Activation of GlcNAc-P-P-Dolichol Synthesis by Mannosylphosphoryldolichol Is Stereospecific and Requires a Saturated α-Isoprene Unit†

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ABSTRACT: Exogenous mannosylphosphoryldolichol (Man-P-Dol) has previously been shown to stimulate UDP-GlcNAc:dolichyl phosphate *N*-acetylglucosamine 1-phosphate transferase (GPT1), the enzyme catalyzing the biosynthesis of *N*-acetylglucosaminylpyrophosphoryldolichol (GlcNAc-P-P-Dol). To define the structural specificity of the mannolipid-mediated activation of GPT1, the ability of a variety of mannosylphosphorylisoprenols to stimulate GlcNAc-lipid biosynthesis in microsomal preparations from retinas of the embryonic chick has been tested. For these comparisons several Man-P-isoprenols were synthesized enzymatically and chemically. The catalytic efficiency of activation expressed as the V_{max}/K_a ratio was substantially higher for Man-P-Dol_{95} than for mannosylphosphorylpolyprenol$_{95}$ (Man-P-$Poly_{95}$), demonstrating that the saturated α-isoprene unit of the dolichyl moiety influences the mannolipid–enzyme interaction. The degree of activation increased with chain length and hydrophobicity of the dolichyl moiety when Man-P-dolichols containing 2, 11, and 19 isoprene units were evaluated. A strict stereospecificity was exhibited as β-Man-P-Dol_{95} provided a 100-fold greater stimulation than the corresponding α-stereoisomer. The recognition of the saturated α-isoprene unit, the influence of chain length, and the strict stereospecificity of the interaction between β-Man-P-Dol and GPT1 extend the description of the mannolipid–enzyme interaction and provide strong new evidence that Man-P-Dol levels can influence the rate of GlcNAc-P-P-Dol synthesis.

The biosynthesis of the core region of asparagine-linked oligosaccharides is initiated in the rough endoplasmic reticulum (RER)[1] by the formation of *N*-acetylglucosaminylpyrophosphoryl dolichol (GlcNAc-P-P-Dol), catalyzed by the enzyme UDP-GlcNAc:dolichyl phosphate *N*-acetylglucosamine 1-phosphate transferase (GPT1) [see reviews, Kornfeld and Kornfeld (1985), Hirschberg and Snider (1987), Waechter (1989), and Lehrman (1991)]. This is followed by a series of reactions leading to the formation of $Glc_3Man_9GlcNAc_2$-P-P-Dol (Oligo-P-P-Dol), the activated form of the precursor oligosaccharide that is transferred to appropriate asparagine residues in nascent polypeptides. While the individual reactions involved in the conversion of GlcNAc-P-P-Dol to Oligo-P-P-Dol in the cytoplasmic and luminal leaflets of the RER have been described extensively, relatively little is known about the regulation of this pathway. Since the biosynthesis of GlcNAc-P-P-Dol is in a sense the "committed step" in this pathway (Lehrman, 1991), factors that affect its formation could influence the rates of Oligo-P-P-Dol biosynthesis and thus the primary N-glycosylation event.

Earlier *in vitro* studies have shown that mannosylphosphoryldolichol (Man-P-Dol), in addition to serving as a mannosyl donor for the conversion of $Man_5GlcNAc_2$-P-P-Dol to $Man_9GlcNAc_2$-P-P-Dol, also acted as an activator of GPT1, resulting in a stimulation in the biosynthesis of GlcNAc-P-P-dolichol in membrane preparations from mammalian cells (Kean, 1982, 1985; Kean & DeBrakeleer, 1986). The stimulatory effect of the mannolipid on GPT1 activity has been subsequently reported by other laboratories (Kaushal & Elbein, 1985; Shailubhai et al., 1988; Carson et al., 1990). Previous studies on the specificity of the interaction between GPT1 and glycosylphosphorylisoprenols revealed that neither mannosylphosphorylretinol (Man-P-retinol) nor glucosylphosphoryldolichol (Glc-P-Dol) functioned in this manner and, moreover, Glc-P-Dol prevented the stimulation by Man-P-Dol (Kean, 1985). The inability of Glc-P-Dol and Man-P-retinol to activate GlcNAc-P-P-Dol synthesis suggests that a specific interaction between the mannosylphosphoryl headgroup, as well as the polyisoprenyl moiety, of Man-P-Dol and GPT1, or an associated regulatory protein component, is required for the stimulatory phenomenon.

The structural specificity of the mannolipid-mediated activation of GPT1 in embryonic chick retinal microsomes has now been extended by examining the influence of the saturated α-isoprene unit and the chain length of the dolichyl moiety, as well as the stereoconfiguration of the mannosyl–phosphoryl linkage in Man-P-Dol. For this investigation a series of β-mannosylphosphorylisoprenols were tested that had been synthesized from selected isoprenyl monophosphates by mannosylphosphorylundecaprenol (Man-P-Undec) synthase, partially purified from membrane fractions isolated from *Micrococcus luteus* (Lennarz & Talamo, 1966; Scher &

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[1] Abbreviations: RER, rough endoplasmic reticulum; GlcNAc-P-P-Dol, *N*-acetylglucosaminylpyrophosphoryldolichol; $(GlcNAc)_2$-P-P-Dol, *N*-acetylglucosaminyl-*N*-acetylglucosaminylpyrophosphoryldolichol; chito, *N*-*N*′-diacetylchitobiose; GPT1, UDP-GlcNAc:dolichyl phosphate *N*-acetylglucosamine 1-phosphate transferase; Man-P-Dol_n, mannosylphosphoryldolichol, where n = the number of carbon atoms in the dolichol chain containing a saturated α-isoprene unit; Man-P-Dol_{10}, mannosylphosphorylcitronellol; Dol-P, dolichyl monophosphate; Man-P-$Poly_n$, mannosylphosphorylpolyisoprenol, where n = the number of carbon atoms in the fully unsaturated polyisoprenol chain; TX-100, Triton X-100; Tes, 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid.

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Lennarz, 1969; Rush et al., 1993). The stereospecificity was examined by comparing the enzymatically synthesized β-anomers with the corresponding α-stereoisomers synthesized by a chemical procedure (Warren & Jeanloz, 1973, 1978).

The results of this investigation indicate that all three structural features of the mannolipid play a role in the stimulation of GPT1. This paper presents the first evidence that the mannolipid–enzyme interaction is stereospecific and involves the saturated isoprene unit of the dolichyl moiety. The recognition of the saturated α-isoprene unit and the stereoconfiguration of the mannosyl–phosphoryl linkage strongly indicate that the Man-P-Dol levels influence the rate of GlcNAc-P-P-Dol formation *in vivo*. The relevance of these findings to the regulation of lipid intermediate biosynthesis is discussed. Some aspects of this study were presented in a preliminary report (Kean et al., 1993).

EXPERIMENTAL PROCEDURES

Materials. UDP-[6-^{3}H]GlcNAc (30 Ci/mmol) was obtained from Du Pont–New England Nuclear (Boston, MA). Tunicamycin was purchased from CalBiochem (San Diego, CA). (*S*)-Citronellol, DEAE-cellulose, chemically hydrogenated dolichol (C_{55}), synthetic GDP-Man (Type III), and dolichyl phosphate, Grade III, C_{80-105} (used as the exogenous substrate in assays for GlcNAc-lipid synthesis), were obtained from Sigma Chemical Co. (St. Louis, MO). Phosphorus trichloride oxide was obtained from Alpha Chemicals (Danvers, MA). Undecaprenyl phosphate, dolichyl phosphate (C_{95}), and polyprenyl phosphate (C_{95}), used for enzymatic synthesis of the various mannolipids tested as activators of GlcNAc-lipid synthesis, were acquired from Dr. Tadeusz Chojnacki, Warsaw, Poland. EcoLume Scintillation cocktail was obtained from ICN Research Products Division (Costa Mesa, CA). All other chemicals and reagents were the highest grade commercially available.

Preparation of Chick Retina Microsomes. Retinas were dissected from the eyes of 15–16-day chick embryos, and microsomes were prepared by differential centrifugation as described previously (Kean, 1985).

Enzymatic Assay of GlcNAc-Lipid Transferase Activities. Assay mixtures contained dolichyl phosphate (19 μM), 27 mM $MgCl_2$, 0.15% Triton X-100 (TX-100), 0.2 M 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid (Tes) buffer, pH 7.4, UDP-[^{3}H]GlcNAc [51 μM, 160–190) × 10^6 dpm/μmol], embryonic chick retina microsomal suspension (100–118 μg of protein), and the indicated concentrations of the appropriate Man-P-polyisoprenols in a total volume of 0.15 mL, as described previously (Kean, 1982, 1985). Dolichyl phosphate and the Man-P-isoprenols were evaporated to dryness with nitrogen and resuspended by vortexing in 1.5% TX-100 at room temperature, while the water-soluble Man-P-citronellol was added to assay mixtures in an aqueous solution. The concentrations of the various mannosylphosphorylisoprenols and dolichyl phosphate were determined by measuring the amount of lipid phosphorus in the stock solutions by the method of Bartlett (1959) or Chen et al. (1956). Initial rates were determined as described previously (Kean, 1985). After incubating at 37 °C for 10 min, the reaction was stopped by the addition of 3 mL of chloroform/methanol (2:1) and the mixture subjected to solvent partitioning according to the method of Folch et al. (1957). The washed organic phase was evaporated to dryness, and the incorporation of radioactivity into the GlcNAc-lipid products was determined by scintillation spectrometry in the presence of 0.5 mL of water and 5 mL of EcoLume scintillation cocktail.

Chemical Synthesis and Purification of Dol-P and Citronellol Monophosphate. Citronellol and dolichol were phosphorylated using phosphorus trichloride oxide and triethylamine in hexane by the procedure of Danilov and Chojnacki (1981) and purified as described earlier (Rush et al., 1993).

Enzymatic and Chemical Synthesis of Various Mannosylphosphorylisoprenols. β-Man-P-polyisoprenols and β-Man-P-Dol_{10} were synthesized enzymatically from GDP-Man and the appropriate polyisoprenyl monophosphate using a partially purified preparation of Man-P-Undec synthase from *M. Luteus* (Rush et al., 1993). The α-stereoisomer of Man-P-Dol_{95} was synthesized by the method of Warren and Jeanloz (1973, 1978). The details of the enzymatic and chemical syntheses, the isolation of the mannolipids, and the structural characterization of the various mannolipid products have been described (Rush et al., 1993).

Characterization of the Enzymatically Labeled Products. The identification of the [^{3}H]GlcNAc-labeled lipids formed by the retina microsomal system and extractable into chloroform/methanol (2:1) was carried out by paper chromatography after mild acid hydrolysis essentially as described previously (Kean, 1991, 1993). After solvent extraction as described above, the enzymatically labeled glycolipids were hydrolyzed [1-propanol/0.02 N HCl (1:2) for 20 min at 100 °C (Turco et al., 1977)]. The neutral glycosyl groups were recovered after mixed-bed ion-exchange chromatography (AG 1-X2, 200–400 mesh, acetate form, and AG-50 X8, 200–400 mesh, hydrogen form) and separated by descending paper chromatography on Whatman No. 1 paper, developed with 1-butanol/pyridine/water (6:4:3) for 14 h. The chromatograms were dried, cut into 1-cm zones, and analyzed for radioactivity by scintillation spectrometry in the presence of 0.5 mL of water and 5 mL of EcoLume. The migration positions of standard GlcNAc and *N,N'*-diacetylchitobiose (chito) were visualized colorimetrically (Trevelyan et al., 1950).

Kinetic Analysis. The apparent activation constants (K_a) and apparent V_{max} values were calculated from Lineweaver–Burk plots after analysis of the data by a basic translation of the Fortran program of Cleland (1979) prepared by Dr. R. Viola (University of Akron, Akron, OH) or by the K.cat program (BioMetalics, Inc., Princeton, NJ). Both programs gave identical results.

Other Analyses. Protein concentration was analyzed by the method of Lowry et al. (1951).

RESULTS

The Influence of Saturation of the α-Isoprene Unit of Man-P-Isoprenols on the Stimulation of GlcNAc-Lipid Synthesis. The requirement for the saturated α-isoprene unit of the dolichyl moiety in Man-P-Dol to activate GlcNAc-lipid synthesis was examined by comparing the stimulation obtained with Man-P-Dol_{95} to that with Man-P-$Poly_{95}$. The latter compound contains a fully unsaturated polyisoprenyl chain. As seen in Figure 1, while maximal stimulation was observed in the presence of Man-P-Dol_{95} at concentrations above 10 μM, Man-P-$Poly_{95}$ was considerably less effective as an activator of GlcNAc-lipid synthesis, not approaching saturation until about 50 μM. In addition, the magnitude of the stimulation in the presence of a saturating concentration of the mannolipid activator was markedly higher with Man-P-Dol compared to Man-P-Poly. Similar results were obtained with the C_{55} derivatives (data not shown).

An analysis of the kinetics of these reactions, summarized in Table 1 comparing the mannolipids containing 11 or 19 isoprene units, clearly demonstrates the importance of the saturated α-isoprene unit in quantitative terms. On the basis

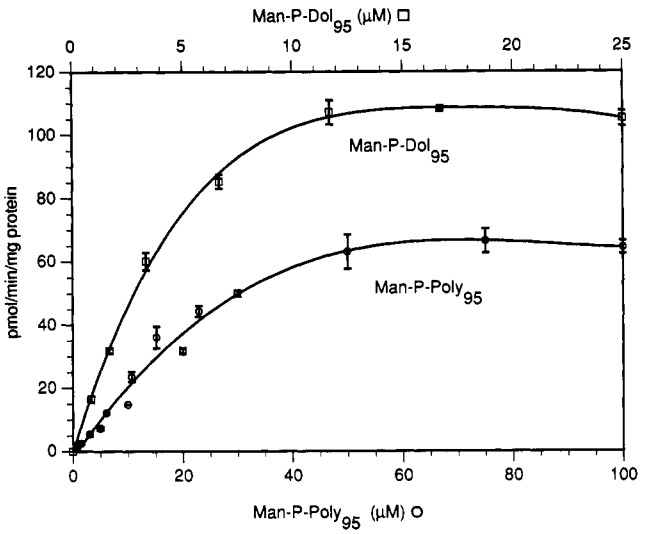

FIGURE 1: Influence of a saturated α-isoprene unit on the stimulation of GlcNAc-lipid synthesis. Incubation mixtures contained 19 μM dolichyl phosphate, 51 μM UDP-[^{3}H]GlcNAc [(160–190) × 10^6 dpm/μmol], 0.2 M Tes buffer (pH 7.4), 27 mM $MgCl_2$, 0.15% TX-100, enzyme (microsomes from the retina of the embryonic chick; 100–118 μg of protein), and the indicated concentrations of either β-Man-P-Dol_{95} (□) or β-Man-P-$Poly_{95}$ (○) in a total volume of 0.15 mL. Incubations were carried out for 10 min at 37 °C, after which the reactions were stopped by the addition of chloroform/methanol (2:1), and GlcNAc-lipid synthesis was measured as described in Experimental Procedures. The error bars refer to the standard deviation at each data point performed in triplicate or duplicate.

Table 1: Kinetics of the Stimulation of GlcNAc-Lipid Synthesis by Various Man-P-Polyisoprenols[a]

mannolipid added	apparent K_a (μM)	apparent V_{max} [pmol/(min·mg of protein)]	V_{max}/K_a
β-Man-P-Dol_{95}	4.2	134	32
β-Man-P-Dol_{55}	5.7	117	21
β-Man-P-Dol_{10}	361	7.8	0.022
α-Man-P-Dol_{90-95}	52	14	0.27
β-Man-P-$Poly_{95}$	31	93	3.0
β-Man-P-$Poly_{55}$	20	129	6.5

[a] Kinetic constants were calculated from Lineweaver–Burk plots of the data presented in Figures 1, 3, and 4 as determined by the program of Cleland (1979) and the K.cat program, as described in Experimental Procedures.

of the V_{max}/K_a ratios obtained from kinetic analysis of these data, the Man-P-Dol derivatives are 3–10 times more stimulatory than the corresponding mannolipid containing fully unsaturated polyisoprenyl moieties.

Evidence That Man-P-Dol and Man-P-Poly Stimulate the Same Reaction. While these studies have shown that there is a greater stimulation in GlcNAc-lipid synthesis by Man-P-Dol compared to Man-P-Poly derivatives, the specificity was not absolute. Two experimental approaches were taken to confirm that Man-P-Poly was stimulating the same reaction as Man-P-Dol in the embryonic chick retinal microsomes. First was the identification of the products of the reactions performed under the influence of either compound. Chromatographic analysis of the enzymatically labeled glycosyl groups released from the glycolipid products by mild acid hydrolysis, illustrated in Figure 2, shows that GlcNAc-P-P-Dol and $(GlcNAc)_2$-P-P-Dol are formed in the presence of both mannolipids and in approximately the same proportions.

In addition to analysis of the carbohydrate groups labeled after stimulation, the response to the presence of tunicamycin was examined. The stimulation of GlcNAc-lipid in the presence of Man-P-Dol or Man-P-Poly was essentially blocked by the addition of tunicamycin added at a concentration of

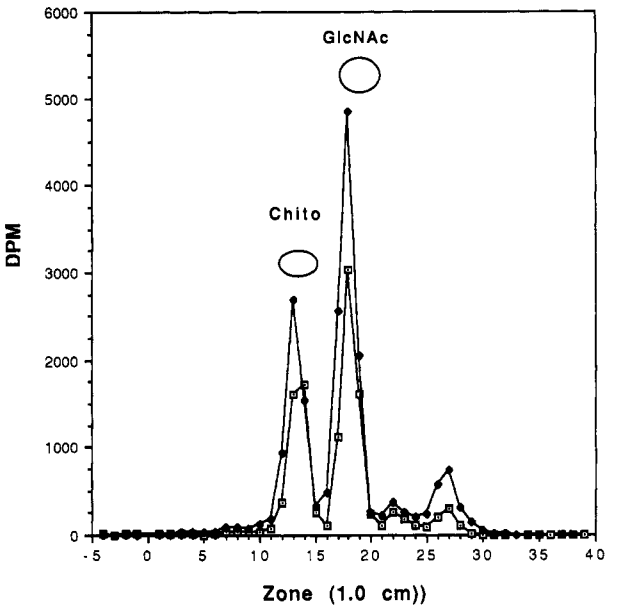

FIGURE 2: Paper chromatographic analysis of the enzymatically labeled glycolipid products. β-Man-P-Dol_{95} (□) (2 nmol) or β-Man-P-$Poly_{95}$ (♦) (15 nmol) was incubated in the same manner as described in Experimental Procedures. After stopping the reaction with chloroform/methanol (2:1) followed by extensive washing by the procedure of Folch et al. (1957), the material in the washed lower phase was evaporated to dryness and hydrolyzed under mild acid conditions, and the products were desalted with mixed anion and cation resins and examined by paper chromatography as described in Experimental Procedures. The dried chromatograms were cut into 1-cm zones and the radioactivity was determined by scintillation spectrometry. The migrations of standard GlcNAc and *N,N'*-diacetylchitobiose (chito) were visualized colorimetrically (Trevelyan et al., 1950).

Table 2: Effect of Tunicamycin on Stimulation by Mannosylpolyisoprenols of GlcNAc-Lipid Synthesis[a]

mannolipid added[b]	tunicamycin (20 μg/mL)	dpm	% of control
Man-P-Dol_{55} (8.1)	–	10700	100
Man-P-Dol_{55} (8.1)	+	122	1.1
Man-P-$Poly_{55}$ (10.2)	–	4810	100
Man-P-$Poly_{55}$ (10.2)	+	68	1.4

[a] Embryonic chick retina microsomes (0.12 mg of protein) were incubated for 10 min at 37 °C with dolichyl phosphate, TX-100, Tes buffer, Mg^{2+}, UDP-[^{3}H]GlcNAc, and the indicated Man-P-polyisoprenols in the presence or absence of tunicamycin in 10 μl of DMSO, and GlcNAc-lipid synthesis was then determined as described in Experimental Procedures. [b] Concentration in μM in parentheses.

20 μg/mL (Table 2). All of these results support the conclusion that the saturated α-isoprene unit of the polyisoprenyl group is required for the maximal stimulatory effect on GPT1 activity in chick retinal microsomes.

Effect of Chain Length and Hydrophobicity of the Dolichyl Moiety on the Stimulation by Man-P-Dolichols. To determine if the length of the polyisoprenyl chain influenced the stimulatory effect of Man-P-Dol on GPT1, the activities of Man-P-Dol compounds with 2, 11, and 19 isoprene units were compared. The results depicted in Figure 3 reveal that the enhancement of GlcNAc-lipid synthesis increased with the chain length and hydrophobicity of the dolichol component. In this comparison, Man-P-Dol_{95} is more stimulatory than Man-P-Dol_{55}. In order to evaluate whether this difference was statistically significant, a third-order regression model was fit to the data. A statistically significant difference was detected in both the more linear ($p = 0.02$) and the quadratic ($p = <0.0001$) portions of the two curves. Both mannolipids containing long-chain polyisoprenyl moieties were substantially

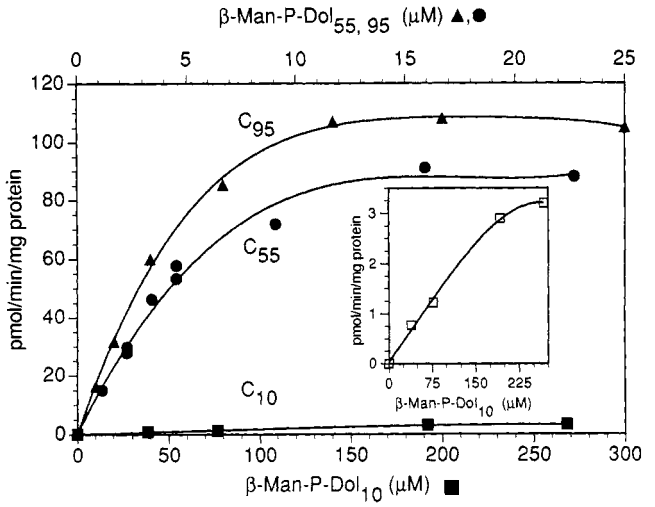

FIGURE 3: Effect of chain length of the polyisoprenyl moiety on the stimulation of GlcNAc-lipid synthesis by Man-P-dolichol. Incubations were carried out in the same manner as described in the legend to Figure 1 and in Experimental Procedures with the indicated concentrations of β-Man-P-Dol$_{95}$ (▲), β-Man-P-Dol$_{55}$ (●), or β-Man-P-Dol$_{10}$ (■). The rate of incorporation of radioactivity into the products was measured as described in Experimental Procedures. The inset is an expanded view of the stimulation achieved with β-Man-P-Dol$_{10}$.

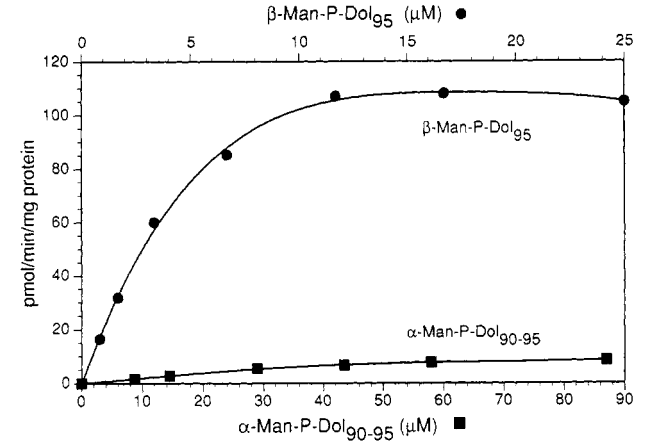

FIGURE 4: Stereospecificity of the stimulation of GlcNAc-lipid synthesis by Man-P-Dol. Incubations were carried out with retina microsomes, as described in the legend to Figure 1, in the presence of the indicated concentrations of either β-Man-P-Dol$_{95}$ (●) or α-Man-P-Dol$_{90-95}$ (■). The incorporation of radioactivity into GlcNAc-lipids was determined as described under Experimental Procedures.

more stimulatory than Man-P-Dol$_{10}$. The stimulatory ability of Man-P-Dol$_{10}$, while limited, is depicted more clearly in the inset to Figure 3. The relationships between dolichyl chain length and ability to enhance GlcNAc-lipid synthesis are also seen in the kinetic data (Table 1), where the V_{max}/K_a ratios indicate that Man-P-Dol$_{95}$ is 1.5-fold more stimulatory than Man-P-Dol$_{55}$ and about 1500-fold more efficient than the water-soluble analog, Man-P-Dol$_{10}$. It is clear from these results that the hydrophobic character of the polyisoprenyl chain of Man-P-Dol enhances the interaction of the mannolipid with GPT1 under the *in vitro* conditions used in these experiments.

Stereospecificity of Man-P-Dolichols for Activation. The stereospecificity of the stimulatory effect of Man-P-Dol on GPT1 was examined by comparing chemically synthesized α-Man-P-dolichols with the corresponding enzymatically synthesized β-mannolipids. As seen in Figure 4 and Table 1, the rate of GlcNAc-lipid synthesis is dramatically stimulated by β-Man-P-Dol, but only a modest increase over the basal level is produced by α-Man-P-Dol even at relatively high concentrations. In this study, the stereoisomers with dolichyl chain lengths of 90–95 carbons were compared. As seen in Table 1, the V_{max}/K_a ratio for β-Man-P-Dol was over 100-fold higher than the corresponding α-linked Man-P-Dol. Thus, the recognition site for Man-P-Dol on GPT1, or a regulatory protein component, exhibits a strict stereospecificity for the anomeric configuration of the mannosyl–phosphoryl linkage.

DISCUSSION

In eucaryotic cells, N-linked oligosaccharides are derived cotranslationally from Glc$_3$Man$_9$GlcNAc$_2$-P-P-Dol in the RER. The biosynthesis of the lipid-bound precursor oligosaccharide is initiated by the formation of GlcNAc-P-P-dolichol. Previous studies have revealed that Man-P-Dol, a mannosyl donor in a later stage of the pathway, may exert a regulatory influence on the GPT1, the enzyme catalyzing the biosynthesis of GlcNAc-P-P-dolichol (Kean, 1982, 1985; Kean & DeBrakeleer, 1986; Kaushal & Elbein, 1985; Shailubhai et al., 1988; Carson et al., 1990).

In this paper, the stimulation of GlcNAc-P-P-Dol synthesis by Man-P-Dol has been further investigated by characterizing the structural specificity for the mannolipid in producing the activation of GlcNAc-lipid synthesis in embryonic chick retinal microsomes. On the basis of these *in vitro* studies, the recognition of Man-P-Dol by GPT1, or a regulatory protein component, requires a saturated α-isoprene unit in the dolichyl moiety for optimal stimulation.

Since fully unsaturated polyprenyl monophosphates are also poor substrates for the enzymes synthesizing Man-P-Dol and GlcNAc-P-P-Dol (Mankowski et al., 1977; Jankowski et al., 1989; Szkopinska et al., 1992; McLachlan & Krag, 1992), a genetic defect in the reductase responsible for the reduction of the terminal isoprene unit would impair lipid intermediate synthesis by multiple effects. Recent studies have also shown that the Man-P-Dol-mediated mannosyltransferases converting Man$_5$GlcNAc$_2$-P-P-Dol to Man$_9$GlcNAc$_2$-P-P-Dol in brain (Rush et al., 1993) and porcine aorta (D'Souza-Schorey et al., 1992) discriminate between Man-P-Dol and Man-P-Poly. All of these effects are consistent with the biochemical phenotype of a putative polyprenol reductase mutant CHO cell line (Stoll & Krag, 1988; Stoll et al., 1988; Rosenwald & Krag, 1990). It has also been shown that substrates containing dolichyl moieties, rather than fully unsaturated polyprenyl groups, are preferred by GPT1, Man-P-Dol synthase, and the mannosyltransferase catalyzing the transfer of mannosyl groups from Man-P-Dol to Ser(Thr) residues in glycoproteins in *Saccharomyces cerevisiae* (Palamarczyk et al., 1980). Recent studies suggest that the α-isoprene unit of dolichol is reduced by an NADPH-dependent reaction involving free polyprenol in rat liver (Sagami et al., 1993).

The influence of chain length, and therefore of hydrophobicity, of the dolichyl moiety of the mannolipid activator was illustrated by the slightly greater, but statistically significant, stimulation of GlcNAc-lipid synthesis by Man-P-Dol$_{95}$ than by Man-P-Dol$_{55}$. Moreover, stimulation by the water-soluble analog Man-P-Dol$_{10}$ was very small compared to that by the mannolipids containing long-chain dolichol units and required substantially higher concentrations. The effect of hydrophobicity could be due to more favorable interaction of the mannolipid with a detergent micelle system *in vitro*. However, defects in the chain-elongation stage of dolichol synthesis could also have severe effects on lipid intermediate synthesis *in vivo* at multiple points in the lipid intermediate pathway. It is also plausible that the chain length of the dolichol could be critical for the transverse diffusion of the dolichyl-saccharides from the cytoplasmic leaflet to the luminal monolayer where Oligo-P-P-Dol synthesis is completed.

Another property of the regulatory response of GPT1 activity is that β-Man-P-Dol was dramatically more effective than the α-stereoisomer. The observation that the enhancement of GlcNA-P-P-Dol synthesis is stereospecific for β-Man-P-Dol represents a strong argument that the stimulation of GPT1 is not an *in vitro* artifact and is physiologically significant.

Although the activation of GPT1 by Man-P-Dol is a potentially important regulatory mechanism controlling the rate and extent of GlcNAc-P-P-Dol biosynthesis, the presence of Man-P-Dol apparently is not obligatory for glycoprotein biosynthesis to occur, as indicated by mutant cells which lack the capacity to synthesize Man-P-Dol yet still synthesize asparagine-linked glycoproteins (Chapman et al., 1980; Stoll et al., 1982). Mutants with a defect in Man-P-Dol synthase, and relatively low levels of endogenous Man-P-Dol, contain levels of GPT1 activity similar to that of the wild-type counterparts (Chapman et al., 1980; Stoll et al., 1982; Stoll & Krag, 1988). While the absence of Man-P-Dol may not be a lethal event, the lesion may not be innocuous since oligosaccharides on glycoproteins synthesized by such mutants are abnormal (Stoll et al., 1982; Thomas et al., 1991). It is of interest that studies with membranes from class E Thy-1-negative mutant mouse lymphoma cells which are defective in Man-P-Dol biosynthesis have provided strong evidence that the phenomenon of the activation by Man-P-Dol of GPT1 activity is an intrinsic trait of the dolichol pathway (Kean & DeBrakeleer, 1986). Retention by the mutant cells of the stimulatory capacity was demonstrated even though the cells had lost the ability to synthesize the activating compound.

It seems reasonable that the regulatory response of GPT1 to Man-P-Dol is a novel feedback mechanism for sensing that a sufficient pool of mannolipid is available for the conversion of $Man_5GlcNAc_2$-P-P-Dol to $Man_9GlcNAc_2$-P-P-Dol, a later stage in Oligo-P-P-Dol biosynthesis. A major question regarding the regulatory effect of Man-P-Dol is whether the interaction with the mannolipid occurs on the cytoplasmic and/or lumenally-oriented domain of GPT1 or a regulatory membrane protein in the RER. Strong evidence has been obtained recently demonstrating that the active sites of GPT1 and the GlcNAc transferase catalyzing the synthesis of $(GlcNAc)_2$-P-P-Dol face the cytoplasmic surface of such vesicles (Abeijon & Hirschberg, 1990; Kean, 1991). Evidence has also been presented suggesting that the stimulatory factor has a similar topography (Kean, 1993). More detailed topological analysis will be necessary to establish this point conclusively.

Future goals are as follows: (1) to establish the molecular mechanism by which Man-P-Dol stimulates GlcNAc-P-P-Dol biosynthesis, and if the activation occurs by a direct interaction with the catalytic subunit of GPT1 or an accessory regulatory subunit, and (2) to determine whether the regulatory interaction occurs when the β-Man 1-P headgroup is oriented toward the cytosolic or luminal face of the RER.

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