A novel mono-branched lipid phosphate acts as a substrate for dolichyl phosphate mannose synthetase

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Dolichyl phosphate mannose synthetase (GDP-mannose: dolichyl-phosphate *O*- β -D-mannosyltransferase; EC 2.4.1.83) is an enzyme that is involved in glycoconjugate biosynthesis and possesses a putatively conserved dolichol binding site. In order to probe the interaction between the enzyme and the dolichol chain, lipid phosphates varying in length and extent of branching have been tested as substrates in crude microsomal preparations from *Saccharomyces cerevisiae*. It was found that phytanyl (3,7,11,15-tetramethylhexadecanyl) phosphate was utilized at 60–70% of the efficiency of the natural dolichyl lipid in transfer of [3,4-³H]mannose from GDP-Man to organic soluble material, whereas addition of *S*-3-methyloctadecanyl phosphate, which is of similar length to the phytanyl analogue but with only one branch, resulted in approximately 25% of the incorporation of the natural substrate. Incubations with the unbranched tetra-

decanyl phosphate and with the short, doubly branched *R*- and *S*-dihydrocitronellyl (3,7-dimethyloctanyl) phosphates exhibited levels of activity similar to incubations with no exogenous acceptor. These results were qualitatively confirmed with experiments on *Escherichia coli* harbouring the *S. cerevisiae* *DPM1* gene. The [³H]mannosylated lipid-linked material from microsomal incubations was purified by anion-exchange chromatography. The major saccharide component recovered after hydrolysis was determined to be mannose, but a mannose-containing disaccharide was also present. It is concluded that branching of lipid phosphates is essential for substrates of dolichyl phosphate mannose synthetase and that significant transfer of mannose occurs even if only branching at C-3 is present.

INTRODUCTION

Dolichol is a polyisoprenol component of cell membranes which contains up to 20 isoprene units, of which only the first is saturated. Its phosphorylated derivatives are involved as cofactors in glycoconjugate biosynthesis, and it is interesting to note that the fully extended polyisoprenyl chain is of much greater length than the 45 Å (4.5 nm) width of the membrane. It has been proposed that the bulk of the chains are located between the leaflets of the lipid bilayer, with the α -terminal ends aligned parallel to the acyl chains of the lipids and the phosphate moiety interacting with the membrane surface. Such an arrangement may be necessary for the observed translocation of mannose from GDP-Man and for enhanced membrane fluidity [1,2].

The identification, through protein sequence similarities, of a potential dolichol binding site in a number of enzymes such as β -mannosyltransferase, *N*-acetylglucosaminyl-1-phosphotransferase, dolichyl phosphate mannose (Dol-*P*-Man) synthetase (GDP-mannose: dolichyl-phosphate *O*- β -D-mannosyltransferase; EC 2.4.1.83), ribophorin I (a putative oligosaccharyltransferase subunit), dolichol kinase and bacterial polysialyltransferases [3–5] is a potential basis for the examination of the structural requirements for lipid binding. Of these enzymes, the simplest cofactor, dolichyl phosphate, is required by Dol-*P*-Man synthetase. Thus it would appear reasonable to initially study this enzyme in order to draw conclusions on the binding requirements of the dolichol binding site.

Dol-*P*-Man synthetase catalyses the transfer of mannose from

GDP-Man to dolichyl phosphate: its product has been demonstrated to be present in a variety of eukaryotic cell extracts [6]. The enzyme has been found to be essential for the normal biosynthesis of N-linked oligosaccharides, glycosylphosphatidylinositol membrane anchors and fungal O-linked manno-oligosaccharides [7]. Dol-*P*-Man donates the final four mannose residues of the Glc₃Man₆GlcNAc₂ tetradecasaccharide precursor [8], which is transferred by oligosaccharyltransferase from dolichyl pyrophosphate to Asn-Xaa-Thr/Ser/Cys tripeptides of nascent polypeptide chains [9,10], all three mannose residues of glycosylphosphatidylinositol membrane anchors [11], and the first unit of fungal O-linked oligosaccharides [12].

In yeast, Dol-*P*-Man synthetase is encoded by the essential gene *DPM1* [13] and is located in the endoplasmic reticulum membrane [14]. The mammalian enzyme is similarly located [15], although the curious presence of Dol-*P*-Man synthetase in the mitochondria has been reported [16]. The topology of the enzyme is controversial, with reports indicating a luminal [17] or a cytoplasmic [18] orientation. Analysis of the *DPM1* gene product sequence has been used to support either orientation [7,17], but new evidence gained from studies with fusion proteins would indicate that the enzyme does not have an N-terminal signal sequence and thus would have a cytoplasmic orientation [7]. Since there is no evidence demonstrating that GDP-Man is translocated across the endoplasmic reticulum membrane [19,20], it would appear that Dol-*P*-Man is in some way the mediator for transfer of activated mannose from the cytoplasm to the endoplasmic reticulum lumen. It has also been suggested, based on studies on the reconstitution of Dol-*P*-Man synthetase into

Abbreviation used: Dol-*P*-Man, dolichyl phosphate mannose.

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Scheme 1 Phosphorylation of lipids

Lipids were phosphorylated according to the method of Perich and Johns [30]. THF, tetrahydrofuran; *m*CPBA, *meta*-chloroperbenzoic acid.

liposomes, that the enzyme itself mediates translocation of Dol-*P*-Man [21,22].

It has been suggested that the dolichyl moiety can be substituted by a phytanyl moiety in substrates for the assay of β -mannosyltransferase and Dol-*P*-Man synthetase [23,24]. In addition, the effects of alterations of the length, the geometries of the double bonds and the C-3 enantiomeric composition of the polyisoprenoid chain and the requirement for saturation of the α -isoprenyl unit of dolichol on the activity of various enzymes requiring dolichol-linked substrates have been examined [25–28]. Extending these previous studies, data are shown here from experiments in which a number of lipid phosphates, varying in length and extent of branching, have been tested as substrates for Dol-*P*-Man synthetase, with subsequent analysis of the products, in order to more fully probe the structural requirements of the dolichol binding site.

EXPERIMENTAL

Reagents

GDP-[3,4-³H]mannose was obtained from New England Nuclear (Stevenage, Herts., U.K.), GDP-mannose, dolichyl phosphate, ammonium acetate, DEAE-Sephacel and 2-mercaptoethanol from Sigma (Poole, Dorset, U.K.), Tris from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.), methanol (h.p.l.c. grade) from Rathburn (Walkerburn, Peebles-shire, Scotland, U.K.), MgCl₂·6H₂O from Aldrich (Gillingham, Dorset, U.K.), Ultima Gold scintillation fluid from Canberra Packard (Pangbourne, Berks., U.K.), ion-exchange resins from Bio-Rad (Hemel Hempstead, Herts., U.K.), and QAE-Sephadex from Pharmacia (Milton Keynes, Bucks., U.K.). Lipid phosphates (other than dolichyl phosphate) were synthesized as described below.

Synthesis of lipid phosphates

S-3-Methyloctadecanyl phosphate was synthesized via the Wittig reaction from triphenylphosphonium lauryl bromide (prepared from lauryl alcohol) and di-*t*-butyl[S-3-methylhexan-6-yl]-phosphate (prepared from *S*-citronellol by phosphorylation and ozonolysis) [29]. Phytol, lauryl alcohol, tetradecanol, *R*-

citronellol and *S*-citronellol were purchased from Aldrich (Gillingham, Dorset, U.K.). Phytanol (3,7,11,15-tetramethylhexadecanol) and *R*- and *S*-dihydrocitronellols (3,7-dimethyloctanols) were prepared by catalytic hydrogenation of the corresponding unsaturated alcohols [23]. Lipids were phosphorylated according to the procedure of Perich and Johns [30] (see Scheme 1). Di-*t*-butyl tetradecanyl phosphate: ¹H n.m.r.: δ (p.p.m.) (500 MHz) (C²HCl₃) 3.93 (2 H, m, α -CH₂), 1.64 (2 H, m, β -CH₂), 1.47 (18 H, s, *t*-Bu), 1.36 (2 H, m), 1.25 (20 H, s), 0.87 (3 H, t, terminal Me); ¹³C n.m.r.: δ (p.p.m.) (125 MHz) (C²HCl₃) 81.82 (d, *J* 7.3 Hz, C), 66.89 (d, *J* 6.6 Hz, CH₂), 31.88 (CH₂), 30.37 (d, *J* 5.2 Hz, CH₂), 30.25 (d, *J* 7.2 Hz, CH₂), 29.84 (d, *J* 4.7 Hz, CH₃, *t*-Bu), 29.61 (d, *J* 3 Hz, CH₂), 29.50 (d, *J* 5.1 Hz, CH₂), 29.29 (CH₂), 29.15 (CH₂), 25.60 (CH₂), 22.62 (CH₂), 14.00 (CH₃); ³¹P n.m.r.: δ (p.p.m.) (121.5 MHz) (C²HCl₃) –12.61; *m/z* (direct chemical ionization, NH₃): (*M*+H)⁺ 407.3290 (calculated for C₂₂H₄₈PO₄, 407.3290). Di-*t*-butyl S-3-methyloctadecanyl phosphate: [α]_D²⁵ +1.19° (c 1 in CHCl₃); ¹H n.m.r.: δ (p.p.m.) (250 MHz) (C²HCl₃) 3.97 (2 H, m, α -CH₂), 1.72 (2 H, m), 1.60 (1 H, m), 1.48 (18 H, s, *t*-Bu), 1.25 (28 H, m), 0.92 (3 H, s, CH₃), 0.88 (3 H, s, CH₃); ¹³C n.m.r.: δ (p.p.m.) (62.5 MHz) (C²HCl₃) 81.80 (d, *J* 7 Hz, C), 65.24 (d, *J* 6 Hz, CH₂), 45.90 (CH₂), 37.36 (CH₂), 37.24 (CH₂), 36.99 (CH₂), 36.93 (CH₂), 31.90 (CH₂), 29.87 (d, *J* 4 Hz, CH₃, *t*-Bu), 29.74 (CH₂), 29.67 (CH₂), 29.58 (CH₂), 29.49 (CH₂), 29.32 (CH), 26.97 (CH₂), 26.88 (CH₂), 26.79 (CH₂), 22.65 (CH₂), 22.56 (CH₂), 19.40 (CH₃), 14.05 (CH₃); ³¹P n.m.r.: δ (p.p.m.) (121.5 MHz) (C²HCl₃) –8.87; *m/z* (fast-atom bombardment; Xe): (*M*+H)⁺ 477.4070 (calculated for C₂₂H₃₈PO₄, 477.4072). Di-*t*-butyl *R*-dihydrocitronellyl phosphate: [α]_D²⁵ +2.8° (c 1 in CHCl₃); ¹H n.m.r.: δ (p.p.m.) (250 MHz) (C²HCl₃) 3.97 (2 H, m, α -CH₂), 1.79–1.05 (28 H, m), 0.85 (9 H, CH₃); ¹³C n.m.r.: δ (p.p.m.) (62.5 MHz) (C²HCl₃) 81.79 (d, *J* 7 Hz, C), 65.20 (d, *J* 7 Hz, CH₂), 39.21 (CH₂), 37.28 (d, *J* 8 Hz, CH₂), 37.12 (CH₂), 29.84 (d, *J* 4 Hz, CH₃), 29.30 (CH), 27.93 (CH), 24.58 (CH₂), 22.64 (CH₃), 22.54 (CH₃), 19.39 (CH₃); ³¹P n.m.r.: δ (p.p.m.) (121.5 MHz) (C²HCl₃) –8.83; *m/z* (fast-atom bombardment; Xe): (*M*+H)⁺ 351.2613 (calculated for C₁₈H₄₀PO₄, 351.2664). Di-*t*-butyl-*S*-dihydrocitronellyl phosphate: [α]_D²⁴ –2.9° (c 1 in CHCl₃); n.m.r. data as for the *R*-enantiomer. N.m.r. data for di-*t*-butyl phytanyl phosphate have been reported previously [23]. High-resolution mass spectra were

recorded at the SERC Mass Spectrometry Centre, Swansea, on a VG ZAB-E high resolution instrument.

Preparation of yeast microsomes

Yeast microsomes were prepared by a modification of the method of Trimble et al. [31]. *Saccharomyces cerevisiae* (strain X-2180A) was grown in 4 litres of 2% (w/v) malt extract and 0.1% (w/v) yeast extract for 9–10 h (exponential phase) at 27 °C. Cells were harvested by centrifugation (6000 g, 20 min, 4 °C) and washed twice with ice-cold Tris/Mg²⁺ buffer (50 mM Tris/HCl, 5 mM MgCl₂, 10 mM 2-mercaptoethanol, pH 7.5). The cells were resuspended in 100 ml of wash buffer, filtered through muslin and disrupted in a French press (276 MPa; 40 000 lbf/in²). Deoxyribonuclease I (10 µg/ml) was added to the homogenate and cellular debris was removed by centrifugation as before. Microsomes were isolated by centrifugation of the supernatant (18 500 g, 50 min, 4 °C). Aliquots were stored at –80 °C prior to assay for Dol-P-Man synthetase. Protein content was measured using a modification [32] of the method of Lowry et al. (Sigma protein assay kit).

Preparation of *E. coli* homogenate

E. coli (strain DH5α) transformed with plasmids pDM6 and YE24 (with and without the *DPM1* gene respectively), a kind gift from Dr. Peter Orlean, were grown overnight in LB medium supplemented with ampicillin (A_{600} 1.8–1.9). The cells were harvested and washed as for yeast (see above) and disrupted with a dispersion tool (20 000 rev./min; Ultra Turrax T-25; Janke and Kunkel) in the presence of deoxyribonuclease I (10 µg/ml). Aliquots of the homogenates were stored at –80 °C prior to assay.

Assay for Dol-P-Man synthetase activity

Dol-P-Man synthetase was assayed at 37 °C by incubation of yeast microsomes (3–4 mg) with 100 µM lipid phosphate, 20 µM GDP-mannose, GDP-[3,4-³H]mannose (0.2 µCi/ml) and 0.5% (v/v) Triton X-100 in Tris/Mg²⁺ buffer. Incubations were performed in Reactivials with continuous stirring in a Pierce ReactiTherm heating/stirring module (Pierce and Warriner, Chester, U.K.). Aliquots (100 µl) were removed at timed intervals into an equal volume of chloroform/methanol (1:1, v/v). The aqueous phase was removed after centrifugation. The organic phase was washed twice with water and incorporation of radioactivity into organic soluble material was measured by scintillation counting (Ultima Gold scintillation fluid; LKB scintillation counter). In the case of *E. coli* clones pDM6 and YE24 the Triton concentration was increased to 1.0% (v/v). The concentrations of lipid phosphates and GDP-Man used in these assays were greatly above the literature values for the K_m s of dolichyl phosphate (0.3 µM) and GDP-Man (0.69 µM) [15].

DEAE-Sephacel anion-exchange chromatography

Anion-exchange chromatography of aqueous and organic soluble radioactive material after Dol-P-Man synthetase assays was accomplished on a column of DEAE-Sephacel (1.2 cm × 11 cm), pre-equilibrated with 99% methanol, by the method of Sasak et al. [33]. After sample application, the column was washed with five column volumes of methanol (0.6 ml/min). Lipid phosphates

were eluted with 10 mM ammonium acetate in 99% methanol (0.6 ml/min) and the column was then washed with 120 ml each of 50 mM and 500 mM ammonium acetate in 99% methanol.

Hydrolysis of lipid-linked mannose

The saccharide components of glycosylated lipid phosphates were released by incubation in 20 mM HCl in 20% (v/v) methanol (20 min, 100 °C). After cooling, the incubation mixture was partitioned with chloroform and the aqueous phase was passed through a column with 200 µl each of Chelex 100 (Na⁺ form, 100–200 mesh), AG50W-X8 (100–200 mesh; H⁺ form), AG3 X4 (100–200 mesh; OH[−] form) and QAE-Sephadex A-25. The saccharide was eluted with five column volumes of water, filtered (Anotop; 0.2 µm filter) and the water removed by rotary evaporation.

Bio-Gel P4 gel filtration chromatography

Gel filtration chromatography of saccharides was performed by the method of Yamashita et al. [34] on a water-jacketed (55 °C) 1.5 cm × 90 cm column of Bio-Gel P4 (< 45 µm). Samples were co-injected with a partial dextran hydrolysate (3 mg; prepared after dialysis against water by treatment with 1 M HCl at 100 °C for 6 h, with subsequent passage through ion-exchange resins as above) and eluted with water. Degassed water was sparged continuously with helium and passed through a column of Chelex 100 (Na⁺ form; 100–200 mesh), AG50W-X8 (100–200 mesh; H⁺ form), AG3 X4 (100–200 mesh; OH[−] form) and Bio-Gel P4 (65 ± 20 µm) prior to the injection loop. The flow rate was maintained at 0.2 ml/min (Waters 6000A chromatography pump). The refractive index of the eluant was monitored using a Waters 410 differential refractometer. Aliquots of each fraction were subject to scintillation counting, and elution positions of radioactive components were compared with those of the glucose oligomers recorded by refractometry and of separately run [³H]glucose.

Thin layer and paper chromatography

T.l.c. was performed on Merck aluminium-backed Kieselgel 60F silica-coated plates (BDH, Poole, Dorset, U.K.). Under system 1, plates were run in n-propanol/14 M ammonium hydroxide/water (2:1:1, by vol.) and developed in 5% (v/v) anisaldehyde in ethanol/sulphuric acid (18:1, v/v) with heating. Under system 2, plates were run in n-propanol/acetone/0.1 M lactic acid (2:2:1, by vol.) and developed in 0.2% (w/v) naphthoresorcinol in ethanol/water/sulphuric acid (18:1:1, by vol.) with heating. Radioactive components were identified after removal of the silica, followed by washing with water and scintillation counting. Analytical paper chromatography of saccharides was performed under system 3 [butanol/pyridine/water (10:3:3, by vol.)] for 36 h at room temperature. Standards were stained with alkaline silver nitrate: chromatograms were first dipped in a solution of 0.1 ml of saturated silver nitrate in 20 ml of acetone and developed in a solution of 2% (w/v) NaOH in methanol/water (19:1, v/v). The presence of radioactive components in 1 cm strips was identified after soaking in water and scintillation counting.

RESULTS

Lipid phosphates as substrates for Dol-P-Man synthetase

As an initial test in the present study, yeast microsomes prepared as described were assayed for Dol-P-Man synthetase activity

Figure 1 Time course analysis of the Dol-*P*-Man synthetase assay with yeast microsomes

Duplicate aliquots were removed at various time intervals and the incorporation into organic-soluble material was determined by scintillation counting. Incubations were run in the presence of no exogenous acceptor (BI), 100 μ M dolichyl phosphate (D) or 100 μ M phytanyl phosphate (P).

Table 1 Comparison of activity of lipid phosphates as substrates for Dol-*P*-Man synthetase

Incorporation of GDP-[3,4-³H]mannose by yeast microsomes into organic-soluble material after 1 h (duplicate aliquots) was determined in experiments with 100 μ M lipid phosphate (where added), 20 μ M GDP-mannose and 0.2 μ Ci/ml-[3,4-³H]mannose. Results are given as means $\pm$ S.D. of two experiments.

Substrate	Incorporation (c.p.m./100 μ l)
Dolichyl phosphate	10378 $\pm$ 517
Phytanyl phosphate	6452 $\pm$ 188
<i>R</i> -Dihydrocitronellyl phosphate	139 $\pm$ 72
<i>S</i> -Dihydrocitronellyl phosphate	191 $\pm$ 27
<i>S</i> -3-Methyloctadecanyl phosphate	2178 $\pm$ 247
Tetradecanyl phosphate	228 $\pm$ 15
No exogenous acceptor	66 $\pm$ 2

using both dolichyl and phytanyl phosphates. Approximately 90% of enzyme activity was recovered in the microsomal pellet after high-speed centrifugation, as measured by incorporation of radioactivity from GDP-[3,4-³H]mannose into material soluble in chloroform/methanol in the presence of phytanyl phosphate. The transfer of ³H-labelled material into organic soluble components was apparently complete within 20 min (Figure 1), with a slight subsequent decline as has been previously noted [7]. Very low incorporation was achieved in the absence of exogenous acceptor.

Phytanyl phosphate was subsequently assayed as a substrate in parallel with other polyisoprenyl phosphates, i.e. dolichyl phosphate and enantiomerically pure *R*- and *S*-dihydrocitronellyl phosphates. All these lipids are saturated at the α -isoprene unit, but only in dolichyl phosphate are the following units unsaturated. Thus dihydrocitronellyl phosphates were synthesized since they exhibit similar structural features to phytanyl phosphate, but have a C₈ backbone ($\sim$ 9.0 Å in length), and the alcohols from which they are derived are commercially available in enantiomerically pure forms. The results indicate that, with approximately equimolar amounts of each substrate, phytanyl

Table 2 Assay of recombinant Dol-*P*-Man synthetase

Incorporation after 1 h into organic soluble material by *E. coli* homogenates (clones pDM6, harbouring a plasmid carrying the *DPM1* gene, and YEp24, which harbours the same plasmid but lacks the gene) was determined in the presence or absence of lipid phosphates. The conditions were as for the experiments in Table 1.

Conditions	Incorporation (c.p.m./100 μ l)	
	pDM6	YEp24
Dolichyl phosphate	1129 $\pm$ 163	116 $\pm$ 10
Phytanyl phosphate	593 $\pm$ 63	75 $\pm$ 24
<i>S</i> -3-Methyloctadecanyl phosphate	336 $\pm$ 70	122 $\pm$ 57
No exogenous acceptor	103 $\pm$ 35	78 $\pm$ 16

phosphate is 60–70% as efficient as dolichyl phosphate as an acceptor. In contrast, the incorporation into organic soluble material in incubations with either of the dihydrocitronellyl phosphates was virtually indistinguishable from that in incubations with no exogenous acceptor (Table 1). Thus it appears that, whereas phytanyl phosphate with a C₁₆ backbone can function efficiently as an acceptor, the dihydrocitronellyl phosphates, possessing only a C₈ backbone, were not glycosylated. Further experiments were therefore performed with lipid phosphates of longer chain length.

In order to probe the effect of branching of the lipid chain, two additional lipid phosphates were synthesized: tetradecanyl phosphate ($\sim$ 16.7 Å backbone) and *S*-3-methyloctadecanyl phosphate ($\sim$ 21.9 Å backbone). The latter has the same C-3 configuration as the natural dolichyl substrate [35], whereas the former is unbranched and is only two methylene groups shorter than the backbone of phytanyl phosphate. Parallel assays with phytanyl phosphate indicated that only a small degree of mannosyl transfer above that of the background is observed in the presence of tetradecanyl phosphate, whereas incubations with *S*-3-methyloctadecanyl phosphate exhibited approx. 30% of the incorporation found in the presence of phytanyl phosphate (Table 1). In addition, preliminary data with phytanyl, *S*-3-methyloctadecanyl and dolichyl phosphates indicated that lipid-mediated transfer of mannose to endogenous protein occurred, as measured by incorporation into phosphotungstic acid-precipitable material.

As additional confirmation that *S*-3-methyloctadecanyl and phytanyl phosphates acted as substrates for the enzyme, *E. coli* clones with either plasmid pDM6 or YEp24 were grown and assayed for enzyme activity. pDM6 contains the yeast *DPM1* gene [8], whereas YEp24 is the same plasmid except that it lacks this insert. The pDM6 homogenate contained Dol-*P*-Man synthetase activity, as expected. Some transfer to endogenous lipids did occur at a low level in the pDM6 homogenate, similar to the levels obtained with the YEp24 homogenate. The results obtained indicated that both *S*-3-methyloctadecanyl and phytanyl phosphates act as substrates of the recombinant enzyme (Table 2).

Analysis of lipid-linked incubation products

The excess incubation mixtures remaining after 1 h time courses in the assays described above were retained for analysis by DEAE-Sephacel chromatography. In order to demonstrate that no mannosylated lipid phosphates were present in the aqueous phase, both the aqueous and organic fractions were analysed.

Figure 2 Analysis of incubation products by DEAE-Sephadex anion-exchange chromatography

Aqueous- (a–g) or organic- (h–n) soluble fractions of incubations of yeast microsomes and GDP-Man in the presence of dolichyl phosphate (a, h), phytanyl phosphate (b, i), *S*-dihydrocitronellyl phosphate (c, j), *R*-dihydrocitronellyl phosphate (d, k), *S*-3-methyloctadecanyle phosphate (e, l), tetradecanyle phosphate (f, m) or no exogenous acceptor (g, n) were applied to a DEAE-Sephadex column. The column was washed with 60 ml of 99% methanol (fractions 1–10), 180 ml of 10 mM ammonium acetate (fractions 11–40) and 120 ml of 50 mM ammonium acetate (fractions 41–60).

Aqueous fractions contained ^3H -labelled material that was eluted with 99% methanol (Figures 2a–2g) and was found by t.l.c. to co-run with mannose (system 1; R_f 0.38). There was no evidence for incomplete partitioning of mannosylated lipid phosphates, since no water-soluble material present in any incubation mixture contained components that eluted with 10 mM ammonium acetate, which were the conditions under which Sasak et al. [33] eluted dolichyl phosphate mannose and retinyl phosphate mannose. Subsequent washing of the column with 500 mM ammonium acetate in 99% methanol removed mainly mannose 1-phosphate, as identified by t.l.c. (system 1; R_f 0.53). Such results with the aqueous fractions are similar to those obtained by

Figure 3 Analysis of saccharides of [3,4- ^3H]mannosylated lipid phosphates by Bio-Gel P4 gel-filtration chromatography

Mannosylated lipid phosphates purified by DEAE-Sephadex anion-exchange chromatography (10 mM ammonium acetate elution) were subject to mild acid hydrolysis as described. The released saccharides were prepared for Bio-Gel P4 chromatography. The elution positions (glucose units) of co-injected glucose oligomers are shown across the top. (a) Dolichyl-linked saccharides; (b) phytanyl-linked saccharides; (c) *S*-3-methyloctadecanyle-linked saccharides.

analysis by paper chromatography in isobutyric acid/15 M ammonium hydroxide/water (59:4:39, by vol.) of incubations of yeast microsomes with phytanypyrophosphoryl-*NN'*-diacetylchitobiose and GDP-Man, which is a substrate for β -mannosyltransferase (I. B. H. Wilson and M. C. Webberley, unpublished work), suggesting degradation certainly of the donor and possibly also of the product.

With the organic soluble fractions, only incubations with dolichyl, phytanyl or *S*-3-methyloctadecanyle phosphate contained significant amounts of ^3H -labelled material. This material was predominantly eluted with 10 mM ammonium acetate in 99% methanol (Figures 2h–2n). Thus the components eluted with 10 mM ammonium acetate were taken to represent lipid-linked mannose phosphates. The organic soluble fractions of the incubations with either of the dihydrocitronellyl phosphates and no exogenous acceptor did not contain any significant amounts of radioactivity eluted under any of the conditions used. All mannosylated lipid phosphates had apparently similar elution volumes.

Analysis of lipid-linked saccharides

Fractions containing putative lipid-linked mannose phosphates were subjected to mild acid hydrolysis (20 mM HCl) and the released water-soluble components were chromatographed on Bio-Gel P4 as described in the Experimental section. Analysis of the hydrolysates derived from incubations with dolichyl, phytanyl and *S*-3-methyloctadecanylethyl phosphates indicated the presence of a major (> 75%) ³H-labelled component with a hydrodynamic volume corresponding to 0.9 glucose unit (Figure 3). With all samples, this peak contained a radioactive component with an *R_F* (t.l.c.; system 2) of 0.57, which is identical to that of mannose. The released phytanyl-linked saccharides were subject to paper chromatography under system 3 and were run with a variety of monosaccharides: the radioactive component co-ran solely with mannose, thus verifying the t.l.c. data. There are other possible components, in particular a potential disaccharide component of 1.5–2.0 glucose units, as judged by P4 chromatography, but there were insufficient quantities for definitive analysis.

DISCUSSION

In this study, fully characterized analogues of dolichyl phosphate have been tested as substrates for Dol-*P*-Man synthetase. Whereas phytanyl phosphate, and to a lesser extent *S*-3-methyloctadecanylethyl phosphate, could replace dolichyl phosphate resulting in the formation of singly mannosylated lipid phosphates as major incubation products, dihydrocitronellyl and tetradecanylethyl phosphates were inactive. Such results suggest a chain-length-dependence for acceptor substrates of Dol-*P*-Man synthetase. The minimum length required in order to efficiently bind the dolichol binding site of Dol-*P*-Man synthetase may be between 8 and 16 methylene groups (i.e. 9–19 Å). Such an hypothesis can be compared with the proposed location of dolichyl phosphate in the membrane [1] and with the length of the proposed binding site of 13 amino acids, which, in an α -helical conformation, is estimated to be 20 Å in length. Jaenicke and co-workers [25,36,37] have suggested a longer minimum length (C_{45–55}), but the reason for the discrepancy between their results with C₂₀ dolichyl phosphate and our results with phytanyl phosphate is unknown.

It has been claimed that citronellyl phosphate mannose (rather than its dihydro derivative) could be formed as a water-soluble product by *Mycoplasma luteus* undecaprenyl phosphate mannose synthetase [38]. However, the formation of the similar dihydrocitronellyl product by yeast microsomes is excluded by the present results from anion-exchange chromatography. In another eukaryotic system, the embryonic chick retina, citronellyl phosphate did not act as a mannosyl acceptor [39], but the same study, at variance with many other investigations, did not find any incorporation above background with phytanyl, retinyl, ficaprenyl and other non-dolichyl polyprenyl phosphates. Indeed, retinyl phosphate had been considered to be a potential natural substrate for Dol-*P*-Man synthetase [40–42], although retinyl, ficaprenyl, solanesyl and other α -unsaturated polyprenyl phosphates are significantly less efficient as cofactors in oligosaccharide biosynthesis than are α -saturated dolichyl phosphates [27,28,42,43]. These lipids are no longer believed to function physiologically in glycoprotein biosynthesis, although the possibility of the occurrence of two lipid phosphate mannose synthetases [15] has not been conclusively discounted.

The role of the α -isoprene unit is of particular interest. The results indicating that *S*-3-methyloctadecanylethyl phosphate is a substrate, whereas tetradecanylethyl phosphate is not, indicate that C-3 methyl substitution is a virtually obligatory structural feature

for a lipid phosphate to be an acceptor for Dol-*P*-Man synthetase. Also, since dolichol is unsaturated except in the α -isoprene unit, the only structural feature that *S*-3-methyloctadecanylethyl and phytanyl phosphates share with a dolichyl moiety is the α -saturated isoprene unit.

The *S*-enantiomer of 3-methyloctadecanylethyl phosphate was synthesized since this is the 'correct' form: dolichols from a variety of natural sources have been found to be > 95% in the *S*-form [35]. Even though rat liver polyisoprenyl phosphatase does not appear to distinguish between the *R*- and *S*-forms [26], the indications from assays of Dol-*P*-Man synthetase, *N*-acetylglucosaminylphosphotransferase and Dol-*P*-Glc synthetase are that the *S*-form is preferred, with lower *V_{max}* values being determined for *R*-dolichyl phosphate [44]. Thus it would appear that the previously noted proposed dolichol binding site, elucidated by sequence similarities between various enzymes of oligosaccharide and glycoprotein biosynthesis [3], may have been adopted to accommodate the optical isomer of dolichol that is the predominant natural form. Indeed, this binding site can be traced back to eubacteria, as noted by Troy with *E. coli* polysialyltransferases [5], and archaeobacteria, since a recently characterized partial sequence of a putative *Sulpholobus acidocaldarius* dolichol pyrophosphatase [45] contains a 13-amino-acid region which could fulfil the consensus criteria. It is possible that the 3-methyl substitution may be necessary in order for the acceptor to be in the correct orientation with respect to the catalytic domain.

In conclusion, the findings of this study demonstrate the importance of the α -isoprene unit in the binding of lipid phosphates to Dol-*P*-Man synthetase. The ability of the novel C₁₉ mono-branched lipid to substitute for its dolichyl homologue would indicate that a minimum chain length and a 3-methyl substitution are the only necessary structural features for mannosyl acceptors, although further branching (as with phytanyl phosphate) increases the efficiency of incorporation. Such results could be applied further to chemoenzymic synthesis, as is being currently pursued [23], or to examination of the translocation of mannose across membranes.

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