Recycling of Dolichyl Monophosphate to the Cytoplasmic Leaflet of the Endoplasmic Reticulum after the Cleavage of Dolichyl Pyrophosphate on the Lumenal Monolayer*

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During protein N-glycosylation, dolichyl pyrophosphate (Dol-P-P) is discharged in the lumenal monolayer of the endoplasmic reticulum (ER). Dol-P-P is then cleaved to Dol-P by Dol-P-P phosphatase (DPPase). Studies with the yeast mutant cwh8Δ, lacking DPPase activity, indicate that recycling of Dol-P produced by DPPase contributes significantly to the pool of Dol-P utilized for lipid intermediate biosynthesis in the cytoplasmic leaflet. Whether Dol-P formed in the lumen diffuses directly back to the cytoplasmic leaflet or is first dephosphorylated to dolichol has not been determined. Incubation of sealed ER vesicles with calf brain with acetyl-Asn-Tyr-Thr-NH2, an N-glycosylatable peptide, to generate Dol-P-P in the lumenal monolayer produced corresponding increases in the rates of Man-P-Dol, Glc-P-Dol, and GlcNAc-P-P-Dol synthesis in the absence of CTP. No changes in dolichol kinase activity were observed. When streptolysin-O permeabilized CHO cells were incubated with an acceptor peptide, N-glycopeptide synthesis, requiring multiple cycles of the dolichol pathway, occurred in the absence of CTP. The results obtained with sealed microsomes and CHO cells indicate that Dol-P, formed from Dol-P-P, returns to the cytoplasmic leaflet where it can be reutilized for lipid intermediate biosynthesis, and dolichol kinase is not required for recycling. It is possible that the flip-flopping of the carrier lipid is mediated by a flipase, which would provide a mechanism for the recycling of Dol-P derived from Man-P-Dol-mediated reactions in N-, O-, and C-mannosylation of proteins, GPI anchor assembly, and the three Glc-P-Dol-mediated reactions in Glc3Man9GlcNAc2-P-P-Dol (DLO) biosynthesis.

When the precursor oligosaccharide is transferred from Glc3Man9GlcNAc2-P-P-Dol (DLO) to appropriate Asn-X-Ser/Thr sequences in nascent polypeptides in the lumen of the endoplasmic reticulum (ER), the glycosyl carrier lipid is released as dolichyl pyrophosphate (Dol-P-P) (1–3). A Dol-P-P phosphatase with a lumenal-oriented active site responsible for converting Dol-P-P to Dol-P has been identified in yeast (CWH8) and mammalian cells (DolPP1) (4, 5). Based on the observation that the cwh8Δ mutant in Saccharomyces cerevisiae has a reduced capacity for synthesizing DLO (6), it appears that the recycling of Dol-P from Dol-P-P released during protein N-glycosylation reactions on the luminal surface contributes significantly to the pool of Dol-P available for lipid intermediate synthesis on the cytoplasmic leaflet of the ER. These results also indicated that the normal rate of biosynthesis de novo cannot fully compensate for the loss of the Dol-P recycled from the luminal surface to the cytoplasmic leaflet pool in the cwh8Δ mutant.

Two plausible mechanisms for the recycling of Dol-P from the lumen of the ER are illustrated in Fig. 1. First, Dol-P may diffuse directly back to the cytoplasmic leaflet and be re-utilized for new rounds of lipid intermediate biosynthesis (Fig. 1, mechanism A). The transverse diffusion of the monophosphate may require the assistance of a flipase to allow the hydrophilic head group to traverse the hydrophobic core of the ER bilayer. Alternatively, Dol-P may first be dephosphorylated, by an as yet to be identified Dol-P phosphatase with a lumenal-oriented active site, prior to flip-flopping in the ER (Fig. 1, mechanism B). This would help to overcome the biophysical constraint of moving the phosphoryl group through the hydrophobic core of the bilayer. Free dolichol could then be phosphorylated by the CTP-dependent dolichol kinase on the cytoplasmic surface to be “activated” for lipid intermediate synthesis (Fig. 1, mechanism B). In this case, dolichol kinase would have a role in the recycling of the glycosyl carrier lipid, and in the de novo pathway by phosphorylating newly synthesized dolichol formed by the reduction of the polyisoprenol precursor (1).

In this study we have incubated sealed ER vesicles with a membrane permeable, N-glycosylatable tripeptide to stimulate the production of Dol-P-P on the luminal monolayer to explore the mechanism by which the carrier lipid is recycled. Following the addition of the acceptor peptide, changes in the level of endogenous Dol-P and/or dolichol on the cytoplasmic surface were assessed by detecting changes in the rates of Man-P-Dol, Glc-P-Dol, and GlcNAc-P-P-Dol synthesis and dolichol kinase activity under conditions that the enzymatic rates were...
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dependent on the amount of endogenous Dol-P or free dolichol, respectively. The potential role of dolichol kinase in the recycling process was also evaluated in a Streptolysin-O permeabilized CHO cell system. The results of these studies indicate that Dol-P diffuses directly to the cytosolic surface after cleavage of Dol-P-P. It appears that virtually no Dol-P is dephosphorylated prior to flip-flopping or diffusing back to the cytosolic monolayer. The possible existence of a Dol-P flip-flop that could facilitate the transbilayer movement of the carrier lipid formed from Dol-P-P, as well as the substantial amount released during Man-P-Dol-mediated reactions in N-, O-, and C-mannosylation of proteins, GPI anchor assembly, and the three Glc-P-Dol-mediated reactions in DLO biosynthesis is discussed.

EXPERIMENTAL PROCEDURES

Materials—[3H]Mannose (60 Ci/mmole), UDP-[3H]glucose (20 Ci/mmole), UDP-[14C]GlcNAc (55 mCi/mmole), and [γ-32P]ATP (~500 Ci/mmole) were purchased from American Radiolabeled Chemicals (St. Louis, MO). GDP-[3H]mannose was synthesized from [3H]mannose, as described previously (7). [γ-32P]CTP was synthesized from [γ-32P]ATP by phosphorylation of CDP using nuclease diphosphate kinase (Sigma) and purified by ion-exchange chromatography on benzyl-DEAE cellulose (Sigma). Bacterial or potato acid phosphatase were obtained from Sigma. Synthetic tripeptides (8) were from Synpep Corp. (Dublin, CA). Dolichol phosphate was synthesized from dolichol (a generous gift from Dr. M. Mizuno, Kuraray Chemical Co., Okayama, Japan) by chemical phosphorylation as described by Danilov and Chojnacki (9).

Preparation of Sealed ER Vesicles from Calf Brain—Sealed, ER enriched, myelin-depleted calf brain microsomes were prepared as described previously (10) except that 10 mM HEPES-NaOH (pH 7.4) was used instead of 0.1 M Tris-HCl (pH 7.4). Microsomes were resuspended in 10 mM HEPES-NaOH (pH 7.4), 0.25 M sucrose, 1 mM EDTA to a membrane protein concentration of 10–20 mg/ml and stored at −20 °C.

Assessment of Integrity of Calf Brain Microsomal Vesicles—The intactness of calf brain microsomal vesicles was determined by measuring the latency of the processing, deoxynojirimycin-sensitive glucosidase I/II activities using [3H]Glc1–3Man3GlcNAc2 as substrate in the presence and absence of Triton X-100 (2 mg/ml) as described in detail elsewhere (11). Based on these latency assays, the vesicles used for the studies described here were 93 ± 4% intact.

Assays for the In Vitro Synthesis of Man-P-Dol, GlcNAc1–2P-P-Dol, Glc-P-Dol, and GlcMan3GlcNAc2-P-P-Dol—Reaction mixtures contained 50 mM Tris-HCl, pH 8.0, 0.25 M sucrose, 5 mM CaCl2, 5 mM MgCl2, 4 mM AMP, calf brain microsomal fraction (0.8 mg membrane protein) and either 5 μM GDP-[3H]Man (4,200 dpm/pmol), 5 μM UDP-[3H]Glc (2,000 dpm/pmol), or 5 μM UDP-[14C]GlcNAc (349 dpm/pmol) in a total volume of 0.1 ml. None of these assay mixtures contained CTP. At the indicated times, the reactions were stopped by the addition of 20 vol of CHCl3/CH3OH (2:1), and the incorporation of radioactivity into each glycolipid product was determined by a multiple extraction procedure described previously (12).

Assay for the Transfer of [3H]Glc1–3Man3GlcNAc2 from [3H]Glc1–3Man3GlcNAc2-P-P-Dol into [3H]Glc1–3Man3GlcNAc2-glycopeptide—Reaction mixtures for prelabeling of [3H]Glc1–3Man3GlcNAc2-P-P-Dol contained 50 mM Tris-HCl, pH 8.0, 0.25 M sucrose, 5 mM CaCl2, 5 mM MgCl2, 4 mM AMP, 5 μM UDP-[3H]Glc (2,000 dpm/pmol), and calf brain microsomal fraction (0.8 mg membrane protein) in a total volume of 0.1 ml. After incubation for 10 min at 37 °C, the indicated concentration of the appropriate peptide (either N-glycosylation acceptor peptide, acetyl-Asn-Tyr-Thr-NH2, or a control peptide, acetyl-Gln-Tyr-Thr-NH2) was added, and the incubation was continued at 37 °C. Following the second incubation, the reactions were terminated by the addition of 20 vol of CHCl3/CH3OH (2:1) and the delipidated membrane residue was sedimented. The insoluble pellet was resuspended in CHCl3/CH3OH (2:1), sedimented by centrifugation, and the lipid extracts were combined. The soluble pellets were analyzed for the incorporation of [3H]glucose into dolichol-linked oligosaccharides as described previously (12). Water soluble products were recovered from the initial organic extracts by partitioning with one-fifth vol of H2O. The aqueous phase was dried by rotary evaporation under reduced pressure at 30 °C, redissolved in 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM CaCl2, 1 mM MgCl2, and 1 mM MnCl2 and fractionated on a 0.5-ml column of concanavalin A-Sepharose (Sigma) according to Welpl et al. (13).

Assay for Dolichol Kinase Activity—Reaction mixtures contained calf brain microsomes (0.8 mg protein), 50 mM Tris-HCl, pH 8.0, 0.25 M sucrose, 50 mM CaCl2, 20 mM UTP, and 40 μM [γ-32P]CTP (2,500 dpm/pmol) in a total volume of 0.1 ml. Following incubation at 37 °C, reactions were stopped by the addition of 20 vol of CHCl3/CH3OH (2:1), and the incorporation of 32P into Dol-32P was determined as described by Burton et al. (14).

Analytical Procedures—Protein concentrations were determined using the BCA protein assay (Pierce) following precipitation of membrane proteins with deoxycholate and trichloroacetic acid according to the Pierce Biotechnology Bulletin, “Eliminate Interfering Substances from Samples for BCA Protein Assay.” Samples were analyzed for radioactivity by scintillation spectrometry in a Packard Tri-Carb 2100TR liquid scintillation spectrometer following the addition of 0.5 ml of 1% SDS and 4 ml of Ecosafone Economical Biodegradable Counting Mixture (Research Products International, Corp., Mount Prospect, IL).

RESULTS

Use of a Membrane-permeable N-Glycosylatable Peptide to Discharge Dol-P-P from Glc3Man3GlcNAc2-P-P-Dol during the Oligosaccharyltransferase (OT)-mediated Reaction on the Lumenal Leaflet of Sealed Calf Brain Microsomes—When the precursor oligosaccharide is transferred from DLO to appropriate asparagine residues in nascent polypeptide substrates, the glycosyl carrier lipid is released as Dol-P-P on the luminal leaflet (1–3). The experiments described below were designed to determine if the Dol-P-P discharged during the primary N-glycosylation reaction is recycled as Dol-P (Fig. 1, mechanism A) after being formed by Dol-P-P phosphatase (4, 5), or if it is completely dephosphorylated and diffuses transversely to the
cytoplasmic monolayer as the free polyisoprenol (Fig. 1, mechanism B). To investigate these two possibilities, the experiments described below with sealed calf brain microsomes were conducted. Sealed calf brain microsomes were preincubated with a membrane-permeable, glycosylatable tripeptide to generate lumenally oriented Dol-P-P. The appearance of either Dol-P or dolichol on the cytoplasmic surface of the microsomal vesicles was then assessed by assaying Man-P-Dol synthase or dolichol kinase activity.

The key to this strategy is that the levels of Dol-P and dolichol are subsaturating and stimulated by exogenous Dol-P and dolichol, respectively, in calf brain microsomes (12, 14). Thus, increases in the amounts of the two substrates in the cytoplasmic leaflet are reflected in increases in the rates of Man-P-Dol synthesis and dolichol phosphorylation.

Initially, the N-glycosylation reaction generating Dol-P-P was followed by assaying the peptide-dependent loss of label from endogenous, prelabeled [3H-Glc]Glc3Man9GlcNAc2-P-Dol in sealed calf brain microsomes when increasing concentrations (0–100 μM) of the acceptor peptide, acetyl-Asn-Tyr-Thr-NH2, were added to the reaction mixture. The results in Fig. 2 (panel A) show that the addition of the acceptor peptide (•) stimulated a peptide-concentration dependent loss of labeled DLO and, presumably, the release of Dol-P-P on the lumenal leaflet. There was a considerably lower loss of labeled oligosaccharide lipid when a control peptide (○) was added (Fig. 2, panel A), probably due to the transfer of the precursor oligosaccharide to endogenous polypeptide acceptors and a low rate of hydrolysis. A time course conducted with 100 μM acetyl-Asn-Tyr-Thr-NH2 indicated that peptide N-glycosylation was complete within 2 min following addition of the acceptor peptide (data not shown).

To confirm that the loss of label from the endogenous, prelabeled DLO resulted from the OT reaction, the appearance of [3H]Glc1–3Man9GlcNAc2-peptide was observed. No increase in concanavalin A-Sepharose-bound [3H]glycopeptide was seen when the control peptide was added (○). These results provide good evidence that the addition of acetyl-Asn-Tyr-Thr-NH2 stimulated the production of Dol-P-P in the lumenal leaflet of sealed calf brain microsomes.

Addition of Acceptor Peptide to Sealed Calf Brain Microsomes Increased the Amount of Endogenous Dol-P Available for Lipid Intermediate Biosynthesis on the Cytoplasmic Leaflet, but Not the Amount of Dolichol Accessible to Dolichol Kinase—The studies described here were conducted to determine if the production of Dol-P-P on the lumenal leaflet resulted in an increase in the pool of either Dol-P or free dolichol in the cytoplasmic monolayer.

Following the addition of the acceptor peptide, the initial rates of the Man-P-Dol synthase reaction and the CTP-mediated formation of Dol-P catalyzed by dolichol kinase were...
assayed under conditions where the initial rates were dependent on the endogenous level of the lipid substrates, respectively. As seen in Fig. 3 (panel A), the initial rate and extent of Man-P-Dol synthesis were significantly stimulated in the absence of CTP following the addition of the acceptor peptide (F), relative to the control peptide (E). In contrast to this result, the initial rate of the dolichol kinase reaction was unaffected when the acceptor peptide was added (Fig. 3, panel B).

Previous studies (14–17) have indicated that there is a common pool of Dol-P which is utilized by all three enzymes synthesizing dolichol-linked monosaccharide intermediates. To test if the Dol-P derived from Dol-P-P was accessible to Glc-P-Dol synthase and UDP-GlcNAc-Dol-P GlcNAc-phosphotransferase (GPT), the rates of Glc-P-Dol and GlcNAc\(_{1–2}\)-P-P-Dol synthesis were compared in sealed vesicles following incubation with acceptor and control peptides in the absence of CTP. The results depicted in Fig. 4 show that stimulation of Dol-P-P formation in the ER lumen by the N-glyosylatable acceptor peptide resulted in a 4–5-fold increase in the synthesis of Glc-P-Dol (panel A, ●) and GlcNAc\(_{1–2}\)-P-P-Dol (panel B, ○) compared with incubation with the control peptide (○).

To eliminate the possibility that the increase in Dol-P in the cytoplasmic leaflet might be due to the coupled “flipping” of free dolichol (Fig. 1, mechanism B), followed by subsequent phosphorylation by dolichol kinase in the presence of a low level of endogenous CTP, the effect of apyrase and exogenous CTP on the acceptor peptide-dependent increase in Man-P-Dol synthesis was investigated. As seen in Table 1, preincubation of intact calf brain microsomes with 10 milliunits of potato apyrase had no effect on the increase in Man-P-Dol synthesis following incubation with acceptor peptide. The failure of incubation with apyrase or CTP (data not included) to affect the acceptor peptide-dependent increase in Man-P-Dol synthesis excludes the possibility that dolichol kinase participates in the recycling of Dol-P in the calf brain in vitro system studied here.
Increased Levels of Dol-P Appear Rapidly in the Cytoplasmic Monolayer following the Addition of the Acceptor Peptide

To estimate the time required for recycled Dol-P to appear on the cytoplasmic monolayer following acceptor peptide addition, intact calf brain vesicles were preincubated with GDP-[3H]Man at 37 °C for 4.5 min. Then either acceptor peptide (●) or control peptide (○) were added and the incubations were continued. At the indicated times, Man-P-Dol synthesis was stopped by the addition of 20 vol of CHCl3/CH3OH (2:1) and the amount of [3H]Man-P-Dol formed was determined as described under “Experimental Procedures.” The inset is an expansion of the 2 min time-frame following acceptor peptide addition.

Bacitracin Addition Blocks the Acceptor Peptide-dependent Increase in Dol-P Accessible to Man-P-Dol Synthase in the Cytoplasmic Monolayer

To provide adducing proof that the increase in the Dol-P pool in the cytoplasmic leaflet is derived from Dol-P generated in the lumenal leaflet during the OT reaction, the effect of bacitracin, an antibiotic that forms a non-reactive complex with polyisoprenyl pyrophosphates in bacteria (18, 19) and calf brain (20), was tested. As seen in Fig. 6 (panel A), the addition of bacitracin (20–40 μg/ml) prevented the increase in the rate of Man-P-Dol synthesis produced by the acceptor peptide. Man-P-Dol synthesis was sensitive to bacitracin in the presence or absence of Triton X-100 (Fig. 6, panels A and B) indicating that bacitracin penetrated the sealed vesicles. Control experiments with intact calf brain microsomes containing prelabeled [3H]Glc1–3Man9GlcNAc2-P-P-Dol showed that bacitracin had no effect on the transfer of the labeled oligosaccharide from [3H]Glc1–3Man9GlcNAc2-P-P-Dol to the N-glycosyl acceptor peptide catalyzed by OT (data not included). These results strengthen the evidence that the increased level of from Dol-P-P generated in the lumenal leaflet during the OT reaction, the effect of bacitracin, an antibiotic that forms a non-reactive complex with polyisoprenyl pyrophosphates in bacteria (18, 19) and calf brain (20), was tested. As seen in Fig. 6 (panel A), the addition of bacitracin (20–40 μg/ml), prevented the increase in the rate of Man-P-Dol synthesis produced by the acceptor peptide. Man-P-Dol synthesis was sensitive to bacitracin in the presence or absence of Triton X-100 (Fig. 6, panels A and B) indicating that bacitracin penetrated the sealed vesicles. Control experiments with intact calf brain microsomes containing prelabeled [3H]Glc1–3Man9GlcNAc2-P-P-Dol showed that bacitracin had no effect on the transfer of the labeled oligosaccharide from [3H]Glc1–3Man9GlcNAc2-P-P-Dol to the N-glycosyl acceptor peptide catalyzed by OT (data not included). These results strengthen the evidence that the increased level of from Dol-P-P generated in the lumenal leaflet during the OT reaction, the effect of bacitracin, an antibiotic that forms a non-reactive complex with polyisoprenyl pyrophosphates in bacteria (18, 19) and calf brain (20), was tested. As seen in Fig. 6 (panel A), the addition of bacitracin (20–40 μg/ml), prevented the increase in the rate of Man-P-Dol synthesis produced by the acceptor peptide. Man-P-Dol synthesis was sensitive to bacitracin in the presence or absence of Triton X-100 (Fig. 6, panels A and B) indicating that bacitracin penetrated the sealed vesicles. Control experiments with intact calf brain microsomes containing prelabeled [3H]Glc1–3Man9GlcNAc2-P-P-Dol showed that bacitracin had no effect on the transfer of the labeled oligosaccharide from [3H]Glc1–3Man9GlcNAc2-P-P-Dol to the N-glycosyl acceptor peptide catalyzed by OT (data not included). These results strengthen the evidence that the increased level of from Dol-P-P generated in the lumenal leaflet during the OT reaction, the effect of bacitracin, an antibiotic that forms a non-reactive complex with polyisoprenyl pyrophosphates in bacteria (18, 19) and calf brain (20), was tested. As seen in Fig. 6 (panel A), the addition of bacitracin (20–40 μg/ml), prevented the increase in the rate of Man-P-Dol synthesis produced by the acceptor peptide. Man-P-Dol synthesis was sensitive to bacitracin in the presence or absence of Triton X-100 (Fig. 6, panels A and B) indicating that bacitracin penetrated the sealed vesicles. Control experiments with intact calf brain microsomes containing prelabeled [3H]Glc1–3Man9GlcNAc2-P-P-Dol showed that bacitracin had no effect on the transfer of the labeled oligosaccharide from [3H]Glc1–3Man9GlcNAc2-P-P-Dol to the N-glycosyl acceptor peptide catalyzed by OT (data not included). These results strengthen the evidence that the increased level of from Dol-P-P generated in the lumenal leaflet during the OT reaction, the effect of bacitracin, an antibiotic that forms a non-reactive complex with polyisoprenyl pyrophosphates in bacteria (18, 19) and calf brain (20), was tested. As seen in Fig. 6 (panel A), the addition of bacitracin (20–40 μg/ml), prevented the increase in the rate of Man-P-Dol synthesis produced by the acceptor peptide. Man-P-Dol synthesis was sensitive to bacitracin in the presence or absence of Triton X-100 (Fig. 6, panels A and B) indicating that bacitracin penetrated the sealed vesicles. Control experiments with intact calf brain microsomes containing prelabeled [3H]Glc1–3Man9GlcNAc2-P-P-Dol showed that bacitracin had no effect on the transfer of the labeled oligosaccharide from [3H]Glc1–3Man9GlcNAc2-P-P-Dol to the N-glycosyl acceptor peptide catalyzed by OT (data not included). These results strengthen the evidence that the increased level of
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| TABLE 2 |
|-----------------|-----------------|
| CTP and apyrase have no effect on the rate of glycan transfer in streptolysin-O-permeabilized untransfected CHO-K1 cells and CHO-K1 cells overexpressing dolichol kinase |
| Control and CHO cells overexpressing dolichol kinase (28) were cultured (10⁶ cells per dish) in F12 medium, permeabilized by incubation with streptolysin-O and preincubated with and without 0.1 unit/ml of apyrase for 20 min (8). Following preincubation, the cells were further incubated for 60 min with 78 mM KC1, 4 mM MgCl₂, 50 mM K-HEPES pH 7.2, 2 mM dithiothreitol, 100 mM UDP-N-acetylglucosamine, 20 mM GDP-mannose, 200 mM UDP-glucose, 2 mM AMP, 100 mM mg/ml caspases (to prevent deglucosylation of glycopeptide), and 50 mM acceptor peptide in the presence and absence of 2 mM CTP. Following the second incubation, the cell monolayers were rinsed twice with ice-cold phosphate-buffered saline and harvested into methanol by scraping with a teflon spatula. Water-soluble triglucosyl glycopeptides were recovered from the dried samples in water, purified by ion-exchange chromatography, hydrolyzed by incubation with Endo H, labeled by reductive amination with 7-amino-1,3-naphthaleneisulfonyl acid and analyzed by FACE as described previously (8, 32). The intensities of fluorescent oligosaccharide species were determined using the Quantity One software supplied with the Bio-Rad Fluor-S Multilutomer and expressed as a percentage of the untreated CHO-K1 cell value (means ± S.E.; n = 4 for CHO-K1, n = 2 for DK-overexpresser). Back- ground as determined by glycopeptide formation in the presence of a control peptide was undetectable, thus no background correction was employed. |
| Cell line | Treatment | Glc₃-Glycopeptide |
|-----------|-----------|------------------|
| CHO-K1    | None      | 103 (± 4)        |
| CHO-K1    | Apyrase   | 103 (± 4)        |
| CHO-K1    | CTP       | 96 (± 9)         |
| DK-Overexpressor | None | 123 (± 14) |
| DK-Overexpressor | Apyrase | 132 (± 0.7) |
| DK-Overexpressor | CTP     | 94 (± 14)        |
| % of untreated CHO-K1 cells |

Dol-P in the cytoplasmic leaflet is derived from Dol-P-P produced during N-glycosylation of the acceptor peptide.

The Addition of Aceptor Peptide Does Not Produce an Increase in the Pool of Free Dolichol Accessible to Dolichol Kinase in SLO-Permeabilized CHO Cells—To verify that the recycling of free dolichol from Dol-P-P was not seen in the microsomal system in vitro due to an essential component being lost or inactivated during the preparation of the sealed microsomes, N-glycosylation of exogenously added acceptor peptides was studied using SLO-permeabilized CHO cells. In these studies permeabilized CHO cells were incubated with the acceptor peptide under conditions which would be expected to affect dolichol kinase activity and then analyzed for peptide N-glycosylation after release of the oligosaccharides by Endo H digestion. Fluorophore-assisted carbohydrate electrophoresis (FACE) was used to monitor Gly₃-Man₃GlcNAc₂ peptide production (Table 2), as well as DLO levels (data not shown), after 1 h incubations to allow calculation of the molar ratios of these molecules under various conditions. From the results in Table 2 it can be seen that neither incubation of permeabilized cells with apyrase, overexpression of dolichol kinase or incubation with high levels of CTP had any significant effect on the rates of acceptor peptide glycosylation. In this experiment, during a 1-h incubation, glycopeptide formation was linear with time, and ~9 mol of glycopeptide were formed per mol DLO at steady state for both CHO-K1 and the DK overexpresser (data not shown), indicating that multiple rounds of lipid intermediate synthesis had occurred. Because it is unlikely that a significant amount of Dol-P is synthesized de novo in the absence of exogenous farnesyl pyrophosphate and isopentenyl pyrophosphate, substantial recycling of Dol-P must be occurring. If recycling of the carrier lipid required phosphorylation of dolichol, it would be expected that degradation of endogenous CTP by apyrase would have decreased the amount of glycopeptide formed, and conversely, the overexpression of dolichol kinase would have increased the rate of acceptor peptide glycosylation. These results are consistent with the conclusion that Dol-P formed from Dol-P-P diffuses directly to the cytoplasmic leaflet.

DISCUSSION

The observation that lipid intermediate biosynthesis and protein N-glycosylation are impaired in the yeast mutant, cwh8Δ, lacking the lumennally oriented Dol-P-P phosphatase (4, 6), suggests that recycling of Dol-P-P from the luminal leaflet contributes significantly to the pool of Dol-P utilized for lipid intermediate biosynthesis. Moreover, because no enzymatic degradation of dolichol has been reported, it is likely that the glyosyl carrier lipid participates in multiple rounds of lipid intermediate biosynthesis. Although it is quite likely that Dol-P is reutilized during DLO assembly, the details of the recycling process have not been elucidated.

In this study, the two potential recycle mechanisms illustrated in Fig. 1 (mechanisms A and B) were evaluated in a sealed microsomal system. The possibility that Dol-P-P diffuses transversely to the cytoplasmic leaflet, where it could be converted to Dol-P, was not explored since the yeast (4) and mammalian (5) Dol-P-P phosphatases have been shown to have lumennally oriented active sites and Dol-P-P accumulates in the cwh8Δ yeast strain (4).

The results presented here support the proposal that Dol-P-P is hydrolyzed to Dol-P on the luminal leaflet and then “flips,” without cleavage to dolichol, to the cytoplasmic leaflet where it can be reutilized for additional rounds of lipid intermediate biosynthesis as shown in Fig. 1 (mechanism A). When Dol-P-P production was stimulated by the addition of an N-glyosylatable acceptor peptide, increases in Man-P-Dol, Glc-P-Dol and GlcNAc-P-P-Dol synthesis were seen under conditions that the rates were dependent on the level of endogenous Dol-P. For each of these enzyme activities, a rapid, initial phase of glycolipid synthesis occurs within 1–2 min, followed by a slower phase lasting 2–20 min.

When bacitracin, which forms an unreactive complex with polisoprenyl pyrophosphates (18–20), was added in combination with the acceptor peptide, it abolished the increase in Dol-P in the cytoplasmic monolayer as assessed by the rate of Man-P-Dol synthesis. This result provides solid evidence that the increased level of Dol-P in the cytoplasmic leaflet is derived from Dol-P-P formed on the luminal surface. No evidence for the phosphorylation of Dol-P on the luminal surface or transverse diffusion of free dolichol was obtained in either the sealed microsomal system or SLO-permeabilized CHO cells.

These results are compatible with a previous report by Spiro and Spiro (21) showing that Glc-P-P-Dol synthesis was stimulated by adding an N-glycosylatable hexapeptide to bovine thyroid vesicles. However, in this study with thyroid microsomes the topological orientation and intactness of the vesicles were not determined conclusively allowing the possibility of increases in Glc-P-P-Dol synthesis occurring by intervescicular reactions. In addition the possible contribution of Dol-P formed from dolichol kinase and endogenous CTP was not evaluated.

We have also reported that the addition of acceptor peptide to SLO-permeabilized CHO cells (8), in which lipid intermedi-
ate consumption had ceased due to inhibition of protein synthesis with cycloheximide, produced a stimulation in lipid intermediate synthesis, consistent with Fig. 1, Mechanism A, but this study was also not designed to address the mechanism of recycling of Dol-P. These results have now been extended by assessing the effect of incubation with either apyrase or CTP and the overexpression of dolichol kinase on the stimulation of lipid intermediate synthesis in the SLO-permeabilized CHO system and eliminate the possibility that any recycled Dol-P was formed from free dolichol via dolichol kinase. In this regard, there have not been any definitive reports of a candidate Dol-P phosphatase in the ER with a luminally oriented active site that would form free dolichol acting in combination with Cw8p/Dolpp1p.

The time course described in Fig. 5 indicates that the t₁/₂ for the release of Dol-P-P, the cleavage to Dol-P and the appearance of Dol-P on the cytoplasmic face of the ER to occur within 2–3 min. This estimate is similar to the estimates for the rates of the transbilayer movement of newly synthesized phosphatidylethanolamine in B. megaterium (22) and glycerophospholipids in rat liver ER (23). Because the unassisted transbilayer movement of polyisoprenyl phosphates in synthetic liposomes is extremely slow (24), these results suggest that mammalian cells may contain an ER membrane protein(s) that mediates the transbilayer movement of Dol-P. A Dol-P flipase would play an important role in recycling of Dol-P formed by at least 12 lipid-mediated reactions. The carrier lipid molecules are discharged during the dolichosaccharyltransferase-catalyzed protein N-glycosylation reaction, protein O- and C-mannosylation reactions, GPI anchor biosynthesis, and the four Man-P-Dol and three Glc-P-Dol-mediated reactions during the synthesis of DLO in the luminal compartment.

The possibility that the ER contains a flipase mediating the flipping of the carrier lipid is currently being investigated by assaying the transport of citronellyl phosphate, Cit-P, a water-soluble analogue of Dol-P. This approach has been used previously to assay Man-P-Dol and Glc-P-Dol flipase activities (25–27). It is plausible that Dol-P could be flipped by a facilitated diffusion process driven by its utilization on the cytoplasmic leaflet for new lipid intermediate synthesis.

The deficiency of the cwh8 yeast mutant in DLO synthesis suggests that the relative contribution of recycling Dol-P-P is quantitatively significant. Based on the glycosylation phenotype of the cwh8 mutant it is possible that the CW8p mutation affects the transbilayer movement of Dol-P formed from Dol-P-P and the other lipid-mediated reactions on the lumenal surface and/or that eventually most of the cellular Dol-P is arrested as Dol-P-P. In any case, the overall importance of the recycling process is emphasized by the evidence that Dol-P availability is a key rate-controlling factor in the biosynthesis of lipid intermediates and, consequently, of protein glycosylation (reviewed in Ref. 1).

Although dolichol kinase apparently is not involved in recycling of dolichol from the ER lumen, it is still possible that dolichol could return to the ER from other subcellular compartments and be rephosphorylated by the CTP-mediated kinase. However, it appears now that the primary function of dolichol kinase is to catalyze the final step in the de novo pathway on the cytoplasmic leaflet. Recent topological studies have identified a cytoplasmic loop that contains part of the CTP-binding site (28). The functional importance of this enzyme is supported by the identification by Kranz et al. (29) of four patients who are homozygous for one of two mutations in dolichol kinase that cause severe clinical phenotypes. Defective Dol-P recycling in the ER could potentially be the cause of another inborn error in Congenital Disorders of Glycosylation in human patients (30, 31).

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