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DPM2 regulates biosynthesis of dolichol phosphate-mannose in mammalian cells: correct subcellular localization and stabilization of DPM1, and binding of dolichol phosphate

Yusuke Maeda, Shuichiro Tomita, Reika Watanabe, Kazuhito Ohishi and Taroh Kinoshita[1]

Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan

[1]Corresponding author
e-mail: tkinoshi@biken.osaka-u.ac.jp

Biosynthesis of glycosylphosphatidylinositol and *N*-glycan precursor is dependent upon a mannosyl donor, dolichol phosphate-mannose (DPM). The Thy-1-negative class E mutant of mouse lymphoma and Lec15 mutant Chinese hamster ovary (CHO) cells are incapable of DPM synthesis. The class E mutant is defective in the *DPM1* gene which encodes a mammalian homologue of *Saccharomyces cerevisiae* Dpm1p that is a DPM synthase, whereas Lec15 is a different mutant, indicating that mammalian DPM1 is not sufficient for DPM synthesis. Here we report expression cloning of a new gene, *DPM2*, which is defective in Lec15 cells. DPM2, an 84 amino acid membrane protein expressed in the endoplasmic reticulum (ER), makes a complex with DPM1 that is essential for the ER localization and stable expression of DPM1. Moreover, DPM2 enhances binding of dolichol phosphate, a substrate of DPM synthase. Mammalian DPM1 is catalytic because a fusion protein of DPM1 that was stably expressed in the ER synthesized DPM without DPM2. Therefore, biosynthesis of DPM in mammalian cells is regulated by DPM2.

Keywords: biosynthesis/dolichol phosphate-mannose/endoplasmic reticulum/glycosylphosphatidylinositol/*N*-glycan

Introduction

Dolichol phosphate-mannose (DPM) acts as a donor for mannosylation reactions occurring on the lumenal side of the endoplasmic reticulum (ER). Due to the lack of a transporter, GDP-mannose, a widely used mannosyl donor, is not available within the ER (Abeijon and Hirschberg, 1992). DPM donates four mannosyl residues in precursors of *N*-linked glycan (Kornfeld and Kornfeld, 1985; Hirschberg and Snider, 1987; Abeijon and Hirschberg, 1992; Herscovics and Orlean, 1993) and all three mannosyl residues in the glycosylphosphatidylinositol (GPI) anchor (Menon *et al*., 1990; Orlean, 1990; Englund, 1993). In *Saccharomyces cerevisiae*, DPM donates the first mannosyl residue in *O*-linked oligosaccharides (Orlean, 1990; Herscovics and Orlean, 1993).

DPM is synthesized from GDP-mannose and dolichol phosphate (Dol-P) on the cytosolic side of the ER by DPM synthase whose activity is detected only in the ER (Czichi and Lennarz, 1977). Synthesized DPM is subsequently translocated to the lumenal side, presumably by a putative flipase (Abeijon and Hirschberg, 1992; Rush and Waechter, 1995), and is used as a mannosyl donor. A temperature-sensitive mutant *dpm1* was isolated from *S.cerevisiae* (Orlean *et al*., 1988). Mutant *dpm1* yeast were defective in DPM synthesis and lethal at a non-permissive temperature. The corresponding gene *DPM1* encodes a 267 amino acid protein that is DPM synthase itself because recombinant Dpm1p expressed in *Escherichia coli* had a DPM synthase activity (Orlean *et al*., 1988).

In mammalian cells, Thy-1-negative lymphoma of complementation class E (Trowbridge *et al*., 1978; Chapman *et al*., 1980) and Chinese hamster ovary (CHO)-derived Lec15 mutant (Stoll *et al*., 1982; Camp *et al*., 1993) cells are known to be defective in DPM synthesis. The defect in DPM synthesis causes accumulation of an immature *N*-linked oligosaccharide precursor bearing five mannose residues (Chapman *et al*., 1980; Stoll *et al*., 1982) and defective synthesis of GPI which results in the defective surface expression of GPI-anchored proteins, such as Thy-1 (Sugiyama *et al*., 1991). Although these cells are defective in the same catalytic step, they belong to different complementation groups as determined by a somatic-cell hybridization experiment (Singh and Tartakoff, 1991), suggesting that at least two proteins participate in DPM synthesis in mammalian cells. Reports that *S.cerevisiae DPM1* complemented both mammalian mutant cells (Beck *et al*., 1990; DeGasperi *et al*., 1990) are consistent with the idea that it is a complete enzyme and suggest that a similar enzyme may be present in mammals. As expected, a mammalian homologue of *DPM1* (Colussi *et al*., 1997; Tomita *et al*., 1998) is responsible for the defect of class E cells (Tomita *et al*., 1998). On the other hand, in contrast to *S.cerevisiae DPM1*, mammalian *DPM1* cDNA did not complement the defective DPM synthesis in Lec15 cells (Tomita *et al*., 1998).

Another CHO cell mutant, Lec35, is defective in usage of DPM (Camp *et al*., 1993). SL15 cDNA restores this defect upon transfection (Ware and Lehrman, 1996). Although it was first reported that SL15 cDNA was cloned based on its ability to complement Lec15 mutant cells (Ware and Lehrman, 1996), a recent communication from the same group indicated that SL15 cDNA does not complement Lec15 cells and that it must have been cloned due to complementation of Lec35 cells that had contaminated Lec15 cells (Ware and Lehrman, 1998). Here we report the cloning of *DPM2*, which is responsible for Lec15 mutation and functions in DPM synthesis.

Results

Expression cloning of rat DPM2

To clarify the defect in Lec15 cells, we first established a transfectant line of Lec15 cells, Lec15.B5, that stably

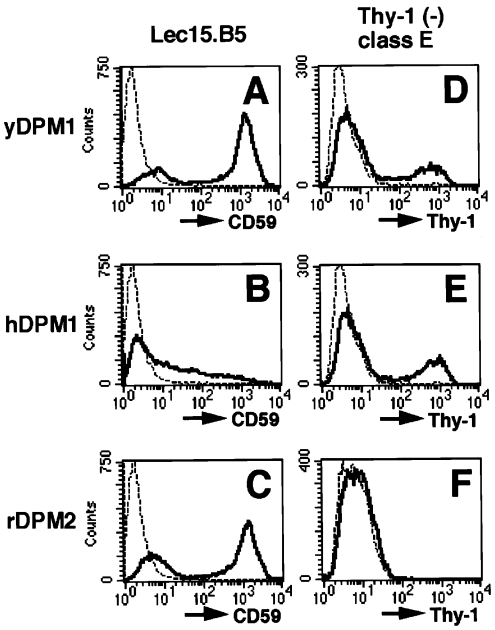

Fig. 1. FACS analysis showing restoration of the surface expression of GPI-anchored proteins on Lec15 and class E cells. Lec15.B5 cells (3×10^6) transfected with 10 μg of plasmids (**A**–**C**) were stained for CD59, and class E cells (10^7) transfected with 20 μg of plasmids were stained for Thy-1 (**D**–**F**). The cells were stained 2 days after transfection. Solid lines represent pME-Py-yDPM1 [(A) and (D)], -hDPM1 [(B) and (E)] and -rDPM2 [(C) and (F)]. Broken lines (A–F) represent pME-Py control plasmids.

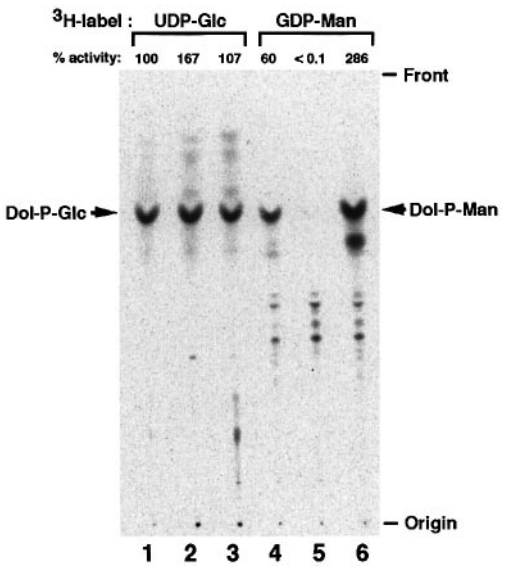

Fig. 2. Restoration of DPM synthesis in Lec15 cells with *DPM2* cDNA. Lec15 cells (10^7 each) were transfected with 30 μg of pME-Py and pME-Py-rDPM2 plasmids and incubated for 2 days. Microsomes from wild-type CHO cells (lanes 1 and 4), and Lec15 transfected with pME-Py (lanes 2 and 5) and with DPM2 (lanes 3 and 6) were incubated with UDP-[^{3}H]glucose (lanes 1–3) and GDP-[^{3}H]mannose (lanes 4–6). The radiolabelled lipids were resolved by TLC. The relative intensities of Dol-P-Glc and DPM spots are indicated, taking that in lane 1 as 100%.

expresses human CD59 precursor peptides as a marker to monitor synthesis of the GPI anchor. CD59 was not expressed on the surface of this transfectant because synthesis of the GPI anchor is defective due to the defective DPM synthesis. The surface CD59 expression would be restored if DPM synthesis was restored. This was confirmed by transfection with *S.cerevisiae DPM1* (Figure 1A). In contrast to the budding yeast *DPM1*, human *DPM1* cDNA induced only a modest surface CD59 expression on Lec15.B5 cells (Figure 1A and B) although it complemented class E mutant as efficiently as *S.cerevisiae DPM1* (Figure 1D and E). Since these results indicated that the gene defective in Lec15 cells is not the *DPM1* gene, we isolated it by means of expression cloning.

From a rat cDNA library, we obtained six plasmids that restored the surface CD59 expression on Lec15.B5 cells. Five clones including 6B12 had the same 5′ end and coding region sequences, length and restriction profile. One (2E5) had the same 5′ end but had an additional sequence within the N-terminal coding sequence (see below). We named the gene *DPM2*. *DPM2* cDNA (clone 6B12) complemented Lec15.B5 (Figure 1C) but not class E mutant (Figure 1F).

To confirm that *DPM2* cDNA complemented defective DPM synthesis in Lec15 cells, we transfected them with control and *DPM2* expression plasmids, and measured synthesis of DPM in their microsomes. As shown in Figure 2, synthesis of DPM which was defective in vector-transfected Lec15 cells (lane 5) was restored in *DPM2*-transfected cells (lane 6). Synthesis of DPM in *DPM2*-transfected cells was 4–5 times higher than that in wild-type CHO cells (Figure 2, lane 4 versus 6). Microsomes from all these cells had comparable activities in terms of synthesis of Dol-P-Glc (Figure 2, lanes 1–3). Therefore, overexpression of DPM2 resulted in overexpression of DPM synthase activity.

Characteristics of the DPM2 gene and its products

Clone 6B12 cDNA consisted of 822 bp (Figure 3A) and had several possible short coding regions. To determine the functional coding region, we made deletion constructs and examined complementation of Lec15.B5 cells. The first 283 bp, encoding a predicted protein of 84 amino acid residues, were necessary and sufficient (data not shown). Clone 2E5 had an insertion of 305 bp between nucleotides 6 and 7 that has GT at the 5′ end and AG at the 3′ end, suggesting that it represents an unspliced intron (not shown in Figure 3). Although there were only three nucleotides upstream of the initiation codon in rat cDNA 6B12, the sequence of a human *DPM2* homologue had an in-frame stop codon upstream of the corresponding ATG codon (see below), suggesting that clone 6B12 had a full open reading frame. We found sequences of human and mouse *DPM2* homologues in the expressed sequence tag (EST) database using the Basic Local Alignment Search Tool (BLAST; Altschul *et al*., 1990), and determined the coding sequences of clones with I.M.A.G.E. Consortium CloneID 129150 (human *DPM2*) and 464740 (mouse *DPM2*) (Lennon *et al*., 1996). The predicted human and mouse DPM2 proteins had 88 and 98% amino acid identity, respectively, with rat DPM2 (Figure 3B). No known proteins in the nr database (National Center for Biotechnology Information) had overall homology with DPM2. As shown in Figure 3C, a hydrophobicity plot (Kyte and Doolittle, 1982) revealed that DPM2 is a very hydrophobic protein and has two putative membrane-spanning regions as defined by the PHDhtm method (Rost *et al*., 1995). DPM2 also had a double lysine sequence

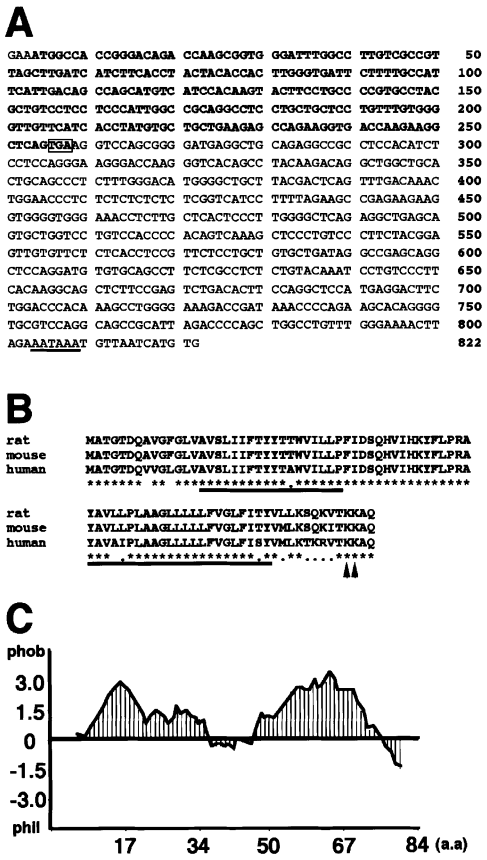

Fig. 3. (**A**) Nucleotide sequence of *rDPM2* cDNA. The coding sequence is shown in bold. The stop codon is boxed. The putative polyadenylation signal sequence is underlined. Nucleotide numbers are on the right. DDBJ/EMBL/GenBank accession numbers of rat, mouse and human *DPM2* cDNAs are AB013359, AB013360 and AB013361, respectively. (**B**) Alignment of rat, mouse and human DPM2 amino acid sequences. Asterisks and dots indicate identical and conserved amino acids, respectively. Two putative transmembrane regions are underlined. Arrowheads indicate a putative ER retention signal. (**C**) Hydropathy profile of rDPM2 drawn according to the Kyte and Doolittle program (Kyte and Doolittle, 1982).

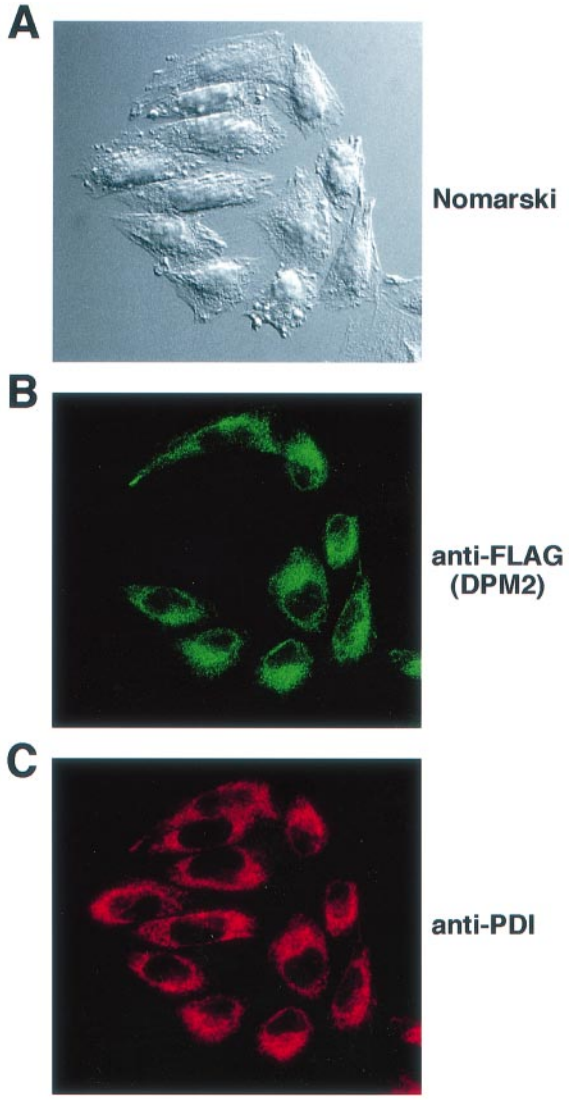

Fig. 4. ER localization of DPM2 protein. An equal mixture of Lec15.B5 and FLAG-tagged rDPM2-transfected Lec15.B5 cells [(**A**), Nomarski)] were double-stained with anti-FLAG antibody (**B**) and anti-PDI (**C**).

near the C-terminus (Figure 3B) which may be an ER retention signal thought to function on the cytoplasmic side (Jackson *et al*., 1990). If this is true, then both the N- and C-termini of DPM2 may face the cytosol. The PHDhtm program also predicted the same orientation. DPM2 did not have a typical dolichol recognition sequence reported in a number of proteins that interact with dolichol (Albright *et al*., 1989; Kelleher *et al*., 1992).

To determine the intracellular expression site of DPM2, its N-terminus was tagged with a FLAG sequence and the fusion construct was stably expressed in Lec15.B5 cells. This fusion protein was active, as shown by complementation of the surface CD59 expression (data not shown). Indirect immunofluorescence staining using anti-FLAG antibody showed a perinuclear and reticular staining profile (Figure 4B) that coincided with the staining profile of a known ER protein, protein disulfide isomerase (PDI) (Figure 4C), suggesting that DPM2 is an ER protein, in agreement with the presence of a putative ER retention signal and the fact that DPM synthesis occurs in the ER (Czichi and Lennarz, 1977).

Lec15 cells are defective in the DPM2 gene

To determine whether *DPM2* is responsible for the mutant phenotype of Lec15 cells, we analysed *DPM2* transcripts by Northern blotting (Figure 5A). A major 1 kb and a minor 1.1–1.2 kb mRNA of *DPM2* in wild-type CHO cells (Figure 5A, lane 2) and a single 1–1.1 kb mRNA in C_6 glioma cells (Figure 5A, lane 1) were detected, whereas no mRNA was detected in Lec15 cells (Figure 5A, lane 3). RT–PCR also showed no detectable band in Lec15 cells (data not shown). We analysed the *DPM2* gene by Southern blot with a hamster cDNA probe (Figure 5B, centre panel). Although the hybridization profile of genomic fragments digested with several restriction enzymes showed no difference between wild-type CHO (C) and Lec15 (L) cells, the intensities of bands were clearly less in the latter. In contrast, the staining intensities with ethidium bromide (left panel) and the intensities of bands rehybridized with a control hamster *PIG-L* cDNA probe (right panel) were comparable between the two cell lines, indicating that the differences in hybridization intensities are specific to

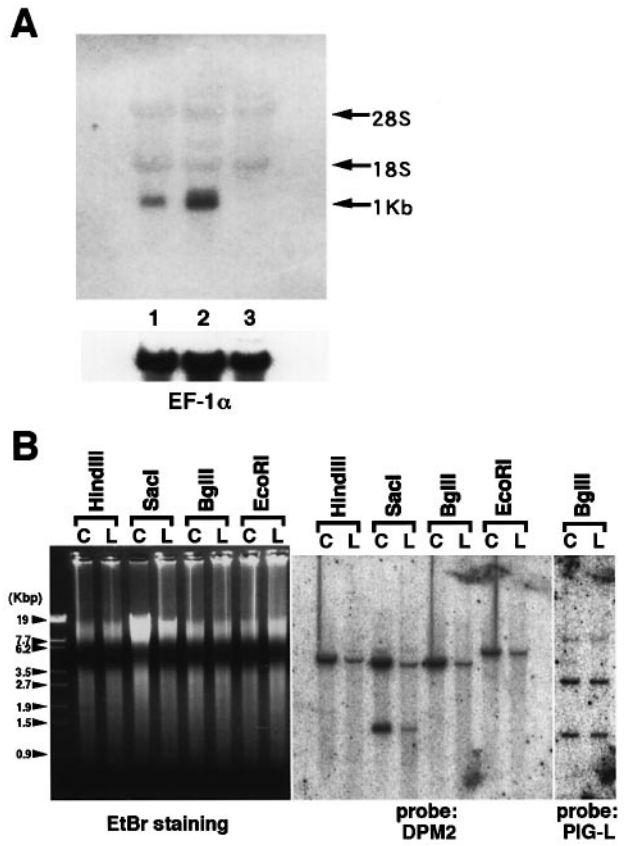

Fig. 5. (**A**) Northern blot analysis of *DPM2*. Poly(A) RNAs from rat C_6 glioma (lane 1), wild-type CHO (lane 2) and Lec15 (lane 3) cells were blotted against hamster *DPM2* cDNA. The same membrane was rehybridized with an EF-1α cDNA. The positions of 28S and 18S rRNAs and the 1 kb position are indicated on the right. (**B**) Southern blot analysis of *DPM2*. DNAs from wild-type CHO (C) and Lec15 (L) cells digested with *Hin*dIII, *Sac*I, *Bgl*II and *Eco*RI were separated on an agarose gel, stained with ethidium bromide (left panel) and transferred to a nylon membrane. The membrane was hybridized with hamster *DPM2* cDNA (centre panel) and rehybridized with hamster *PIG-L* cDNA (right panel). The positions of size markers are indicated on the left.

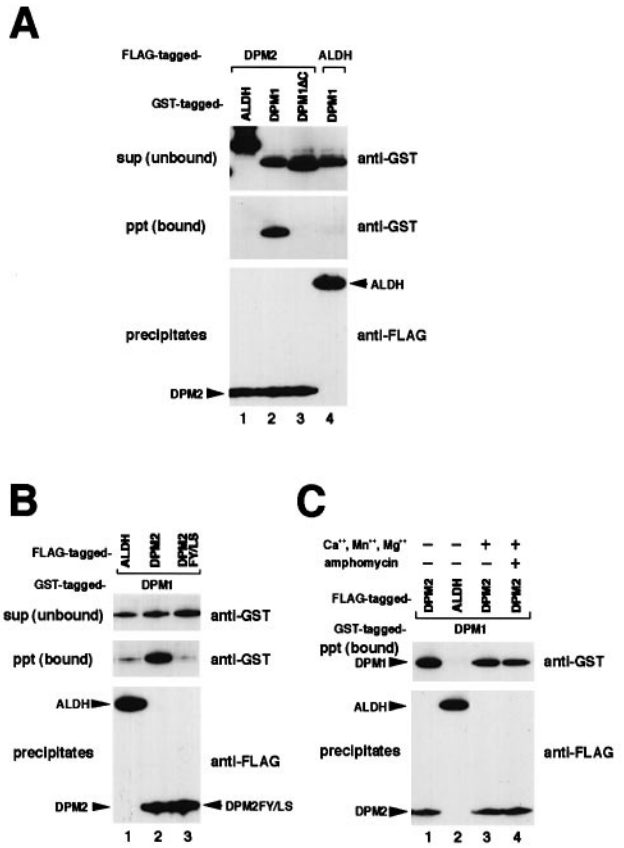

Fig. 6. Western blot analyses of association of DPM1 with DPM2. (**A**) FLAG-tagged DPM2 (lanes 1–3) or ALDH (lane 4) were co-transfected with each of GST-tagged ALDH (lane 1), DPM1 (lanes 2 and 4) and DPM1ΔC (lane 3) into Lec15 cells. Immunoprecipitates with anti-FLAG beads (middle and lower panels) and the supernatant (upper panel) were analysed by SDS–PAGE/ Western blotting against anti-GST (upper and middle panels) and anti-FLAG (lower panel) antibodies. (**B**) FLAG-tagged ALDH (lane 1), DPM2 (lane 2) and DPM2FY/LS (lane 3) were co-transfected with GST-tagged DPM1 into Lec15 cells. Western blots were done as described in (A). (**C**) FLAG-tagged DPM2 (lanes 1, 3 and 4) and ALDH (lane 2) were co-transfected with GST-tagged DPM1 into Lec15 cells. Metal ions alone (lane 3) or with amphomycin (lane 4) were added to the extract before immunoprecipitation. Metal ions are required for amphomycin (Banerjee *et al*., 1981). The final concentrations were 5 mM for $MnCl_2$ and $MgCl_2$, 10 mM for $CaCl_2$ and 600 μg/ml for amphomycin. Immunoprecipitation and Western blotting were carried out as described in (A).

DPM2. This may be due to a loss of one allele, and the detected bands may represent an inactive *DPM2* allele that does not make a transcript.

DPM2 protein associates with DPM1 protein

Because DPM1 and DPM2 participate in the synthesis of DPM, the possibility that these proteins associate with each other to form an enzyme complex was tested. Human DPM1 tagged with glutathione *S*-transferase (GST) and rat DPM2 tagged with FLAG at their N-termini were constructed. They were functional because they complemented the mutant phenotypes of class E and Lec15 cells, respectively (data not shown). As a control, rat microsomal aldehyde dehydrogenase (ALDH) that has a transmembrane domain and that resides in the ER membrane (Masaki *et al*., 1994) was also tagged with FLAG or GST. We transfected various combinations of these expression plasmids into Lec15.B5 cells and analysed the digitonin-solubilized cells by immunoprecipitation with anti-FLAG beads followed by Western blotting with anti-GST and anti-FLAG antibodies. As shown in Figure 6A, about half of the GST–DPM1 (lane 2) was co-precipitated with FLAG-tagged DPM2. In contrast, GST–ALDH (lane 1) was not co-precipitated. In a similar experiment, GST–DPM1 was not co-precipitated with FLAG-tagged ALDH (lane 4). These results indicated that DPM1 and DPM2 proteins physically associated with each other.

To localize the region of DPM1 necessary for its function and association with DPM2, we constructed various deletion mutants of *DPM1* cDNA and transfected them into class E cells. A DPM1ΔC mutant in which 24 C-terminal amino acids were deleted did not restore the mutant phenotype of class E cells (data not shown). As shown in lane 3 of Figure 6A, association of DPM1ΔC with DPM2 was not detected in Lec15 cells, indicating that the C-terminal region of DPM1 was essential for association with DPM2. We also constructed a mutant DPM2, DPM2FY/LS, in which Phe21 and Tyr23 were changed to Leu and Ser, respectively. These mutations,

which may not change the secondary structure of DPM2 as expected by PHDhtm analysis, abolished the abilities to restore the surface CD59 expression in Lec15.B5 cells (data not shown) and to associate with DPM1 (Figure 6B), suggesting that this binding was necessary for DPM synthase activity and that the first transmembrane domain of DPM2 is involved in association with DPM1.

We then examined the effect of amphomycin on the binding of DPM1 and DPM2 (Figure 6C). Amphomycin is known to inhibit the synthesis of monosaccharide-lipids, such as DPM, Dol-PP-GlcNAc and Dol-P-Glc (Kang *et al*., 1978; Banerjee *et al*., 1981). Association of DPM1 with DPM2 was not affected by the same concentration of amphomycin (Figure 6C, lane 4) that completely inhibited DPM synthesis *in vitro* (data not shown), suggesting that inhibition of DPM synthesis is due to the interaction between dolichol monophosphate and amphomycin, as reported previously (Banerjee, 1989, 1994), and not to destruction of the association between DPM1 and DPM2.

DPM2 is essential for the ER localization and the stable expression of DPM1

The fact that DPM2 associates with DPM1 suggested that a function of DPM2 is to localize DPM1 at the correct site, i.e. the ER membrane. To examine whether this is true, we studied the subcellular localization of DPM1 in Lec15 cells and Lec15 cells permanently transfected with *DPM2* cDNA. We co-transfected Lec15 and *DPM2*-transfected Lec15 cells with GST–DPM1 and GST–ALDH expression plasmids. After culture, we disrupted them and fractionated a nuclei-free sample into the ER, Golgi, plasma membrane and cytoplasm by sucrose density gradient centrifugation. GST–ALDH was used as an ER marker. As shown in Figure 7A, in *DPM2*-transfected Lec15 cells, GST–DPM1 and GST–ALDH were detected in the same fractions that were well separated from those containing the plasma membranes and Golgi, indicating that DPM1 resided mainly in the ER (right panel). In contrast, in Lec15 cells which lacked DPM2, DPM1 was detected mainly in fraction 2 that contained the plasma membranes and Golgi but only a small amount of the ER marker ALDH (left panel). Therefore, the localization of DPM1 to the ER was dependent upon DPM2 expression.

In Western blotting and immunofluorescence microscopic analyses, we noticed that the DPM1 expression level in Lec15 cells is always lower than that in wild-type CHO and *DPM2*-transfected Lec15 cells. Figure 7A also shows that less DPM1 was present in Lec15 than in *DPM2*-transfected Lec15 cells. To confirm this, we co-transfected GST, GST–ALDH and GST–DPM1 into Lec15 and DPM2-expressing Lec15 cells. Both cell lysates contained similar amounts of GST and GST–ALDH proteins, whereas Lec15 cells expressed only one-fifth as much GST–DPM1 as DPM2-expressing Lec15 cells (Figure 7B), indicating that the expression level of DPM1 protein was dependent upon DPM2. To see whether this dependency was due to a DPM2-mediated regulation at the transcript level, the *DPM1* transcript was examined in wild-type CHO and Lec15 cells by Northern blotting. As shown in Figure 7C, the quantities of *DPM1* transcripts were similar, eliminating this possibility.

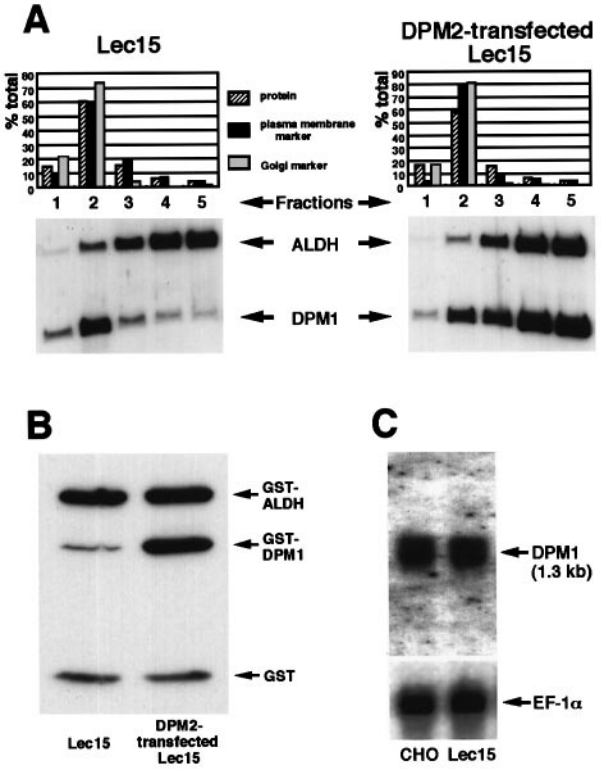

Fig. 7. (**A**) DPM2-dependent ER localization of DPM1. Lec15.B5 (left panels) and DPM2-transfected Lec15.B5 cells were transfected with GST-tagged DPM1 and ALDH. Their membranes were fractionated by sucrose density gradient centrifugation. Fractions were characterized by assaying for protein content and organelle-specific marker enzymes (alkaline phosphodiesterase I for the plasma membrane and α-mannosidase II for the Golgi apparatus) (upper panels), and by Western blotting against anti-GST antibody (lower panels). The enzyme activities in fractions are shown as a percentage of total activities. (**B**) DPM2-dependent stable expression of DPM1. Lec15.B5 (left lane) and DPM2-transfected Lec15.B5 (right panel) cells were transfected with a pre-mixture of pME-Py-GST (1.5 μg), pME-Py-GST-ALDH (1.5 μg) and pME-Py-GST-DPM1 (20 μg) plasmids, cultured for 2 days. The lysates from these transfectants were Western blotted against anti-GST antibody. (**C**) Northern blot analysis of *DPM1*. Poly(A) RNAs from wild-type CHO (left lane) and Lec15 (right lane) cells were blotted against human *DPM1* cDNA. The membrane was rehybridized with EF-1α cDNA (lower panel).

DPM1 has a catalytic activity without DPM2, if it is stably expressed in the ER

Transfection of a *DPM1* cDNA into Lec15.B5 cells induced surface CD59 expression weakly (Figures 1B and 8A), indicating that DPM1 alone has some enzymatic activity. Since transfection of a *DPM1* cDNA into Lec15 resulted in only a low level expression of DPM1 (Figure 7B), we tried to obtain high expression of DPM1 without DPM2. For this, we made a chimeric construct in which the C-terminus of GST–DPM1 (GD1) was fused with a transmembrane region of ALDH that contains an ER retention signal (Masaki *et al*., 1994). For comparison, GD1 was fused with DPM2. Surface CD59 expression, expression levels of the chimeric DPM1 proteins and DPM synthase activities were assessed by fluorescence-activated cell sorting (FACS) (Figure 8A), Western blotting with anti-GST antibody (Figure 8B) and an *in vitro* DPM synthase assay using microsomal membranes (Figure 8C), respectively. Expression of GD1 itself was very low, requiring longer exposure for detection (Figure 8B, lane 2), whereas two

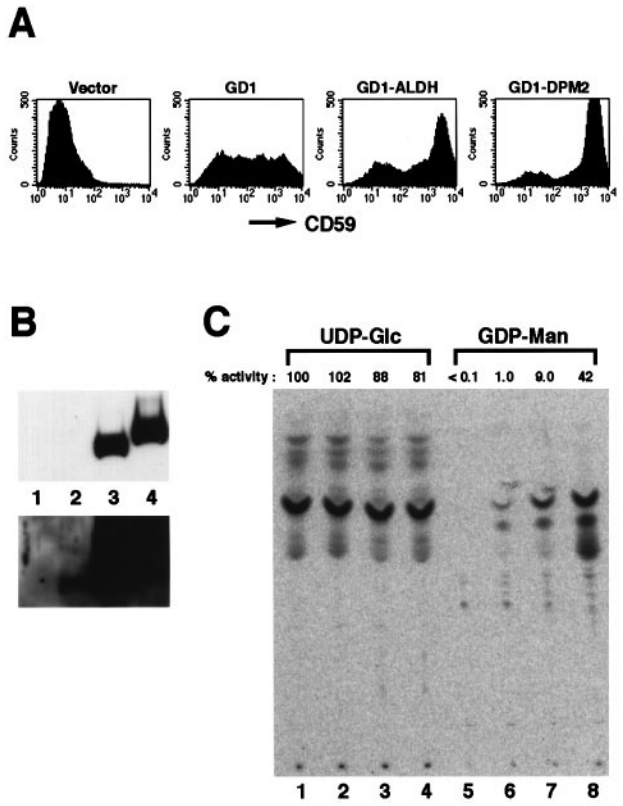

Fig. 8. DPM synthase activities of DPM1 chimeric proteins. Lec15.B5 cells (7.5×10^6) were transfected with 20 μg of pME-Py (Vector), pME-Py-GST-hDPM1 (GD1), pME-Py-GD1-ALDH or pME-Py-GD1-rDPM2 and cultured for 2.5 days. One-tenth of the transfected cells were used for FACS analysis to examine the surface CD59 expression (**A**) and the rest were used to prepare the membrane fractions. To assess GST-tagged protein, membranes (400 μg of proteins) were solubilized and immunoprecipitated with glutathione beads, then Western blotted against anti-GST antibody (**B**). Upper panel, short exposure; lower panel, longer exposure. The rest of the membrane (100 μg proteins) was used to measure activities of DPM and Dol-P-Glc synthases (**C**). Lanes 1 and 5, vector; lanes 2 and 6, GD1; lanes 3 and 7, GD1–ALDH; lanes 4 and 8, GD1–DPM2.

fusion proteins were stably expressed at high levels (Figure 8B, lanes 3 and 4). These fusion proteins resided in the ER (data not shown). GD1 caused a partial restoration of the surface CD59 expression (Figure 8A) and very weak DPM synthase activity (Figure 8C, lane 6), as expected. The two fusion proteins caused complete restoration of CD59 expression (Figure 8A) and had considerable DPM synthase activities (Figure 8C, lanes 7 and 8), indicating that DPM1 has a catalytic activity without DPM2, if it is stably expressed in the ER. However, GD1–DPM2 (Figure 8C, lane 8) had several times higher activity than GD1–ALDH (Figure 8C, lane 7) and was comparable with that of wild-type CHO cells when normalized by Dol-P-Glc synthesis (Figure 2 versus 8C). The higher activity of GD1–DPM2 might be due to association of endogenous DPM1 with the DPM2 portion. Another possibility is that DPM2 has another function, for example it enhances binding of Dol-P, causing an enhanced enzymatic activity (see below).

DPM2 enhances binding of Dol-P to DPM synthase

Although DPM1 itself has some enzymatic activity, i.e. it binds Dol-P, we speculated that hydrophobic DPM2

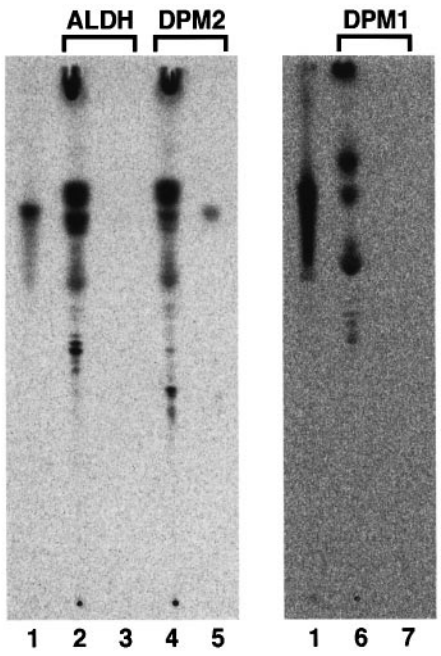

Fig. 9. Association of Dol-P with DPM synthase. Transfectants of Lec15 cells with FLAG-tagged ALDH (lanes 2 and 3), DPM2 (lanes 4 and 5) and DPM1 (lanes 6 and 7) were established and metabolically labelled with [5-^{3}H(*N*)]mevalonolactone. Labelled lipids were analysed as described in Materials and methods. Lane 1, control Dol-P; lanes 2, 4 and 6, total labelled lipids; lanes 3, 5 and 7, immunoprecipitates with anti-FLAG beads.

enhances binding of Dol-P to the enzyme. To test this, we stably transfected Lec15 cells with FLAG-DPM1, -DPM2 and -ALDH, and incubated them with [5-^{3}H]mevalonolactone in the presence of mevastatin (compactin), an inhibitor of HMG-CoA reductase, to label polyisoprenoid lipids. Digitonin extracts of these cells were immunoprecipitated with anti-FLAG beads, and the precipitates and the total cell extracts were extracted with chloroform/methanol (2:1) and analysed by thin-layer chromatography (TLC) (Figure 9). Similar radiolabelled products were seen in total cell extracts of these transfectants (Figure 9, lanes 2, 4 and 6). The immunoprecipitates from the *DPM2* transfectant (Figure 9, lane 5) contained a single ^{3}H-labelled spot migrating similarly to control Dol-P (Figure 9, lane 1). In contrast, the immunoprecipitates from the ALDH transfectant (Figure 9, lane 3) and the DPM1 transfectant (Figure 9, lane 7) did not contain a detectable amount of Dol-P, indicating that binding of Dol-P to DPM synthase was enhanced by the presence of DPM2.

Discussion

A major finding in this study is that synthesis of DPM in mammalian cells is mediated by two proteins, i.e. catalytic DPM1 and regulatory DPM2. This is in contrast to biosynthesis of DPM characterized in three eukaryotic microorganisms. In *S.cerevisiae* (Orlean *et al*., 1988), *Ustilago maydis* (Zimmerman *et al*., 1996) and *Trypanosoma brucei* (Mazhari-Tabrizi *et al*., 1996), a single component known as Dpm1p has DPM synthase activity. Singh *et al*. (1991) reported genetic evidence that two genes regulate DPM synthesis in mammalian cells. They

demonstrated that a somatic cell hybrid of two mutant cells, mouse class E lymphoma and CHO Lec15 cells, both lacking DPM, restored DPM synthesis (Singh *et al*., 1991). We recently found that the mammalian homologue of *S.cerevisiae DPM1* is the gene defective in the class E mutant cells (Tomita *et al*., 1998). Here we show that Lec15 cells are defective in another gene *DPM2*. Therefore, the genetic evidence is now clearly correlated with the biochemical evidence.

DPM2 is a hydrophobic protein consisting of 84 amino acids. It contains two putative transmembrane domains and a double lysine sequence near the C-terminus that is a putative ER localization signal (Figure 3). Consistent with these characteristics, DPM2 is expressed in the ER membrane (Figures 4 and 7). We found that DPM2 associates with DPM1 and that DPM2 is required for ER localization of DPM1 (Figures 6 and 7). Introduction of two amino acid substitutions into the first transmembrane domain of DPM2 resulted in a loss of association with DPM1, suggesting that association occurs within the membrane. Although mammalian DPM1 does not have a typical transmembrane domain, it resides on the membrane even in the absence of DPM2 (Tomita *et al*., 1998) but was localized, presumably non-specifically, to various membranes other than the ER (Figure 7A). In contrast, in the presence of DPM2, DPM1 was localized to the ER, indicating that association with DPM2 is essential for proper localization of the catalytic component DPM1.

The amount of DPM1 protein was also dependent upon DPM2, i.e. the level of DPM1 protein in the absence of DPM2 was much lower than that in the presence of DPM2 (Figure 7B). Transcriptional regulation of *DPM1* is unlikely because the amount of *DPM1* mRNA in Lec15 cells was nearly equal to that in wild-type CHO cells (Figure 7C). Co-translational regulation of DPM1 by DPM2 is also unlikely because DPM1, which is a peripheral membrane protein (our unpublished result), is presumably translated in the cytoplasm whereas DPM2 is presumably translated on the ER membrane and is expressed in the ER. It is most likely that the post-translational stability of DPM1 is regulated by DPM2. A lack of ER localization of DPM1 coincided with a lowered level of DPM1 expression in Lec15 cells, suggesting that DPM2-dependent localization of DPM1 to the ER is important for its stability. Consistent with this, DPM1–ALDH and DPM1–DPM2 fusion proteins, both of which have an ER retention signal, were more highly expressed than free DPM1 in Lec15 cells (Figure 8B). Another possible mechanism of stabilization is that association of DPM2 prevents degradation of DPM1 by inducing a conformational change or by masking a region susceptible to a degradation process. Such a mechanism might also contribute to the enhanced stabilities of DPM1–ALDH and DPM1–DPM2 fusion proteins.

In addition to the essential roles of DPM2 for ER localization and stability of DPM1, DPM2 enhances binding of Dol-P to the enzyme. When FLAG-tagged DPM2 was expressed in Lec15 cells and immunoprecipitated, Dol-P was co-precipitated (Figure 9). In contrast, when FLAG-tagged DPM1 was expressed and immunoprecipitated, Dol-P was not detected in the precipitates. This may simply depend upon the amount of DPM1 protein. Another possibility is that Dol-P may have a higher affinity for DPM synthase complex than DPM1 itself owing to its affinity for DPM2. We have not studied the enzyme kinetics of DPM synthase in the presence and absence of DPM2. Once the two types of enzyme are isolated, their K_ms for Dol-P should be determined.

DPM1 proteins of various eukaryotic organisms are divided into two groups (Colussi *et al*., 1997). The first group includes DPM1 proteins of *S.cerevisiae*, *U.maydis* and *T.brucei*. The second includes DPM1 proteins of mammals, *Schizosaccharomyces pombe* and the nematode *Caenorhabditis briggsiae* (Colussi *et al*., 1997). Members within each group share 60–70% amino acid identity, whereas amino acid identities between the two groups were only 30–40%. A major structural difference between the two groups is the presence of a transmembrane domain at the C-terminus only in the first group. It was reported that although most part of the C-terminal hydrophobic region of *S.cerevisiae* Dpm1p was dispensable for enzyme activity and growth, the entire C-terminal portion could not be eliminated (Zimmerman and Robbins, 1993). So, Dpm1 proteins in the first group are typical integral membrane proteins. In contrast, those in the second group lack a typical transmembrane domain and is peripheral membrane proteins (Tomita *et al*., 1998). Recombinant proteins of the first group made in *E.coli* had DPM synthase activity, indicating that DPM1 proteins of the first group are DPM synthase itself (Orlean *et al*., 1988; Mazhari-Tabrizi *et al*., 1996; Zimmerman *et al*., 1996). *DPM1*s from the first group of organisms complemented a temperature-sensitive mutant of *S.cerevisiae, dpm1*. Moreover, *S.cerevisiae DPM1* complemented both class E and Lec15 mutants as well as the *S.pombe dpm1* mutant (Colussi *et al*., 1997). These results indicate that DPM1 proteins in the first group were stably expressed in cells of various organisms and acted as DPM synthases. In contrast, human and *S.pombe DPM1* did not complement the temperature sensitivity of *S.cerevisiae dpm1* (Colussi *et al*., 1997). If human DPM1 is stably expressed, it has considerable DPM synthase activity even without DPM2 (Figure 8), suggesting that the lack of complemention may be due to mainly inefficient expression of DPM1. Although it is not certain at the moment whether other organisms in the second group have DPM2, the present finding that stable expression of human DPM1 requires DPM2 and the fact that human *DPM1* cDNA complemented *S.pombe dpm1* (Colussi *et al*., 1997) are consistent with the idea that *S.pombe* has a DPM2 homologue.

The results of this study indicate that DPM1 is intrinsically unstable. This is beneficial to prevent any mislocalized DPM1 from synthesizing DPM at incorrect sites. In CHO cells, DPM2 is limited relative to DPM1 because overexpression of DPM2 in Lec15 cells caused a 4- to 5-fold higher synthesis of DPM than in wild-type CHO cells (Figure 2). It seems, therefore, that DPM synthase activity is determined primarily by DPM2 rather than the DPM1 component because excess free DPM1 is labile. There are reports that isoproterenol and oestrogen treatment which enhanced protein *N*-glycosylation and glycoprotein synthesis also enhanced DPM synthase activity in mammalian cells and tissues (Banerjee *et al*., 1987; Carson *et al*., 1990). Another report showed that DPM stimulated biosynthesis of GlcNAc-P-P-dolichol by allosterically enhancing an activity of the *N*-acetylglucosamine

phosphate transferase (Kean, 1985). These lines of evidence suggest that protein glycosylation may be regulated *in vivo* under various conditions through regulation of DPM synthase activity. The two-component DPM synthase system may be necessary for skilful regulation of DPM synthesis in mammalian cells. Further studies on the regulation of DPM1 and DPM2 will provide a much more comprehensive understanding of the regulation of protein glycosylation.

Materials and methods

Cells and culture

Wild-type CHO-K1, and Lec15.2 and Lec35.2 mutants (gifts from Dr M.A.Lehrman, Texas Southwestern Medical Center, Dallas, TX) (Camp *et al*., 1993) were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum. Their transfectants were selected and maintained in 600 μg/ml G-418 and/or 6 μg/ml puromycin. The mouse lymphoma BW5147 and its Thy-1-negative class E mutant cells (gifts from Dr R.Hyman, Salk Institute, San Diego, CA) were cultured as described (Tomita *et al*., 1998). Lec15.B5 cells were established from Lec15 by transfecting 25 μg of pME-DAF-Neo-CD59 (Nakamura *et al*., 1997) followed by limiting dilution in G-418. Lec15.B5D7 and Lec15.B5A6 cells were established from Lec15.B5 by co-transfection of 20 μg of pME-Py-FLAG-rDPM2 (for B5D7) or pME-Py-FLAG-ALDH (for B5A6), and 2.5 μg of pGKPuro (a gift from Dr T.Yagi, National Institute for Physiological Science, Japan) (Watanabe *et al*., 1995) followed by limiting dilution in puromycin. Lec15.FD1C10 cells that express FLAG-tagged hDPM1 were established by transfection of 30 μg of pME-neo-FLAG-hDPM1 into Lec15 cells, followed by limiting dilution in G-418.

Plasmids

pME-Py-hDPM1 and -yDPM1 were constructed by subcloning cDNAs containing the full coding region of human *DPM1* (Tomita *et al*., 1998) and yeast *DPM1* (Orlean *et al*., 1988; Tomita *et al*., 1998), respectively, into pME-Pyori18Sf– expression vector (Ohishi *et al*., 1996) originally derived from pME18Sf– (a gift from Dr K.Maruyama). pME-Py-rDPM2 is identical to the clone 6B12 obtained by expression cloning. To fuse GST and FLAG at the N-termini of hDPM1, rDPM2 and ALDH, we first subcloned the *Xho*I–*Not*I fragment of pMEEB-GST-PIG-A and pMEEB-FLAG-PIG-A (Watanabe *et al*., 1996) into the *Xho*I–*Not*I site of pME-Pyori18Sf– to obtain pME-Py-GST-PIG-A and pME-Py-FLAG-PIG-A, both of which had a *Sal*I site that connects the tags with PIG-A. pME-Py-GST (and -FLAG) -hDPM1, -rDPM2 and -ALDH were obtained by replacing PIG-A of pME-Py-GST-PIG-A (and pME-Py-FLAG-PIG-A) with the coding region of each gene. In these plasmids, the *Sal*I sites (GTCGAC which is translated into Val and Asp) following the GST and FLAG tags were followed by human *DPM1* cDNA which begins at the initiation codon, rat *DPM2* cDNA bearing an additional three bases, 'TCC', 5′ to the initiation codon, and *msALDH* cDNA (Watanabe *et al*., 1996), respectively.

pME-neo-FLAG-hDPM1 was constructed by subcloning the FLAG-hDPM1 region from pME-Py-FLAG-hDPM1 into pME-neo (Watanabe *et al*., 1996). pME-Py-GST-hDPM1ΔC was obtained by replacing the last 24 amino acids at the C-terminus of GST–hDPM1 with Thr and Arg (ACGCGT). pME-Py-FLAG-rDPM2FY/LS was obtained by converting the sequence 'TTC ACC TAC' which encodes 'Phe–Thr–Tyr' to the sequence 'TTG ACT AGT' which encodes 'Leu–Thr–Ser' by means of site-directed mutagenesis. pGL3-FLAG-rDPM2 was constructed by replacing the luciferase gene of pGL3-Control vector (Promega) with the FLAG-rDPM2 region of pME-Py-FLAG-rDPM2. pGL3-FLAG-rDPM2 that uses an SV40 promoter and induces a lower expression level than pME-Py-FLAG-rDPM2 was used for expression comparable with pME-Py-FLAG-ALDH. pME-Py-GD1-ALDH was constructed by connecting the 3′ end of the coding region of GST–hDPM1 with a sequence ACG CGT followed by a sequence encoding Trp450 to stop485 of *msALDH* (Masaki *et al*., 1994). pME-Py-GD1-rDPM2 was constructed by connecting the 3′ end of the coding region of GST–hDPM1 with a sequence ACG CGC followed by the full *rDPM2* coding sequence beginning at the initiation codon.

Transfection

Wild-type CHO and Lec15 cells and their transformants (suspended in 0.4 ml of culture medium with the indicated amounts of DNA) were electroporated at 260 V and 960 μF. Thy-1-negative class E cells (10^7) suspended in 0.8 ml of culture medium with 20 μg of DNA were electroporated at 350 V and 250 μF. Electroporations were done in a Gene Pulser (Bio-Rad).

Fluorescence staining of cell surface Thy-1 and CD59

Cells were stained for Thy-1 and for CD59 as described (Watanabe *et al*., 1996).

Cloning of rat DPM2 cDNA

A total of 2×10^8 Lec15.B5 cells were mixed with 240 μg each of rat glioma cDNA library (Nakamura *et al*., 1997) and pcDNA-PyT(ori-) plasmids (Nakamura *et al*., 1997) in HEPES-buffered saline, and were electroporated in 12 cuvettes. Two days later, transfected cells were stained with biotinylated anti-CD59 monoclonal antibody 5H8 in combination with phycoerythrin-conjugated streptavidin (Nakamura *et al*., 1997) and ~800 cells with restored surface CD59 expression were collected by a cell sorter (FACS-Vantage). From these cells, 2.7×10^4 independent plasmid clones were recovered by Hirt's method (Hirt, 1967). Pooled plasmids (20 μg) were retransfected with 180 μg of pcDNA-PyT(ori-) plasmids into 1.2×10^8 Lec15.B5 cells as described above in eight cuvettes. After another cycle of cell sorting and recovery of plasmids, 1248 independent plasmid clones were analysed and six positive clones were obtained.

Northern and Southern blot analyses

For Northern blot analysis of DPM2, mRNAs (12.5 μg) prepared using Trizol (Gibco-BRL) and oligo(dT) column (Pharmacia) were separated on a 0.7% agarose gel, then transferred to a nylon membrane (Amersham). This membrane was hybridized with a hamster *DPM2* cDNA probe and rehybridized with a human elongation factor-1α (EF-1α) cDNA probe (Uetsuki *et al*., 1989). Hamster *DPM2* cDNA was prepared by RT–PCR using total RNA of Lec35 cells, 5′ primer (5′-TCA TCT TCA CCT ACT ACA CCR CYT GG) and 3′ primer (5′-CTT YYT CTY GGC TTC TAA AAG GAT). After sequence confirmation, the cDNA was radiolabelled.

For Southern blot analysis, 6 μg of genomic DNA digested with a restriction enzyme was separated on a 0.7% agarose gel and transferred to a nylon membrane. The membrane was hybridized in Church phosphate buffer (Church and Gilbert, 1984) with a radiolabelled hamster *DPM2* cDNA probe which was obtained by RT–PCR using 5′ primer (5′-GGA GCG TCG ACT CCA TGG CCA CCG GGA CAG ACC A) and 3′ primer as described above. It was rehybridized with a hamster *PIG-L* cDNA probe (Nakamura *et al*., 1997). For Northern blot analysis of *DPM1*, mRNAs (5 μg) and a human *DPM1* cDNA probe were used.

Immunofluorescence microscopic analysis

Equal mixtures of Lec15.B5 and Lec15.B5D7 (FLAG-tagged rDPM2-transfected Lec15.B5) cells cultured on 14 mm diameter glass coverslips were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 1 h, and incubated in 5% bovine serum albumin in PBS for 10 min. They were then incubated with anti-FLAG antibody M2 (Kodak), fluorescein isothiocyanate-conjugated donkey anti-mouse IgG antibodies (Chemicon International), rabbit anti-PDI antibodies (a gift from Drs R.Masaki and A.Yamamoto, Kansai Medical School, Osaka, Japan) and rhodamine-conjugated donkey anti-rabbit IgG antibodies (Chemicon International), and studied under a confocal laser scanning microscope (Olympus).

Assay of DPM and Dol-P-Glc synthases activities

Cells were destroyed hypotonically by a Teflon homogenizer in a buffer (20 mM Tris–HCl pH 7.4, 10 mM NaCl, 2 μg/ml leupeptin and 1 mM p-APMSF) on ice. After removal of cell debris and nuclei by centrifugation at 1500 *g* for 10 min, membranes were collected by centrifugation at 100 000 *g* for 1 h and suspended in a reaction buffer consisting of 50 mM HEPES–KOH pH 7.4, 25 mM KCl, 5 mM $MgCl_2$ and 5 mM $MnCl_2$. Dol-P (10 μg; Sigma), which was first added to a tube in chloroform/methanol (2:1) solution and dried under a nitrogen stream, then the membranes (100 μg of protein) and GDP-[^{3}H]mannose (0.16 μM, 0.8 μCi, American Radiolabeled Chemicals) or UDP-[^{3}H]glucose (0.25 μM, 1 μCi, American Radiolabeled Chemicals) were mixed vigorously in a final volume of 100 μl of reaction buffer. The mixture was incubated for 10 min at 37°C and added with 0.5 ml chloroform/methanol (2:1) to stop the reaction. Lipids were extracted with chloroform/methanol (2:1), washed once with 0.5 ml of chloroform/methanol (2:1)-saturated water and then evaporated. The dried materials were

extracted with 30 µl of chloroform/methanol (2:1), and the extracts were separated by TLC on Kieselgel 60 (Merk) with a solvent system of chloroform/methanol/H_2O (10:10:3). The radiolabelled lipids were analysed by Image Analyzer BAS 1500 (Fuji Film Co., Tokyo, Japan) after 2–4 days exposure.

Analysis of the protein complexes

Lec15 cells co-transfected with 15 µg of pME-Py-GST-hDPM1 and 10 µg of pGL3-FLAG-rDPM2 were cultured for 2 days and solubilized with lysis buffer A (1% digitonin, 10 mM triethanolamine, 150 mM NaCl, 10 mM iodoacetoamide, 1 mM EDTA, 2 µg/ml leupeptin and 1 mM APMSF). After removal of insoluble material by centrifugation at 18 000 *g* for 5 min and at 100 000 *g* for 1 h, the soluble fraction was mixed with M2 anti-FLAG beads (Kodak) and agitated for 2 h. The mixture was separated into unbound fraction (the supernatant) and immunoprecipitates by centrifugation at 4000 *g* for 2 min. The unbound fraction was then mixed with glutathione beads (Pharmacia) for 2 h, centrifuged and the supernatant was discarded. Both precipitates were washed with the lysis buffer, eluted with a sample buffer and analysed by SDS–PAGE/Western blotting against goat anti-GST antibody (Pharmacia) visualized by horseradish peroxidase-conjugated anti-goat IgG antibody (Organon Teknika) plus chemiluminescence reactions (Dupont). The membranes were stripped in a buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 0.1 M 2-mercaptoethanol) at 50°C for 30 min and reprobed with anti-FLAG antibody M2. Amphomycin was a gift from Drs V.Eckert and R.T.Schwarz, University of Marburg, Germany.

Isolation of subcellular fractions

Lec15 and Lec15.B5D7 cells (1.5×10^7) were electroporated with 50 µg of pME-Py-GD1 and 4 µg of pME-Py-GST-ALDH in two cuvettes, cultured for 2 days, suspended in 3 ml of buffer containing 0.25 M sucrose, 10 mM HEPES–NaOH (pH 7.5), 1 mM dithiothreitol (DTT), 1 mM AEBSF and 2 µg/ml leupeptin, disrupted by a Dounce homogenizer (Wheaton, type A) with 60 strokes, and treated with 2 U/ml DNase for 20 min at 4°C. After centrifugation at 10 000 *g* for 15 min at 4°C, the post-nuclear supernatants were fractionated by discontinuous sucrose gradient centrifugation as described (Vidugiriene and Menon, 1993). Proteins, and plasma membrane and Golgi enzyme activities in fractions were measured as previously described (Storrie and Madden, 1990). GST-tagged proteins were located by incubating with glutathione beads for 2 h followed by SDS–PAGE/Western blotting analysis.

Metabolic labelling of polyisoprenoids and Dol-P binding assay

Lec15.B5D7, Lec15.B5A6 and Lec15.FD1C10 cells (1.2×10^6) were cultured for 3 days in the medium containing 40 µM mevastatin (Sigma), 0.3 mM mevalonic acid lactone (Sigma) and 200 µCi (0.5 µM) of RS-[5-^{3}H(*N*)]mevalonolactone (American Radiolabeled Chemicals), washed twice with cold PBS, harvested using a cell scraper with 1.5 ml of lysis buffer A and agitated for 1 h at 4°C (Rosenwald *et al*., 1990). After removal of insoluble materials by centrifugation at 18 000 *g* for 5 min and at 100 000 *g* for 1 h, 500 µl of the soluble fraction was used for immunoprecipitation with anti-FLAG beads and 150 µl was used for detection of total labelled polyisoprenoids. These materials were extracted with 0.5 ml of chloroform/methanol (2:1) and analysed by TLC as described above. [1-^{3}H]Dol-P was purchased from American Radiolabeled Chemicals.

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