its leaky phenotype (17), the lysate of Lec15 mutant cells synthesized about 20% as much Dol-P-Man as the lysate of wild-type CHO cells (Fig. 6). Yeast DPM1 restored Dol-P-Man synthase of Lec15 mutant as reported (8), whereas hDPM1 had no enhancement effect (Fig. 6). Transfection of the same expression vector of hDPM1 cDNA into wild-type CHO cells doubled the Dol-P-Man synthase activity (data not shown), indicating that the expression vector was functional in CHO cells.

To confirm the inability of hDPM1 to complement Lec15, we cultured wild-type CHO cells, Lec15, and Lec15 hDPM1 and DPM1 transfectants in swainsonine for 4 days, before staining them with fluorescein isothiocyanate-conjugated phytohemagglutinin-E4 for complex oligosaccharides. The staining intensity of wild-type CHO decreased by 75% after incubation in swainsonine, whereas that of Lec15 did not change as expected (Fig. 7, panels A and B). In about a half of the DPM1-transfected Lec15 cells, the staining intensity was reduced by 80% after incubation in swainsonine, indicating complementation (panel C). On the other hand, hDPM1-transfected Lec15 cells maintained a mutant phenotype (panel D).

A Mutation in mDPM1 Is Responsible for Class E Mutant

A mDPM1 cDNA encoded a protein of 260 amino acids having 91% identity in amino acids with hDPM1 protein (Fig. 8A). Nucleotide sequence analysis of products of reverse transcriptase-polymerase chain reaction from class E cells demon-
strated that all eight clones had a T to C substitution at nucleotide 365 that caused a change of serine 122 to phenylalanine. To test whether this missense mutation causes a loss of function, we transfected the mutant hDPM1 cDNA into class E mutant cells. The cDNA bearing the mutation did not restore the surface expression of Thy-1 while the wild-type cDNA restored it (Fig. 8B), indicating that this base substitution is an inactivating mutation and that the class E cell is a mutant of the mDPM1 gene.

A Possible Role of the Protein Defective in Lec15 Cells

The above results indicate that synthesis of Dol-P-Man in mammalian cells is mediated by a mammalian homologue of Dpm1p and a product of the gene defective in Lec15 cells. hDPM1 protein may be either a cytoplasmic protein or a peripheral membrane protein as described above. If the former is true, the protein defective in Lec15 cells may be required for association of hDPM1 protein with the membrane. In this case, Dol-P-Man synthesis activity would be complemented if the cytoplasm of Lec15 cells that may contain a soluble DPM1 homologue is mixed with the membranes of class E cells that may contain the protein necessary for membrane association of DPM1 homologue. This mixture, however, was inactive (data not shown), indicating that the latter case may be true. To confirm this, we tested whether hDPM1 is membrane-bound even when expressed in Lec15 cells. As shown in Fig. 9, transfected GST-tagged hDPM1 was expressed in the membrane of Lec15 cells (lane 4) but not in the cytoplasm (lane 3), similarly to transfection into wild-type CHO cells (lanes 1 and 2). Therefore, it appeared that the protein defective in Lec15 cells is not required for membrane association of mammalian DPM1.

DISCUSSION

We have cloned a human homologue of the yeast Dol-P-Man synthase gene DPM1 and termed it hDPM1. An hDPM1 cDNA complemented mouse Thy-1-negative class E mutant cells that are defective in Dol-P-Man synthesis (Figs. 2, 4, and 5). However, the hDPM1 cDNA did not complement another Dol-P-Man synthesis mutant, CHO Lec15 (Figs. 6 and 7). To determine whether class E cells are defective in mouse DPM1 homologue, we cloned it, analyzed its sequence in class E cells, and found an inactivating mutation (Fig. 8). This indicates that the defective Dol-P-Man synthesis in class E cells is due to the mutation in mDPM1. Somatic cell hybridization demonstrated that class E and Lec15 cells are of different complementation groups (5). These results together indicate that two genes, a DPM1 homologue and one defective in Lec15 cells, are necessary for synthesis of Dol-P-Man in mammalian cells.

In contrast to hDPM1 cDNA, yeast DPM1 DNA complemented both class E and Lec15 mutants (8, 14) (Figs. 2 and 4–7). Since yeast Dpm1p has Dol-P-Man synthase activity by itself, it is not surprising that yeast DPM1 complements both mutants. A major structural difference between yeast and human Dpm1p proteins is that yeast Dpm1p has a hydrophobic carboxyl-terminal segment, whereas human DPM1 protein lacks it (Fig. 3). The hydrophobic segment in Dpm1p may serve as a transmembrane domain (6) and in addition it may act as an acceptor site for Dol-P. hDPM1 protein does not have a hydrophobic segment for a typical transmembrane domain, nevertheless, it associated with the membrane even in Lec15...
cells (Fig. 9), suggesting that hDPM1 may be a peripheral membrane protein. The protein defective in Lec15 cells, therefore, does not play a role in association of hDPM1 with the membrane. So, a possible role of that protein may be in a step of Dol-P-Man synthesis, such as binding of Dol-P. It is noticeable, however, that an expression level of GST-tagged hDPM1 protein in Lec15 mutant was much lower than that in wild-type CHO cells (Fig. 9). This suggests another possible role of the protein defective in Lec15 cells, i.e. it may be necessary for stable expression of hDPM1 in the endoplasmic reticulum. This has to be formally shown by a complementation experiment when the gene defective in Lec15 mutant is cloned because it is possible that some nonrelevant mechanism operating in this particular Lec15 cell line caused rapid degradation of GST-tagged hDPM1 and/or its mRNA.

Recently, a hamster SL15 cDNA was cloned (22). SL15 encodes a protein of 248 amino acids bearing at least two putative transmembrane domains and a double-lysine endoplasmic reticulum retention signal near the carboxyl terminus, suggesting that it is an endoplasmic reticulum transmembrane protein (22). SL15 is a candidate that is necessary, together with mammalian DPM1, for Dol-P-Man synthesis, because it has been reported to suppress the Lec15 mutation. It is yet to be determined whether the Lec15 mutation is actually due to a defect in SL15 or some other protein.

The synthesis of Dol-P-Man in yeast and mammalian cells, therefore, differs; Dpm1p alone is sufficient in S. cerevisiae, whereas at least two gene products are necessary in mammalian cells. DPM1 homologues were cloned from a protozoa, *Trypanosoma brucei brucei* and a fungus *Ustilago maydis* (23, 24). The overall structure of *T. b. brucei* DPM1 protein is very similar to that of yeast Dpm1p, i.e. 267 amino acids having a hydrophobic putative transmembrane domain near the carboxyl terminus. *U. maydis* DPM1 protein is of 294 amino acids. It also has a hydrophobic putative transmembrane domain near the carboxyl terminus. These two DPM1 proteins have Dol-P-Man synthase activity in *vitro*. So, only mammalian DPM1 protein requires an additional protein and lacks the carboxy-terminal hydrophobic segment. Moreover, *S. cerevisiae* Dol-P-Man synthase is active in the presence of nonionic detergent (6, 7), whereas mammalian Dol-P-Man synthase is inactive (25). Furthermore, amino acid identities among three microbial Dpm1 proteins are 49–60%, whereas those between mammalian DPM1 and three microbial ones are only about 30%. Therefore, mammalian DPM1 diverged from three microbial DPM1s.

A consensus sequence for cAMP-dependent protein kinase-mediated phosphorylation is conserved in yeast, *Ustilago* and *Trypanosoma* DPM1 proteins, i.e. RRVIS (6), RRIS (24), and RRFIS (23), respectively. The corresponding sequence in hDPM1 was RKKIS (amino acids 161–165). Although the double arginine sequence conserved in three other DPM1 proteins is changed to arginine-lysine in hDPM1, it would still be consistent with a phosphorylation site (26, 27). It was reported that Dol-P-Man synthase activities of microsomes prepared from rat, bovine, and hen cells were enhanced by 30–80% upon phosphorylation with cAMP-dependent kinase (28). Whether this regulation of Dol-P-Man synthesis is mediated through phosphorylation of DPM1 is yet to be determined.

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Note Added in Proof—Colussi et al. recently reported cloning of a human DPM1 cDNA (Colussi, P. A., Taron, C. H., Mack, J. C., and Orlean, P. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 7873–7878).

REFERENCES

1. Kornfeld, R., and Kornfeld, S. (1985) *Ann. Rev. Biochem.* 54, 631–664
2. Herscovics, A., and Orlean, P. (1993) *FASEB J.* 7, 540–550
3. Menon, A. K., Mayor, S., and Schwarz, R. T. (1990) *EMBO J.* 9, 4249–4258
4. Orlean, P. (1990) *Mol. Cell. Biol.* 10, 5796–5805
5. Singh, N., and Tartakoff, A. M. (1991) *Mol. Cell. Biol.* 11, 391–400
6. Orlean, P., Albright, C., and Robbins, P. W. (1988) *J. Biol. Chem.* 263, 17499–17507
7. Schützbach, J. S., Zimmerman, J. W., and Forsey, W. T. (1993) *J. Biol. Chem.* 268, 24190–24196
8. Beck, P. J., Orlean, P., Albright, C., Robbins, P. W., Gething, M. J., and Sambrook, J. F. (1990) *Mol. Cell. Biol.* 10, 4612–4622
9. Trowbridge, I. S., Hyman, R., and Mazarakis, C. (1978) *Cell* 14, 21–32
10. Chapman, A., Fujimoto, K., and Kornfeld, S. (1980) *J. Biol. Chem.* 255, 4441–4446
11. Sugiyama, E., DeGasperi, R., Urake, M., Chang, H. M., Thomas, L. J., Hyman, R., Warren, C. D., and Yeh, E. T. H. (1991) *J. Biol. Chem.* 266, 12119–12122
12. Stoll, J., Robbins, A. R., and Krag, S. S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 79, 2296–2300
13. Zeng, Y. C., and Lehrman, M. A. (1990) *J. Biol. Chem.* 265, 2296–2305
14. DeGasperi, R., Thomas, L. J., Sugiyama, E., Chang, H. M., Beck, P. J., Orlean, P., Albright, C., Waneck, G., Sambrook, J. F., Warren, C. D., and Yeh, E. T. H. (1990) *Science* 250, 898–901
15. Adams, M. D., Kelley, J. M., Gocayne, J. D., Duhmick, M., Polymersopouls, M. H., Xiao, H., Merrill, C. R., Wu, A. Olde, B., Moreno, R. F., Kerlavage, A. R., McCombie, W. R., and Venter, C. (1991) *Science* 253, 1651–1656
16. Watanabe, R., Kinoshita, T., Masaki, R., Yamamoto, A., Takeda, J., and Inoue, E. T. H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 3010–3014
17. Colussi, P. A., Taron, C. H., Mack, J. C., and Orlean, P. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 7873–7878.