Three enzymes involved in oligosaccharide-lipid assembly in Chinese hamster ovary cells differ in lipid substrate preference

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Abstract Initial steps in N-linked glycosylation involve formation of a large oligosaccharide structure on a lipid carrier, dolichyl phosphate. We have previously characterized Chinese hamster ovary (CHO) glycosylation mutants (Lec9 cells) that utilize the polyisoprenoid lipid polyprenyl phosphate rather than dolichyl phosphate in these glycosylation reactions. Polyprenyl phosphate differs from dolichyl phosphate only in the degree of saturation of its terminal isoprenyl unit. Our goal was to determine whether the glycosylation defect of Lec9 cells could be explained simply by knowing lipid substrate preferences of the enzymes involved in the assembly of oligosaccharide-lipid (OSL) intermediates. In this study, we have used in vitro assay systems to compare the ability of dolichyl phosphate and polyprenyl phosphate to act as substrates for three glycosyl transferase enzymes involved in OSL assembly. In order to insure that we were only examining lipid substrate preferences of the enzymes and not other potential defects present in Lec9 cells, we used membranes prepared from wild-type cells in these in vitro reactions. Our results indicate that one of the enzymes, mannosylphosphoryldolichol (MPD) synthase, exhibited a significant preference for the dolichol substrate. Glucosylphosphoryldolichol (GPD) synthase, on the other hand, showed no binding specificity for the dolichol substrate, although the enzyme used the dolichol substrate at a twofold higher rate. N,N'-diacetylchitobiosylpyrophosphoryldolichol (CPD) synthase was able to use either lipid substrate with equal efficiency. These results suggest that not all glycosyl transferases in this pathway show a preference for dolichol derivatives. Moreover, in conjunction with other studies from our laboratory, these results help explain the glycosylation phenotype of Lec9 cells; that is, they synthesize less OSL and the structure of the major OSL is $Man_5GlcNAc_2$-P-P-lipid rather than $Glc_3Man_9GlcNAc_2$-P-P-lipid.—**McLachlan, K. R., and S. S. Krag.** Three enzymes involved in oligosaccharide-lipid assembly in Chinese hamster ovary cells differ in lipid substrate preference. *J. Lipid Res.* 1994. **35:** 1861–1868.

Supplementary key words dolichyl phosphate • polyprenyl phosphate • glycosylation mutants

The biosynthesis of asparagine-linked glycoproteins in eukaryotic cells is a complex process, initiated by the synthesis of a lipid carrier, dolichyl phosphate, in the endoplasmic reticulum. An oligosaccharide intermediate containing 14 monosaccharides is then assembled onto this lipid in a stepwise manner (**Scheme 1**). Once assembled, the oligosaccharide is transferred to an Asn residue in the consensus sequence. Asn-X-Ser/Thr in a nascent polypeptide in the endoplasmic reticular lumen. The oligosaccharide is subsequently processed by a variety of enzymes located in both the endoplasmic reticulum and Golgi, resulting in formation of the mature carbohydrate structure (1).

Previously, our laboratory isolated and characterized glycosylation mutants in CHO cells that were defective in their ability to synthesize dolichol (2, 3). Instead, these mutants, termed Lec9, synthesize and use polyprenol and its derivatives in all their glycosylation reactions. Polyprenol is the immediate precursor of dolichol, differing from the latter only in the saturation of the terminal isoprenyl unit of the molecule (in dolichol it is saturated; in polyprenol, unsaturated). As a result of this defect, Lec9 cells are severely impaired in their glycosylation activities; OSL intermediates are synthesized in lower amounts and are altered in structure when compared to wild-type cells. Also, Lec9 cells underglycosylate protein in comparison with wild type cells (4).

In order to understand why the Lec9 defect in lipid synthesis has such a profound effect on the cell's glycosylation pathway, we have compared the ability of dolichol and polyprenol derivatives to act as substrates for several of

Abbreviations: chitobiose, N,N'-diacetylchitobiose; CHO, Chinese hamster ovary; CM2:1, chloroform–methanol 2:1; CPD, N,N'-diacetylchitobiosylpyrophosphoryldolichol; CMW, chloroform–methanol–water; GlcNAc 1-P transferase, UDP-N-acetylglucosamine:dolichol phosphate N-acetylglucosamine 1-phosphate transferase; GPD, glucosylphosphoryldolichol; GPP, glucosylphosphorylpolyprenol; MPD, mannosylphosphoryldolichol; MPP, mannosylphosphorylpolyprenol; OSL, oligosaccharide-lipid; TLC, thin-layer chromatography.

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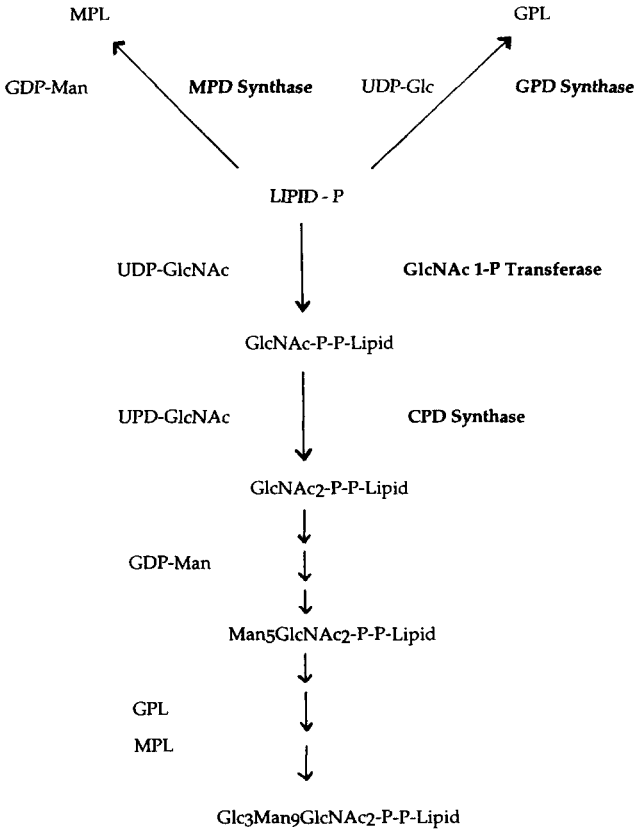

Scheme 1. Assembly of oligosaccharide-lipid. In these reactions, the lipid (L) could be either dolichol or polyprenol. Multiple arrows indicate multiple steps.

the glycosyl transferases involved in OSL assembly (see Scheme 1). In order to ensure that we were only examining lipid substrate preferences of the enzymes and not other potential defects present in Lec9 cells, we used membranes prepared from wild-type cells as the source of the activity of these glycosyltransferases. Previously, we showed that GlcNAc 1-P transferase, which catalyzes the initial step in the assembly pathway, namely the formation of GlcNAc-PP-dolichol, is significantly impaired in its ability to use polyprenyl phosphate compared to dolichyl phosphate. The enzyme had a 10-fold lower apparent K_m and a 10-fold higher apparent V_{max} using dolichyl phosphate as a substrate compared to polyprenyl phosphate (5).

In this paper, we report studies on the substrate specificity of three additional enzymes, namely CPD synthase, MPD synthase, and GPD synthase (see Scheme 1). CPD synthase catalyses the second step in oligosaccharide lipid assembly, namely incorporation of a second GlcNAc residue from UDP-GlcNAc to form GlcNAc-GlcNAc-PP-lipid. Very little information is currently known about this enzyme, most likely due to its membrane-associated nature and the difficulty in obtaining reasonable quantities of the GlcNAc-PP-lipid substrates with which to assay the enzyme. Formation of CPD has been observed in in vitro experiments using microsomes prepared from porcine aorta (6), hen oviduct (7, 8), embryonic pig liver (9), yeast (10), and rat liver (11), in addition to CHO cells (12). However, no detailed analysis of the substrate specificity of the enzyme has been carried out to date.

MPD synthase activity catalyzes the formation of MPD from GDP-mannose and dolichyl phosphate. Similarly, the GPD synthase enzyme catalyzes formation of GPD from UDP-glucose and dolichyl phosphate. The lipid-linked mannose acts as donor for four of the nine mannose residues found in the full-length OSL structure, while the lipid-linked glucose acts as donor for all three glucose residues in the structure (13). The predominant OSL structure found in the Lec9 glycosylation mutants contains only five mannoses -$Man_5GlcNAc_2$-PP-lipid (4). The synthesis of this truncated OSL intermediate rather than the full length OSL intermediate could be due to lack of formation of the appropriate monoglycosylated lipids resulting from the inability of the MPD and GPD synthase enzymes to use polyprenyl phosphate as a substrate. We have observed Man-P-polyprenol and Glc-P-polyprenol in the mutant cells (3), indicating that the synthase enzymes have some ability to utilize polyprenyl phosphate. However, we felt that a careful examination of the lipid substrate specificities was necessary in order to completely understand the Lec9 lipid phenotype. Another possibility for the appearance of the shortened OSL intermediate is that the transferase enzymes responsible for incorporation of the sugars from the monoglycosylated lipid donors are incapable of transferring sugars attached to polyprenyl phosphate. This possibility will be addressed further in the discussion.

In this article we describe experiments addressing the substrate specificities of the three above-mentioned enzymes. Interpretations relating to the Lec9 glycosylation phenotype will be discussed.

MATERIALS AND METHODS

Materials

UDP-N-acetyl [^{3}H]glucosamine and UDP-[^{3}H]glucose were purchased from New England Nuclear and GDP-[^{14}C]mannose was synthesized as described (14). Polyprenol was isolated from cherry leaves (15) and was shown to contain a mixture of isomers ranging from C_{85} to C_{105}, based on analysis by reverse-phase HPLC (3). The C_{95} isomer was the predominant species (data not shown). This polyprenol preparation was completely susceptible to MnO_2 oxidation (3), indicating there was no contaminating dolichol present (15). Polyprenyl phosphate was prepared chemically as described (16). Dolichyl phosphate and cold sugar nucleotides were from Sigma Chem-

ical Company. Silica gel TLC plates were from J. T. Baker Chemical Company. Biogel P4 and AG 1X-8 formate resins were obtained from Bio-Rad Laboratories; DEAE cellulose resin was from Schleicher and Schuell. Sugar standards for gel filtration chromatography were prepared as described (17). All other chemicals were from standard commercial sources.

Cell culture and cell membrane preparation

Membranes prepared from wild-type WTB cells (18) were used as the enzyme source for CPD synthase, MPD synthase, and GPD synthase in all assays described here. Membranes prepared from 3E11 cells (19) provided the source of GlcNAc 1-P transferase activity, which was used in order to synthesize the GlcNAc-PP-lipid substrates for the CPD synthase assays. Cell culture and membrane preparations were as described (20). Membranes prepared from WTB cells were resuspended in Tris-buffered saline, pH 7.4, at protein concentrations >10 mg/ml, and stored on ice at 4°C for up to 48 h. GPD synthase and CPD synthase activities were found to be unstable upon storage for times exceeding 48 h. Thus, assays were always performed immediately after preparation of each batch of membranes. Membranes from 3E11 cells were resuspended at a protein concentration of 10 mg/ml in 20 mM Tris-HCl, pH 7.4, containing 20% sucrose and 5 mM $MgCl_2$, and stored at −20°C.

Synthesis and purification of GlcNAc-PP-lipids

GlcNAc-PP-dolichol and GlcNAc-PP-polyprenol were synthesized using GlcNAc 1-P transferase activity from 3E11 membranes. Reactions were carried out in 20 mM glycine, pH 9, as described (5) except the concentration of UDP-N-acetyl[^{3}H]glucosamine was 2 mCi/mmol, and the assay time was 20 min. Multiple reactions were performed in order to generate enough lipid substrates for the CPD synthase assay.

It was necessary to synthesize the GlcNAc-PP-lipids with a tracer level of radioactivity in order to quantitate the amount produced, hence the use of 2 mCi/mmol UDP-N-acetyl [^{3}H]glucosamine rather than unlabeled UDP-N-acetylglucosamine. This is a specific activity 250-fold lower than that used in the CPD synthase assays, and in these assays, all data points were corrected for counts that originated from the GlcNAc-PP-lipid substrates.

GlcNAc-PP-lipids from multiple reactions were pooled, concentrated under nitrogen, and purified by thin-layer chromatography (TLC) in a chloroform–methanol–water (CMW) 60:25:4 solvent system. Material eluted from the TLC plate was then applied to a DEAE-cellulose column, and GlcNAc-PP-lipids were eluted in 20 ml chloroform–methanol (CM) 2:1 containing 100 mM ammonium acetate.

Assay conditions for CPD synthase

Assays were performed in 20 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$, 0.2 M KCl, 0.15 M NaCl, 0.1% Triton X-100, 50 μM UDP-N-acetyl[^{3}H]glucosamine, 500 mCi/mmol, and 100 μg membrane protein. GlcNAc-PP-dolichol or GlcNAc-PP-polyprenol were first resuspended in 0.017% Triton X-100, and the amount of lipid substrates initially resuspended was determined by liquid scintillation counting of a small portion of the suspension. The GlcNAc-PP-lipids were assayed in concentrations ranging from 0.05 to 5 μM. Because the GlcNAc-PP-lipid substrates contained some contaminating lipid phosphate (see Results section for an explanation of this), membrane protein was first preincubated in buffer containing the GlcNAc-PP-lipids and 5 μM cold GDP-mannose in order to remove any contaminating lipid phosphate from the final reaction. Preincubations were carried out for 5 min at 37°C, after which time the radiolabeled sugar nucleotide, salt, and additional detergent were added. Assays were then performed for a further 5 min at 37°C, stopped by the addition of 1 ml CM 2:1, extracted with 0.15 M NaCl containing 0.01 M HCl, and radioactivity incorporated into the organic phase was determined by liquid scintillation counting.

Product analysis of CPD synthase

The reaction products (organic phase) of assays performed with 0.1, 0.5, 1, and 2 μM GlcNAc-PP-dolichol, and 0.1 and 1 μM GlcNAc-PP-polyprenol were individually dried under nitrogen and resuspended in 1 ml 0.1 M HCl in 80% tetrahydrofuran. Samples were incubated for 2 h at 50°C, neutralized by the addition of 1 ml 0.1 M NaOH containing 20 mM Na_2HPO_4, dried, and resuspended in 1 ml 0.1 M Tris-Cl, pH 8, containing sugar standards. Samples were applied to a Bio-Gel P4 column, and 100 × 0.6 ml fractions were collected. Column fractions were assayed for elution of the sugar standards as described (21, 22) and the elution position of the reaction products was determined by liquid scintillation counting.

Assay conditions for MPD and GPD synthases

Assays for MPD synthase were carried out in 20 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, 10 mM $MgCl_2$, 10 μM GDP-[^{14}C]mannose, 50 mCi/mmol, 0.015% NP40, and 40 μg membrane protein in a volume of 50 μl. All reactions were for 4 min at 37°C. Dolichyl and polyprenyl phosphates were assayed in concentrations ranging from 5 to 250 μM. Lipid phosphates were first resuspended in 0.1% NP40, which was subsequently diluted to give a final concentration of 0.015% in the assay mix. The amount of lipid phosphates initially resuspended was determined for each assay (23). Assays were quenched by the addition of 1 ml CM2:1, extracted with 4 mM $MgCl_2$, followed by 4 mM $MgCl_2$–CH_3OH 2:1,

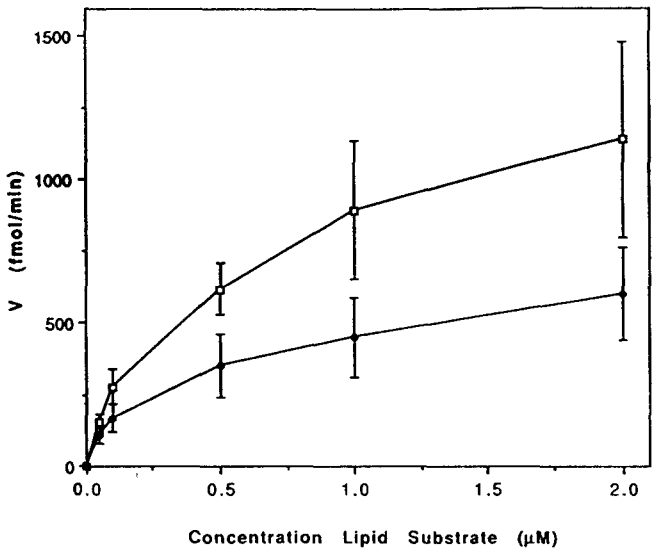

Fig. 1. N,N'-Diacetylchitobiosylpryophosphoryldolichol synthase activity. The assays were carried out as described in Materials and Methods. All data points have been corrected for counts in the organic phase originating from the substrate GlcNAc-PP-lipids (See Materials and Methods). Additionally, assays with GlcNAc-PP-dolichol at concentrations of 0.5 μM and higher have been corrected for contamination with competing GlcNAc 1-P transferase activity, as described in the text. All data points were then expressed in terms of rate of reaction in pmols/min. The curves shown represent the mean of three independent experiments, and in each experiment all points were measured in duplicate. Open symbols, GlcNAc-PP-dolichol. Filled symbols, GlcNAc-PP-polyprenol.

and counted as described for the CPD synthase assay above. GPD synthase activity was assayed essentially as above, except that the sugar nucleotide donor in this case was 10 μM UDP-[^{3}H]glucose, 310 mCi/mmol, and 80 μg membrane protein was used.

Analysis of products from MPD synthase and GPD synthase reactions

Reaction products (organic phase) were pooled where necessary, dried under nitrogen, resuspended in 40 μl CM2:1 and applied to 5 × 10 cm silica gel TLC plates. Previously prepared [^{14}C]MPD or [^{3}H]GPD were applied to the plates as external standards where applicable. Chromatograms were developed in chloroform–methanol–ammonia–water 65:35:2:2; sample lanes were cut into 0.5-cm slices and counted.

Other methods

Membrane protein concentration was determined by the method of Lowry et al. (24), using bovine serum albumin as a standard.

RESULTS

N,N' diacetylchitobiose-PP-dolichol synthase

In order to assay CPD synthase activity, it was first necessary to prepare the appropriate lipid-linked substrates for the enzyme, namely GlcNAc-PP-dolichol and GlcNAc-PP-polyprenol. These substrates are themselves the products of the first step in the pathway for OSL assembly, formation of which is catalyzed by the enzyme GlcNAc 1-P transferase. These substrates were synthesized enzymatically, using membranes prepared from 3E11 cells as the source of the GlcNAc 1-P transferase activity. 3E11 membranes exhibit approximately 15-fold higher levels of transferase activity than wild-type membranes (19), and therefore provide an excellent source of this enzyme. In order to make as much of each of these lipid-linked substrates as possible, we incubated 3E11 membranes for 20 min under optimal assay conditions using 50 μM lipid phosphate substrates (5). GlcNAc-PP-lipids were purified by TLC and DEAE-cellulose chromatography as described in Materials and Methods.

We determined the optimal assay conditions for CPD synthase as described in Materials and Methods. **Figure 1** shows the enzyme activity with both GlcNAc-PP-dolichol and GlcNAc-PP-polyprenol assayed at concentrations ranging from 0.05 to 2 μM. This figure clearly shows that only a minimal difference in substrate preference exists between these two substrates. Kinetic parameters for the enzyme were calculated from the data in this figure, and are shown in **Table 1.** CPD synthase had an apparent K_m of 0.2 μM and an apparent V_{max} of 0.8 pmol/min for the dolichol-linked substrate. For the polyprenol-linked sub-

TABLE 1. Kinetic parameters for synthase enzymes[a]

Enzyme	Substrate	K_{mapp}	V_{maxapp}
		μM	*pmol/min*
Chitobiose synthase	GlcNAc-PP-dolichol	0.2 ± 0.02	0.79 ± 0.04
	GlcNAc-PP-polyprenol	0.24 ± 0.04	0.58 ± 0.06
MPD synthase	Dolichyl phosphate	64.3 ± 9.6	28.5 ± 1.79
	Polyprenyl phosphate	41.0 ± 3.0	2.9 ± 0.14
GPD synthase	Dolichyl phosphate	18.0 ± 3.2	7.6 ± 0.4
	Polyprenyl phosphate	14.0 ± 4.0	4.6 ± 0.7

[a]The data shown in Figs. 1 and 2 were replotted using the Enzfitter program (Elsevier Biosoft) to determine the kinetic parameters for each of the synthase enzymes.

strate the values were 0.24 μM and 0.6 pmol/min for apparent K_m and apparent V_{max}, respectively. These data indicated that CPD synthase does not exhibit a strong preference for the dolichol-linked substrate over the polyprenol-linked substrate. This result is in striking contrast to the results for the GlcNAc 1-P transferase enzyme, the first enzyme in the pathway (5).

One of the concerns in studying CPD synthase was to ensure that we were actually observing the formation of chitobiosyl-PP-dolichol and chitobiosyl-PP-polyprenol in these assays. Early product analyses indicated that our substrate preparations were contaminated with unlabeled prenyl phosphates. As the membrane preparations also contained GlcNAc 1-P transferase, this contamination resulted in the formation of GlcNAc-PP-lipid in addition to the chitobiosyl-PP-lipid product (data not shown). All attempts to remove the prenyl phosphate contamination were unsuccessful.

In order to circumvent this problem, CPD synthase assays included a preincubation of membranes with GlcNAc-PP-lipids in the presence of cold GDP-mannose. The rationale behind this particular approach was that contaminating lipid phosphate would be converted to mannosylphosphoryl-lipid due to the action of MPD synthase activity also present in the membrane preparations. Thus, on addition of radiolabeled UDP-GlcNAc and other assay components, the only substrate available for incorporation of [^{3}H]GlcNAc would be GlcNAc-PP-lipid. Hence, we could investigate CPD synthase activity specifically.

In order to assess the validity of this assumption, we analyzed the products of CPD synthase assay reactions using different levels of either GlcNAc-PP-dolichol or GlcNAc-PP-polyprenol as described in Materials and Methods. The results of this analysis indicated that for CPD synthase assays using the polyprenol substrate, chitobiose-PP-polyprenol was the predominant product formed. However, at high concentrations of the dolichol substrate, we found that dolichyl phosphate contamination was still significant (GlcNAc-PP-dolichol accounted for 20–30% of the reaction product; data not shown). Therefore, to ensure that we measured only CPD production in these assays, the data presented in Fig. 1 and Table 1 have been corrected based on the product analysis described here.

MPD and GPD synthases

Substrate curves for MPD synthase activity with dolichyl phosphate compared to polyprenyl phosphate are shown in **Fig. 2A.** The data in this figure show a substantial difference in the rate of the reaction between the two lipid substrates, with dolichyl phosphate clearly being the preferred substrate. Kinetic parameters for the enzyme were determined from this data, and are shown in Table 1. The enzyme had an apparent K_m of 64 μM and an apparent V_{max} of 29 pmol/min for dolichyl phosphate, compared with an apparent K_m of 41 μM and an apparent V_{max} of 2.9 pmol/min for polyprenyl phosphate. The lower apparent K_m for polyprenyl phosphate was somewhat surprising. However, both values were within the same order of magnitude and therefore the difference between the two may not be significant. The difference in apparent V_{max} between the two lipid substrates was 10-fold, which indicates that this enzyme is able to utilize dolichyl phosphate at a much higher rate than polyprenyl phosphate.

Fig. 2B shows the results obtained from assays of GPD synthase activity. The rate of the reaction with the GPD

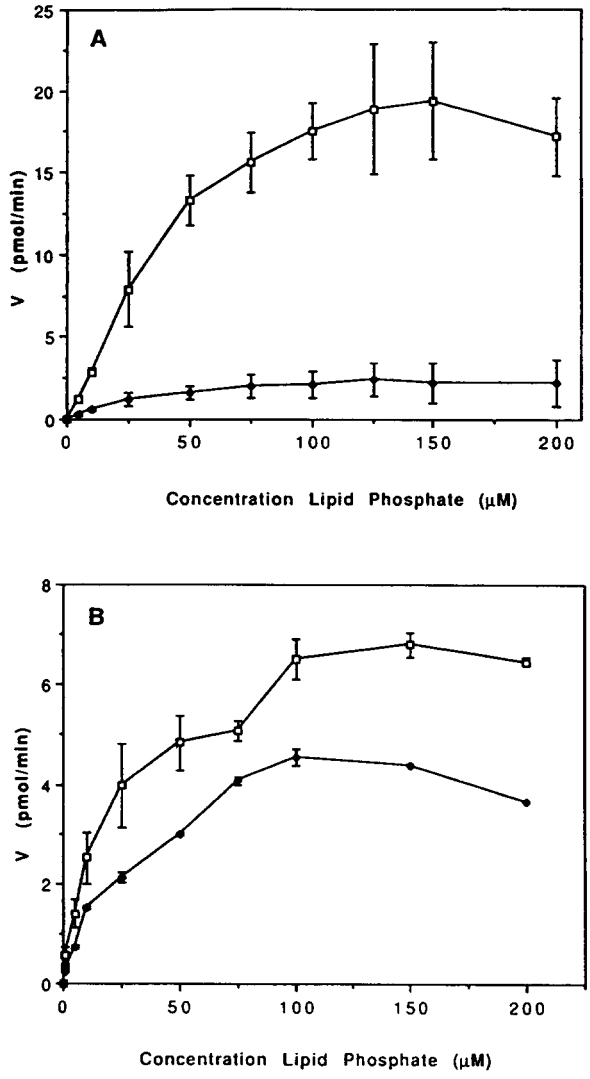

Fig. 2. MPD and GPD Synthase Activities. All assays were carried out as described in Materials and Methods. All data points have been expressed in terms of rate of reaction in pmol/min. The curves shown represent the mean of three independent experiments, and in each experiment all points were measured in duplicate. Open symbols, dolichyl phosphate. Filled symbols, polyprenyl phosphate. A: MPD Synthase; B: GPD Synthase.

synthase enzyme appeared to be twofold faster with dolichyl phosphate compared to polyprenyl phosphate. Kinetic parameters for the enzyme were determined from this data, and are shown in Table 1. The enzyme had an apparent K_m of 18 μM and an apparent V_{max} of 7.6 pmol/min for dolichyl phosphate, while apparent K_m and apparent V_{max} values for polyprenyl phosphate were 14 μM and 4.6 pmol/min, respectively. The apparent K_m values for the two substrates are within experimental error and can be assumed to be identical, indicating that the only difference between these substrates is in their rate of utilization by the enzyme.

The only products we anticipated being produced in these assays were the lipid-linked sugars MPD, MPP, GPD, and GPP. In order to ensure that this was in fact the case, and that neither the lipid-linked sugars formed nor the radiolabeled sugar nucleotides used in these assays were being incorporated into any other OSL structures, we examined the products produced in assays with both dolichyl phosphate and polyprenyl phosphate of both MPD synthase and GPD synthase by TLC as described in Materials and Methods. The results of this analysis indicated that in all cases a single product was formed that comigrated with the appropriate lipid-linked sugar standard. A representative chromatogram to illustrate this point is shown in **Fig. 3.**

One possible explanation for the difference in reaction rate observed between dolichyl phosphate and polyprenyl phosphate could be the availability of the sugar nucleotide substrate in the course of the assay. For example, GDP-mannose breakdown has been observed previously (25). We therefore analyzed the aqueous fractions (containing unincorporated sugar nucleotide) from both MPD synthase and GPD synthase assays by ion-exchange chromatography. The results of this analysis indicated that the sugar nucleotide was stable under the assay conditions used here, and therefore sugar nucleotide degradation was not a factor contributing to the observed differences in enzyme activity between dolichyl and polyprenyl phosphate (data not shown).

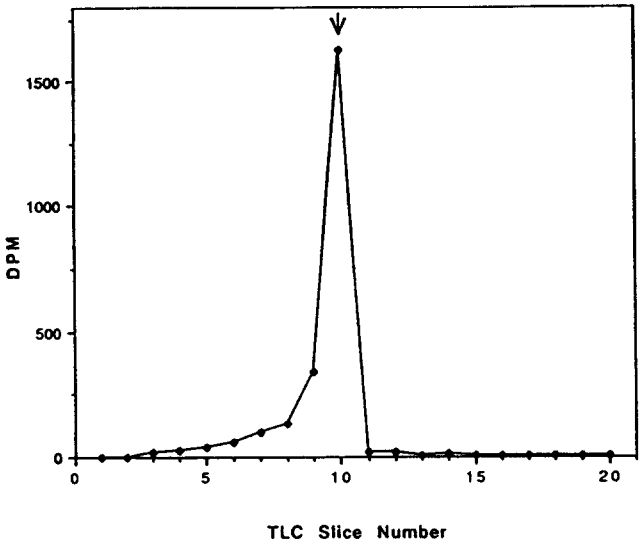

Fig. 3. Product analysis for MPD synthase reactions. MPD synthase assays using varying concentrations of dolichyl phosphate and polyprenyl phosphate were carried out as described. The organic phases from the reactions were pooled where necessary and applied to a 5 × 10 cm silica TLC plate, along with a [^{14}C]MPD standard. Plates were run in C-M-NH_4OH-W 65:35:2:2; sample lanes were cut into 0.5-cm slices, and were counted. The profile shown is that of the products from a reaction containing dolichyl phosphate at a concentration of 150 μM. The migration position of radiolabeled MPD standard is indicated by the arrow.

DISCUSSION

In this study we have examined the substrate specificity of three enzymes involved in the assembly of OSL, namely, CPD synthase, MPD synthase, and GPD synthase. CPD synthase, which catalyzes the second step in the pathway of OSL synthesis, is an enzyme about which little is known. Formation of CPD has been observed previously in other in vitro systems, most notably rat liver (11) and hen oviduct (7). In these experiments, incorporation of labeled sugar from UDP-GlcNAc by membrane preparations in the presence of exogenously added dolichyl phosphate was followed. As these membrane preparations contained both GlcNAc 1-P transferase and CPD synthase, a mixture of both GlcNAc-PP-dolichol and CPD were produced. Detergent solubilization of both of these activities from porcine aorta (6) and yeast (10) was described; however, no further purification of the enzymes was described.

The assay system we described in this paper allowed us to look specifically at CPD synthase activity in membranes by adding the product of the GlcNAc 1-P transferase enzyme, namely GlcNAc-PP-dolichol. Our results were reproducible and showed that this enzyme had no preference for one lipid substrate over the other (i.e., GlcNAc-PP-dolichol vs. GlcNAc-PP-polyprenol) (Fig. 1).

This finding was initially somewhat surprising to us, considering the extensive difference in substrate specificity we observed for GlcNAc 1-P transferase (5). However, our results may reveal some interesting characteristics of the nature of these enzymes' interactions with their substrates. The GlcNAc 1-P transferase enzyme presumably has a binding site for the lipid substrate that specifically requires saturation of the alpha-isoprene unit for tight binding and utilization. On the other hand, CPD synthase presumably has a binding site for the carbohydrate residue and specifically requires it to be GlcNAc. The identity of the lipid moiety attached to the carbohydrate appears not to be important. This is an interesting concept, as formation of chitobiosyl lipid, or indeed any other lipid species smaller than $Man_5GlcNAc_2$-PP-lipid,

has never been detected in vivo under steady-state labeling conditions either in mutant or wild-type CHO cells incubated with tritiated mevalonate (26). This observation leads us to speculate that perhaps the other transferases involved in elongating the initially formed GlcNAc-PP-lipid to the $Man_5GlcNAc_2$-PP-lipid intermediate also do not have a preference for the lipid species to which these carbohydrates are attached. Rather, these glycosyl transferases may have specificity only for the carbohydrate structure and linkage between the sugars. This hypothesis is not unprecedented, as it has previously been demonstrated that processing enzymes such as alpha-mannosidase and glucosidase I can hydrolyze simple artificial substrates provided they contain the correct sugar moiety in the appropriate linkage (27, 28). If this hypothesis is accurate, this could explain why the Lec9 mutant cells are able to synthesize $Man_5GlcNAc_2$-PP-lipid; once the initial step has taken place (i.e., formation of GlcNAc-PP-polyprenol) the activities of the next six glycosyl transferases are unaffected by the growing oligosaccharide being linked to polyprenol rather than dolichol.

Obviously, the above argument does not hold for all glycosyl transferases involved in oligosaccharide assembly; otherwise, Lec9 cells would be capable of synthesizing $Glc_3Man_9GlcNAc_2$-PP-polyprenol. The fact that the major OSL species they form is $Man_5GlcNAc_2$-PP-polyprenol indicates that some of the enzymes acting later in the pathway must be impaired in their ability to use polyprenyl derivatives. Two enzymes involved in these later steps are MPD synthase and GPD synthase, which catalyze formation of the monoglycosylated lipids used as sugar donors for the remaining glycosyl transferases. Some previous studies on these enzyme activities in yeast (29, 30) and rat liver microsomes (31) have been published. While these papers addressed the effect of lipid chain length on enzyme activity more specifically, they also provided evidence to suggest that polyprenyl phosphate is a less effective substrate than is dolichyl phosphate. However, these studies were not performed under optimal experimental conditions, nor were measurements made over a range of substrate concentrations.

Our work described in this paper on the CHO enzymes also indicated that MPD synthase had a clear preference for the dolichol substrate. On the other hand, the data for the GPD synthase (Fig. 2B) indicated this enzyme had a minimal difference in activity between dolichyl and polyprenyl phosphate. Why the difference in activity is not as pronounced for GPD synthase as for MPD synthase is unclear.

Our data indicated that the MPD synthase enzyme was unable to utilize polyprenyl phosphate as effectively as dolichyl phosphate, and thus one might anticipate that the Lec9 mutants would therefore synthesize less mannosyl-phosphoryllipid compared to wild-type cells. In actuality, labeling studies (2, 3) indicated comparable amounts of both monoglycosylated species are produced in both cell types. Detection of these monoglycosylated lipid species in the mutant cells could be expected if the transferase enzymes responsible for their incorporation into OSL were unable to transfer polyprenol-linked sugars. In fact, we have recently shown that dolichyl-P-mannose:$Man_5GlcNAc_2$-PP-dolichol mannosyltransferase and dolichyl-P-glucose:$Man_9GlcNAc_2$-PP-dolichol glucosyltransferase prefer dolichol-linked sugars rather than polyprenol-linked sugars as substrates (32). This preference for dolichol-linked substrates was also seen by Rush et al. (33) using a pig brain membrane system.

Previous data using bacterial membranes (31) indicated that in glycosylation reactions in prokaryotes the unsaturated (polyprenyl) lipids are preferred. Taken together, our data in vitro (5, 32, this paper) and in vivo using Lec9 cells (2, 3) indicate that some but not all mammalian lipid-linked glycosylation reactions require saturated lipid substrates (dolichol) for maximal efficiency. Although it is unclear why both types of lipids exist in nature, it is clear that eukaryotes utilize saturated lipids preferentially and therefore have had to evolve the ability to convert the unsaturated polyprenol to the saturated dolichol.

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