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## MINI REVIEW

# The ins(ide) and outs(ide) of dolichyl phosphate biosynthesis and recycling in the endoplasmic reticulum

**Barbara Schenk[2], Fabiana Fernandez[2], and Charles J. Waechter[1,3]**

[2]Institute for Microbiology, ETH Zurich, 8092 Zurich, Switzerland and [3]Department of Biochemistry, University of Kentucky College of Medicine, Lexington, KY 40502, USA

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**The precursor oligosaccharide donor for protein N-glycosylation in eukaryotes, $Glc_3Man_9GlcNAc_2$-P-P-dolichol, is synthesized in two stages on both leaflets of the rough endoplasmic reticulum (ER). There is good evidence that the level of dolichyl monophosphate (Dol-P) is one rate-controlling factor in the first stage of the assembly process. In the current topological model it is proposed that ER proteins (flippases) then mediate the transbilayer movement of Man-P-Dol, Glc-P-Dol, and $Man_5GlcNAc_2$-P-P-Dol from the cytoplasmic leaflet to the lumenal leaflet. The rate of flipping of the three intermediates could plausibly influence the conversion of $Man_5GlcNAc_2$-P-P-Dol to $Glc_3Man_9GlcNAc_2$-P-P-Dol in the second stage on the lumenal side of the rough ER. This article reviews the current understanding of the enzymes involved in the de novo biosynthesis of Dol-P and other polyisoprenoid glycosyl carrier lipids and speculates about the role of membrane proteins and enzymes that could be involved in the transbilayer movement of the lipid intermediates and the recycling of Dol-P and Dol-P-P discharged during glycosylphosphatidylinositol anchor biosynthesis, N-glycosylation, and O- and C-mannosylation reactions on the lumenal surface of the rough ER.**

*Key words:* topology/rough endoplasmic reticulum/flippase/polyisoprenyl phosphate/recycling/cis-isoprenyltransferase

## Introduction

The structures of polyisoprenoid glycosyl carrier lipids and their roles in the biosynthesis of complex glycoconjugates are well established in prokaryotes (Lennarz and Scher, 1972; Bugg and Brandish, 1994) and eukaryotes (Waechter, 1989; Kornfeld and Kornfeld, 1985; Burda and Aebi, 1999). Undecaprenyl monophosphate (Undec-P) functions as an essential glycosyl carrier lipid primarily in the cytoplasmic membrane of bacteria, and dolichyl monophosphates (Dol-P) serve a similar function in yeast and mammalian cells in the C- and O-mannosylation and N-glycosylation of proteins and the biosynthesis of glycosylphosphatidylinositol (GPI) anchors in the endoplasmic reticulum (ER). The information on the enzymology and molecular biology of these complex biosynthetic pathways has increased impressively over the past 20 years, but many questions remain unanswered. There are still significant gaps in the understanding of the factors regulating the rate of biosynthesis of the carrier lipids, the glycolipid intermediates, and the lipid-mediated assembly processes. Just to cite two examples, virtually nothing is known about the precise function of the Lec35 gene product (Ware and Lehrman, 1996, 1998) or the mechanisms involved in the transbilayer movement of this class of polar glycolipid intermediates.

In this article, the structural features of prokaryotic and eukaryotic glycosyl carrier lipids and the current understanding of the enzymatic mechanisms and regulation of their biosynthesis are reviewed. In addition, a model for the possible roles of the *CWH8* gene product (van Berkel *et al.*, 1999; Rush *et al.*, 2000), and other pertinent enzymes and membrane proteins (flippases) in the recycling of Dol-P and Dol-P-P released on the lumenal surface of the ER during the various lipid-mediated glycosylation reactions is presented. An attempt has been made to recognize and connect past contributions that formed the foundation for the more recent developments in these topics. Some related aspects of the structure and function of dolichol and the mechanistic details of *trans*- and *cis*-isoprenyltransferases (OIS-IPTase) have also been reviewed recently (Krag, 1998; Ogura and Koyama, 1998).

## Structures of polyisoprenoid glycosyl carrier lipids in prokaryotic and eukaryotic cells

It has been nearly 40 years since Undec-P was the first polyisoprenyl phosphate shown to function as a glycosyl carrier lipid in O-antigen, peptidoglycan, capsular polysaccharide, teichoic acid, and mannan biosynthesis in bacteria (Lennarz and Scher, 1972). Undec-P contains 11 isoprene units and is fully unsaturated. The number of isoprene units and their stereoconfiguration are depicted as $\omega$-$t_2$-$c_8$-P in Table I. As indicated, $\omega$ denotes the distal terminal isoprene unit of the polyisoprenol; t the trans isoprene units derived from farnesyl pyrophosphate (F-P-P); c the *cis* isoprene units derived directly from isopentenyl pyrophosphate (I-P-P); and S denotes the position of saturated isoprene units. Using this nomenclature, the number and the stereoconfiguration of the isoprene units in other polyisoprenyl phosphates known to function as glycosyl carrier lipids in pro- and eukaryotic cells are listed in Table I. As seen in this list, the

[1]To whom correspondence should be addressed

**Table I.** Structure of polyprenyl and dolichyl monophosphates used as glycosyl carrier lipids in prokaryotic and eukaryotic cells

| | Stereochemical structure | Number of isoprene units |
|---|---|---|
| Eubacteria | ω-$t_2$-$c_8$-P | 11 (C55) |
| Mycobacteria | ω-$t_1$-$c_8$-P | 10 (C50) |
| | $S_4$-$c_3$-P | 7 (C35) |
| | $S_4$-$t_2$-$c_1$-P | |
| *Trypanosoma brucei* | ω-$t_2$-$c_{7/8}$-S-P | 11/12 (C55/60) |
| *Crithidia fasciculata* | ω-$t_2$-$c_7$-S-P | 11 (C55) |
| Archaea | ω-$t_2$-$c_7$-S-P | 11 (C55) |
| Yeasts | ω-$t_2$-$c_{10–13}$-S-P | 14–17 (C70–85) |
| Mammals | ω-$t_2$-$c_{15–17}$-S-P | 19–21 (C95–105) |

Numbers in parenthesis = total numbers of carbon atoms.
c = *cis* isoprene unit.
t = *trans* isoprene unit.
S = saturated α-isoprene unit.
ω = distal terminal isoprene unit.

polyisoprenyl phosphate glycosyl carrier lipids are a family of membrane lipids varying in the number and stereoconfiguration of their linearly linked isoprene units. The chain length is species-specific with 11 isoprene residues commonly found in eubacterial and archaebacterial cells (Higashi *et al.*, 1967; Lechner *et al.*, 1985). Mycobacteria are unusual among eubacteria, having one containing 7 isoprene units (Wolucka and de Hoffmann, 1998), and another with 10 isoprene units (Wolucka *et al.*, 1994). Trypanosamatids utilize dolichyl phosphates as glycosyl carrier lipids with chain lengths of 11 or 12 isoprene units (Low *et al.*, 1991; Quesada-Allue and Parodi, 1983).

Eukaryotes contain dolichols, which differ from undecaprenol and other fully unsaturated polyprenols in that the chain length is generally longer and the α-isoprene unit is saturated. The yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* contain dolichols with 14–17 isoprene units (Quellhorst *et al.*, 1998). In mammalian cells the predominant dolichols range from 18–21 isoprene units (Rip *et al.*, 1985). Dolichols, the longest aliphatic molecules synthesized in animal cells, are considerably longer than the fatty acyl chains of glycerophospholipids. The structures of the (C90–105) Dol-Ps found in mammalian cells are denoted as ω-$t_2$-$c_{15–17}$-S-P in Table I.

Experiments conducted in many laboratories have established that the chain length and the presence (or absence) of a saturated α-isoprene unit in polyisoprenyl phosphate substrates are critical for their recognition by the enzymes that glycosylate them and utilize their glycosyl derivatives as sugar donors (Szkopinska *et al.*, 1992; Kean *et al.*, 1994; McLachlan and Krag, 1992, 1994; Rush *et al.*, 1993, 1997; D'Souza-Schorey *et al.*, 1994; Dotson *et al.*, 1995 and other references cited in these papers). All of these reports show that, in general, each glycosyltransferase prefers the natural polyisoprenyl monophosphate as the acceptor lipid substrate. However, the functional significance of why the chain length increased and the α-isoprene units of the eukaryotic glycosyl carrier lipids (dolichols) became saturated during evolution remains an intriguing question.

## Biosynthesis of the glycosyl carrier lipids Undec-P and Dol-P

### *Initiation and chain elongation stage catalyzed by cis-isoprenyltransferases*

Numerous laboratories have documented the presence of *cis*-IPTase activity in crude microsomal fractions from a variety of animal tissues (Daleo *et al.*, 1977; Grange and Adair, 1977; Wong and Lennarz, 1982; Crick *et al.*, 1991; Ericsson *et al.*, 1992a), yeast (Adair and Cafmeyer, 1987; Szkopinska *et al.*, 1996), and soluble fractions from hen oviduct (Wellner and Lucas, 1979) and Ehrlich ascites tumor cells (Adair *et al.*, 1984). In contrast to the microsomally associated *cis*-IPTases in mammalian tissues, bacterial *cis*-IPTases are apparently soluble proteins (Keenan and Allen, 1974; Baba and Allen, 1980). In all of these enzyme systems chain elongation can be initiated with F-P-P in the presence of I-P-P (Figure 1). In the bacterial systems eight *cis*-isoprene units are added to F-P-P, forming ω-$t_2$-$c_8$-P-P, which is then converted to the "active" form of the carrier lipid by a pyrophosphate phosphatase (Goldman and Strominger, 1972). A recent report indicates that the biosynthesis of the unusual heptaprenyl phosphate is derived from ω-$t_4$-P-P, and the synthesis of decaprenyl phosphate is initiated by a reaction involving the F-P-P stereoisomer, ω-t-c-P-P, and I-P-P in mycobacteria (Crick *et al.*, 2000).

In the eukaryotic systems the chain elongation stage is catalyzed by *cis*-IPTases that add 12–18 isoprene units to F-P-P, forming the pyrophosphorylated intermediates, ω-$t_2$-$c_{12–18}$-P-P. Although it has been proposed that F-P-P synthase in yeast may be associated with *cis*-IPTase (Szkopinska *et al.*, 1997), a direct interaction between the enzymes has not yet been demonstrated. The chain elongation process can also be initiated with the all trans-stereoisomer of geranylgeranyl pyrophosphate (ω,*t,t,t,*-GG-P-P) or ω,*t,t,c*-GG-P-P (Crick *et al.*, 1991; Ericsson *et al.*, 1992b) and I-P-P *in vitro*. Presumably, the "nascent" allylic pyrophosphate intermediate becomes firmly integrated into the cytoplasmic leaflet of the ER early in the elongation stage and continues to be extended until the *cis*-IPTases amazingly recognize that the chain is the correct length (Figure 1). The studies reviewed herein indicate that the conversion of F-P-P to the appropriate fully unsaturated, long chain polyprenyl pyrophosphate (Poly-P-P) end product is catalyzed by a single enzyme in bacteria and yeast. Further work will be required to determine if this is true in animal cells. The biosynthesis of Dol-Ps is completed by the terminal reactions described below.

### *Structures and subcellular localization(s) of eukaryotic cis-IPTases*

In early work on this class of isoprenyltransferases, *cis*-IPTase activities were partially purified and characterized from *Micrococcus luteus* (formerly called *Micrococcus lysodeiktikus*), *Lactobacillus plantarum*, and *Escherichia coli* (Keenan and Allen, 1974; Baba and Allen, 1980; Fujisaki *et al.*, 1986). Cloning of the corresponding cDNAs over the past few years has now provided more definitive information on the structures of pro- and eukaryotic *cis*-IPTases (Table II). The first *cis*-IPTase

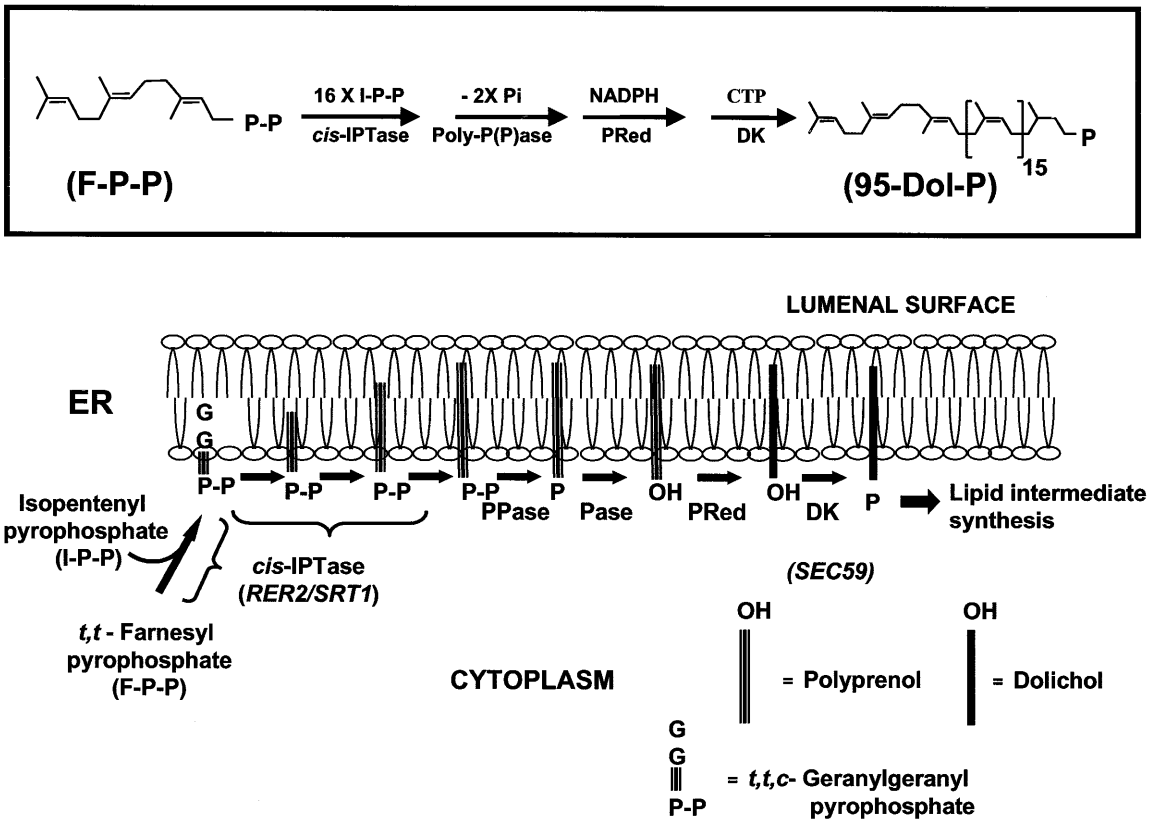


**Fig. 1.** Topological model for the enzymatic reactions leading to Dol-P biosynthesis *de novo* on the cytoplasmic face of the ER. The enzymatic reactions converting farnesyl pyrophosphate (F-P-P, structure shown), to the end product, 95-dolichyl monophosphate (95-Dol-P, structure shown) are illustrated in the upper box. *cis*-IPTase = *cis*-isoprenyltransferase; Poly-P-(P)ase = polyprenyl mono-and pyrophosphate phosphatase; PRed = polyprenol reductase; DK = dolichol kinase.RER2/SRT1 refers to yeast genes encoding separate *cis*-IPTases.

**Table II.** Gene family encoding *cis*-IPTase activities

| Organism | Gene | Gene product | Reference |
|---|---|---|---|
| *S. cerevisiae* | *RER2* | polyprenyl pyrophosphate synthase | Sato *et al.*, 1999 |
| *S. cerevisiae* | *SRT1* | polyprenyl pyrophosphate synthase | Sato *et al.*, 1999; Schenk *et al.*, 2001 |
| *S. pombe* | | hypothetical protein | GenBank 4038613 |
| *A. thaliana* | DPS | polyprenyl pyrophosphate synthase | Cunillera *et al.*, 2000; Oh *et al.*, 2000 |
| *M. luteus* | UPS | undecaprenyl pyrophosphate synthase | Shimizu *et al.*, 1998 |
| *E. coli* | UPS | undecaprenyl pyrophosphate synthase | Apfel *et al.*, 1999; Kato *et al.*, 1999 |
| *C. elegans* | | hypothetical protein | GenBank 3877579 |
| *D.melanogaster* | | hypothetical protein | GenBank 7290854 |

(Undec-P-P synthase) to be cloned was from *M. luteus* (Shimizu *et al.*, 1998). More recently, the cloning of Undec-P-P synthases from *E. coli* (Apfel *et al.*, 1999; Kato *et al.*, 1999), a *cis*-IPTase from *Arabidopsis thaliana* (Cunillera *et al.*, 2000; Oh *et al.*, 2000) and two *cis*-IPTases from *S. cerevisiae* (Sato *et al.*, 1999; Schenk *et al.*, 2001) have been reported. Homologous loci were found in many organisms, ranging from archae-bacteria to *C. elegans* and to humans. Even though *trans*- and *cis*-IPTases both catalyze a head-to-tail condensation reaction between I-P-P and an allylic pyrophosphate intermediate, they apparently do not share any significant sequence homology (Shimizu *et al.*, 1998). It is noteworthy that the conserved DDXXD motif, characteristic for *trans*-IPTases (Chen *et al.*, 1994) and shown to be required for catalytic activity (Wang and Ohnuma, 1999), is not present in the *cis*-IPTases.

As recently reported in this journal (Schenk *et al.*, 2001), the alternative *cis*-IPTase in *S. cerevisiae* extends the Poly-P-P intermediate to chain lengths similar to mammalian dolichols.

Important clues to the domains that determine how many isoprene units are added by each *cis*-IPTase may be found by comparing the bacterial (C55), *S. cerevisae* (*RER2*, C75), *SRT1* (C95), and *Arabidopsis* (C120), and ultimately the mammalian enzymes that elongate F-P-P to different chain lengths. The structure of the mammalian enzyme(s) has not yet been reported, but it will be interesting to see if those *cis*-IPTases are structurally more closely related to *SRT1* and the *Arabidopsis* enzyme than to *RER2* and the bacterial Undec-P-P synthases. It will also be fascinating to learn how the bacterial *cis*-IPTases, most of which are apparently soluble enzymes, terminate the chain elongation process after adding the correct number of isoprene units with fairly high fidelity.

The mammalian *cis*-IPTases appear to be bound firmly to microsomes, and the rat brain enzyme is highly enriched in heavy microsomes (Crick *et al.*, 1991). Studies with rat liver suggest that peroxisomes may also be able to synthesize Poly-P-P from I-P-P and F-P-P, presumably with a *cis*-IPTase that is distinct from the ER enzyme (Ericsson *et al.*, 1992a). Clearly, the localization and topological arrangements of the *cis*-IPTases involved in Dol-P biosynthesis are important subjects that warrant further investigation.

The initial substrates, I-P-P and F-P-P, are soluble cytosolic intermediates, but the Poly-P-P intermediates and final products are extremely hydrophobic lipids that presumably are embedded in the ER bilayer. It is expected that the active sites of the *cis*-IPTases are located at the boundary of hydrophilic and hydrophobic environments in the ER. In this regard, it has been demonstrated that *cis*-IPTase activity has a protease-sensitive site on the cytoplasmic face of liver microsomes (Adair and Cafmeyer, 1983). Although no membrane-spanning helices are predicted by the structures of the yeast *cis*-IPTases, they are, nevertheless, membrane-bound. Further work will be required to determine the exact nature of the membrane association of eukaryotic *cis*-IPTases.

*Terminal steps in* de novo *pathway*

The terminal step in the *de novo* pathway for Undec-P biosynthesis in bacteria simply requires the cleavage of the pyrophosphate bond in Undec-P-P (Goldman and Strominger, 1972). In the current model for yeasts and mammalian cells, the final Poly-P-P intermediate is dephosphorylated prior to the reduction of the α-isoprene unit. Many laboratories have reported microsomal polyisoprenyl mono- and pyrophosphate phosphatase activities that could catalyze these steps (Kato *et al.*, 1980; Wedgwood and Strominger, 1980; Appelkvist *et al.*, 1981; Belocopitow and Boscoboinik, 1982; Scher and Waechter, 1984; Wolf *et al.*, 1991). There is, however, no definitive proof yet for the Poly-P-P and Poly-P phosphatases, which presumably have active sites exposed on the cytoplasmic leaflet of the ER.

As shown in Figure 1, the α-isoprene unit of the free polyprenol is reduced subsequently by a microsomal reductase with NADPH serving as the reductant (Sagami *et al.*, 1993). Although Chinese hamster over (CHO) mutants that contain defects in α-reduction have been characterized (Stoll *et al.*, 1988), there is virtually no information on the properties and structure of this enzyme. Because the recognition of the saturated α-isoprene unit is vital for the synthesis and function of the lipid intermediates as glycosyl donors, the reductase plays an indispensable role in this biosynthetic pathway. It will be extremely important to learn more about the enzymatic α-reduction of the long chain polyprenol and to establish if this is the only mechanism for the formation of the saturated α-isoprene unit in dolichols.

If the saturated α-isoprene unit of dolichol occurs only at the free isoprenol level, the terminal step in the *de novo* pathway would be catalyzed by dolichol kinase. This CTP-mediated kinase was first detected in microsomes from several mammalian tissues by Charlie Allen and his co-workers by following the conversion of [$^3$H]dolichol to [$^3$H]Dol-P in the presence of unlabeled CTP (Allen *et al.*, 1978), and in brain microsomes by assaying the transfer of $^{32}$P from [γ-$^{32}$P]CTP to endogenous dolichol (Burton *et al.*, 1979). In the latter study the Dol-P formed via the kinase was shown to be formed in a membrane site, where it is available for lipid intermediate biosynthesis. The calf brain kinase is highly enriched in heavy microsomal fractions (Scher and Waechter, 1984), and the active site of the rat liver enzyme faces the cytoplasm (Adair and Cafmeyer, 1983). In *S. cerevisiae*, the *SEC59* gene encodes an essential polypeptide component of dolichol kinase although it has not been conclusively established that it is the catalytic subunit (Heller *et al.*, 1992).

Clearly, significant progress has been made in the last two decades on these reactions, but considerably more work will be required to answer many critical questions about the enzymology, topology, and molecular biology of the terminal steps, dephosphorylation–reduction–rephosphorylation (Figure 1), in Dol-P biosynthesis.

## Regulation of Dol-P and lipid intermediate biosynthesis

The critical role of lipid-mediated glycosylation in: (1) the proper folding and intracellular translocation of N-linked glycoproteins (Helenius and Aebi, 2001), (2) protein O- and C-mannosylation, and (3) GPI-anchor assembly emphasizes the importance of elucidating all of the mechanisms regulating Dol-P and lipid intermediate biosynthesis.

In a number of experimental model systems (Harford *et al.*, 1977; Lucas and Levin, 1977; Harford and Waechter, 1980; Hubbard and Robbins, 1980; Carson *et al.*, 1981; Spiro and Spiro, 1986; Rosenwald *et al.*, 1990) it has been shown that the level of Dol-P in the ER is one important rate-controlling factor in the biosynthesis of glycolipid intermediates and consequently protein N-glycosylation. These results emphasize the need to understand all of the factors regulating the biosynthesis of Dol-P.

Changes in dolichol kinase have been reported in mammalian cells (Burton *et al.*, 1981; Volpe *et al.*, 1987; Eggens, 1988) and developing sea urchin embryos (Rossignol *et al.*, 1981); shifts in the metabolic balance of the phosphorylation of dolichol and the dephosphorylation of Dol-P(P) have been implicated in controlling the level of the Dol-P pool (Scher *et al.*, 1985; Bhat *et al.*, 1991). However, developmental increases in *cis*-IPTase activity represent the best correlation with the induction of Dol-P biosynthesis and protein N-glycosylation in mammalian cells. The induction of the *cis*-IPTase system catalyzing the elongation stage of Dol-P biosynthesis (Figure 1) has been shown to precede large developmental increases in Dol-P and lipid intermediate biosynthesis and protein N-glycosylation in embryonic rat brain (Crick and

Waechter, 1994), proliferating murine B lymphocytes (Crick and Waechter, 1994) and cAMP-treated JEG-3 choriocarcinoma cells (Konrad and Merz, 1996). The recent cloning of cDNAs for several *cis*-IPTases from bacteria and yeast should accelerate the progress in understanding their regulation and how they are associated with the ER.

The rate of formation of GlcNAc-P-P-Dol, Man-P-Dol and Glc-P-Dol in the first stage of the pathway (Figure 2) is influenced by the level of Dol-P in the ER, and there is evidence that dolichylsaccharide intermediate biosynthesis is subject to control by feedback control mechanisms. Kean and co-workers (Kean, 1985; Kean *et al.*, 1994) have published a series of articles documenting the regulation of UDP-GlcNAc:Dol-P N-acetylglucosaminyl 1-P transferase (GPT), the enzyme responsible for GlcNAc-P-P-Dol biosynthesis, by Man-P-Dol. More recently, the same laboratory has reported evidence that Man-P-Dol synthesis is enhanced by GlcNAc-P-P-Dol and that GlcNAc-P-P-Dol inhibits its own synthesis by product inhibition of GPT (Kean *et al.*, 1999). Similarly, $(GlcNAc)_2$-P-P-Dol inhibits the enzyme catalyzing the transfer of GlcNAc from UDP-GlcNAc to GlcNAc-P-P-Dol.

At least three factors could affect the rate of conversion of $Man_5GlcNAc_2$-P-P-Dol to $Glc_3Man_9GlcNAc_2$-P-P-Dol on the lumenal surface in the second stage of the pathway. In addition to the level of the lipid-mediated mannosyl- and glucosyltransferases, the lumenal reactions would be influenced by the rate at which $Man_5GlcNAc_2$-P-P-Dol, Man-P-Dol, and Glc-P-Dol diffuse transversely from the cytoplasmic face, where they are synthesized, to the lumenal monolayer. The transbilayer movement of these three intermediates could be accelerated by a "mass action" effect as they are consumed in the lumenal mannosylation and glucosylation reactions. Similarly, it is also reasonable that the preceding lipid-mediated reactions on the lumenal leaflet could be driven by the consumption of $Glc_3Man_9GlcNAc_2$-P-P-Dol, the lipid end product of the pathway, during the primary N-glycosylation reactions.

The regulation of lipid intermediate biosynthesis is also likely to include other novel mechanisms. In this regard, Doerrler and Lehrman (1999) have recently reported that the unfolded protein response (UPR) stimulates the conversion of $Man_{2-5}GlcNAc_2$-P-P-Dol to $Glc_3Man_9GlcNAc_2$-P-P-Dol. This is another mechanism that enables the UPR to maintain efficient protein folding in the ER in addition to *de novo* synthesis of chaperones. In view of all this information, it is clear that the regulation of $Glc_3Man_9GlcNAc_2$-P-P-Dol biosynthesis is complex and multifaceted, and there is certainly much more to be learned on this topic.

## Topological model for the biosynthesis, utilization in the lipid intermediate pathway for protein N-glycosylation and recycling of Dol-P-P/Dol-P

The current topological model for lipid intermediate biosynthesis and protein N-glycosylation was first outlined in detail by

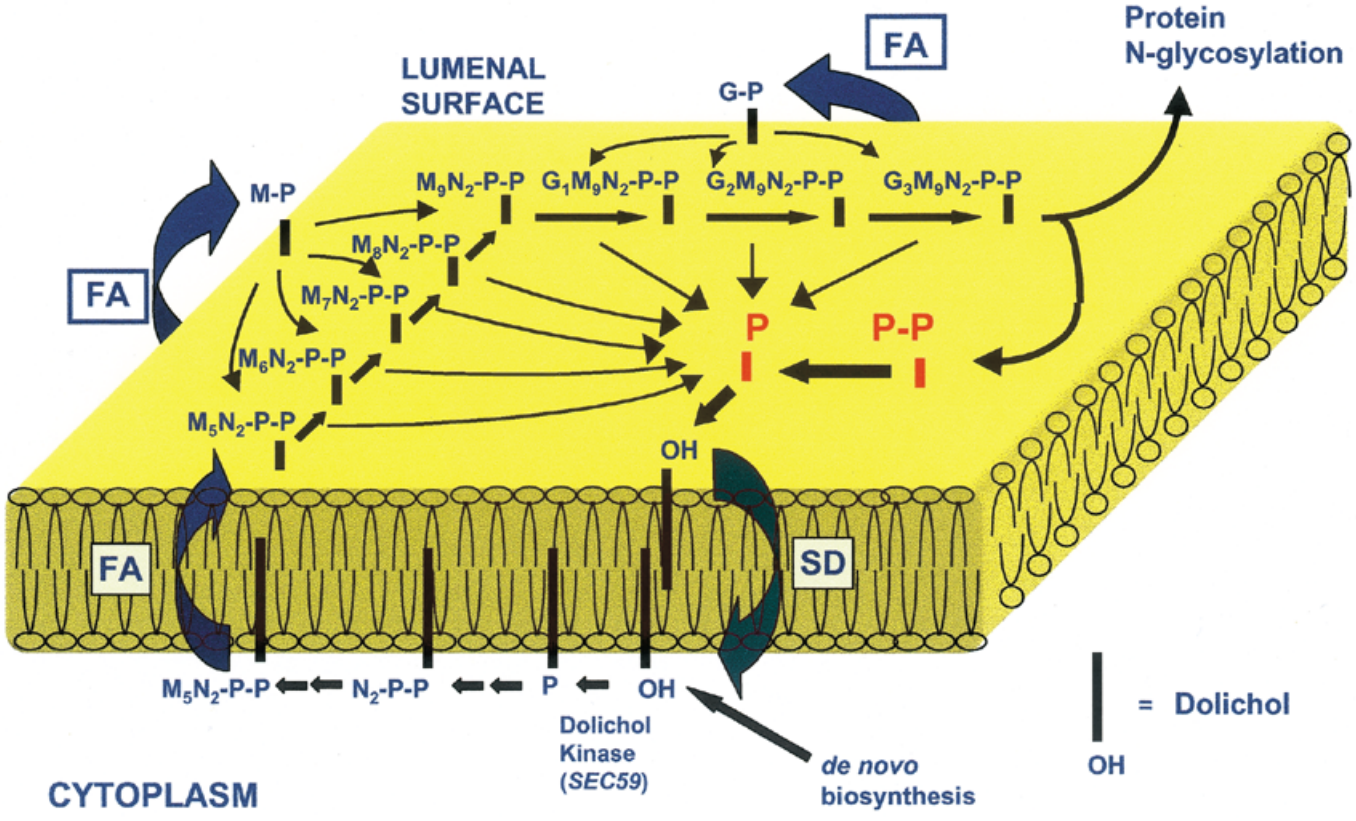


**Fig. 2.** Topological model for lipid intermediate synthesis, translocation and the proposed role for Dol-P-P/Dol-P phosphatases in the recycling of Dol-P-P/Dol-P in the ER. FA = flippase assisted; SD = simple diffusion; N = N-acetylglucosamine; M = mannose; G = glucose.

Hirschberg and Snider (1987). The impressive work of Snider and his co-workers made a major contribution to the principal features of this early model. Snider and colleagues determined the topological orientation of the active sites of most of the biosynthetic enzymes and the various intermediates primarily by assessing protease sensitivity of the active sites and the inextractability of ConA–lipid intermediate complexes in sealed microsomal vesicles. The Hirschberg and Snider review and related articles (Lennarz, 1987; Abeijon and Hirschberg, 1992) stimulated considerable interest in this critical aspect of the dolichol pathway and provided a clear perspective on the technical complications of these topological studies and their interpretation. In Figure 2, the previous model is extended to include the entry of F-P-P into the *de novo* biosynthesis of Dol-P on the cytoplasmic face of the ER, and the proposed recycling of Dol-P-P/Dol-P formed on the lumenal monolayer.

Newly synthesized Dol-P is available for the synthesis of Man-P-Dol, Glc-P-Dol and GlcNAc-P-P-Dol, the precursor for $Man_5GlcNAc_2$-P-P-Dol, on the cytoplasmic leaflet of the ER. In the second stage of the pathway, the synthesis of $Glc_3Man_9GlcNAc_2$-P-P-Dol is completed after these three intermediates diffuse transversely to the lumenal leaflet. Recent work by Lehrman and co-workers (Anand *et al.*, 2001) has established that the Lec35 gene product is involved in all of the lipid-mediated reactions in the second stage of the pathway.

The model in Figure 2 proposes that the transbilayer movement of the polar phosphoglycosyl head groups of these three intermediates is flippase-assisted. Different experimental approaches indicate that the "flip-flopping" of this type of polyisoprenoid glycolipid in pure lipid bilayers does not occur at a biologically significant rate (Hanover and Lennarz, 1979; McCloskey and Troy, 1980). Theoretical calculations also argue that these translocation events are thermodynamically unfavorable (Lennarz, 1987), but the existence of "flippases" operating in this pathway is still hypothetical. The transport of water-soluble analogues of Man-P-Dol (Rush and Waechter, 1995) and Glc-P-Dol (Rush *et al.*, 1998) into sealed ER vesicles has been shown to be mediated by membrane proteins that may be involved in the transbilayer movement of these intermediates.

As illustrated in Figure 2, seven Dol-Ps and one Dol-P-P are discharged during each round of $Glc_3Man_9GlcNAc_2$-P-P-Dol synthesis and protein N-glycosylation. It should be noted that additional Dol-P molecules are also formed on the lumenal surface as a result of reactions leading to GPI-anchor synthesis and protein O- and C-mannosylation (Table III). In addition to these biosynthetic reactions, Dol-P/Dol-P-P can also be produced on the lumenal leaflet by the hydrolysis of oligosaccharide-P-P-Dol intermediates (see excellent review by Moore, 1999). Thus, another major question open to speculation is if these Dol-P and Dol-P-P molecules are recycled and utilized again for additional rounds of lipid intermediate biosynthesis. If so, what enzymatic and protein-mediated translocation mechanisms are required for this scheme?

Here we suggest two plausible basic and testable sequences of events. First, Dol-P-P and Dol-P could be dephosphorylated on the lumenal surface, and the free polyisoprenol could diffuse transversely in an unassisted manner (Figure 2, SD). After returning to the cytoplasmic leaflet, Dol-P would be reformed by dolichol kinase. The *CWH8* gene product is a phosphatase candidate that could sequentially convert Dol-P-P to dolichol in yeast on the lumenal surface (see below).

Alternatively, the Dol-P-P and Dol-P discharged during the glycosylation reactions on the lumenal monolayer, could "flip-flop" to the cytoplasmic leaflet. The transbilayer movement of Dol-P-P and Dol-P would presumably occur by a protein-assisted (flippase) mechanism. The necessity of a flippase is suggested by studies conducted with spin-labeled analogues that indicate that the rate of unassisted transverse diffusion of polyisoprenyl monophosphates in lipid bilayers is extremely slow (McCloskey and Troy, 1980). In this scheme, a Dol-P-P pyrophosphatase with a cytoplasmically oriented active site would be required to regenerate Dol-P to be reutilized for lipid intermediate synthesis. The presence of an ER enzyme with an active site oriented toward the cytoplasm that is capable of converting Dol-P-P to Dol-P has not yet been conclusively demonstrated.

## Proposed roles for Dol-P-P/Dol-P phosphatase in the recycling model

The model for the recycling of Dol-P-(P) in Figure 2 clearly requires lipid phosphatases that can convert Dol-P-P to Dol-P and dephosphorylate Dol-P. These activities have been documented in microsomal fractions from yeast (Hosaka and Yamashita, 1984; Morlock *et al.*, 1991; Carman, 1997) and many mammalian tissues (Idoyaga-Vargas *et al.*, 1980; Wedgwood and Strominger, 1980; Appelkvist *et al.*, 1981; Burton *et al.*, 1981; Rip *et al.*, 1981; Belocopitow and Boscoboinik, 1982; Scher and Waechter, 1984; Frank and Waechter, 1998), but their structures, specificity, subcellular locations, and

**Table III.** Lipid-mediated reactions releasing Dol-P or Dol-P-P on the lumenal surface of the ER in yeasts and mammals

| Lipid-mediated reactions | Number of lumenally oriented Dol-P(P)s formed for each reaction sequence |
|---|---|
| $M_5N_2$-P-P-Dol → $G_3M_9N_2$-P-P-Dol | 7 |
| $G_3M_9N_2$-P-P-Dol → $G_3M_9N_2$-(Asn)Protein | 1 |
| GPI-anchor biosynthesis | 3 (mammals and trypanosomes)<br>4 (yeast) |
| Protein(Trp) C-mannosylation | 1 |
| Protein(Ser/Thr) O-mannosylation | 1 (yeast) |
| Hydrolysis of Oligo-P-P-Dol | 1 |

topological orientation of their active sites have not been rigorously characterized. One $Mg^{2+}$-independent lipid phosphatase that actively hydrolyzes Dol-P has been purified from pig brain and shown to be a 34-kDa membrane glycoprotein (Frank and Waechter, 1998). This enzyme also actively dephosphorylates phosphatidate, indicating that Dol-P may not be the primary physiological substrate *in vivo*. The *LPP1* and *DPP1* genes in *S. cerevisiae* encode $Mg^{2+}$-independent lipid phosphatases that are capable of converting Dol-P-P to Dol-P and dephosphorylating Dol-P (Faulkner *et al.*, 1999). Because disruption of these genes has no effect on growth, Dol-P levels or protein N-glycosylation, it is unlikely that these enzymes are responsible for the dephosphorylation of Dol-P-(P) *in vivo*.

It was reported by van Berkel *et al.* (1999) that the *CWH8* gene, which encodes an ER transmembrane protein, was required for normal levels of lipid intermediates and protein N-glycosylation in yeast. The presence of a phosphate-binding domain suggested that it could be involved in Dol-P-(P) metabolism.

Preliminary evidence has been obtained recently that the *CWH8* gene in yeast encodes a Dol-P-Pase that is a good candidate to be involved in the recycling model in Figure 2 (Rush *et al.*, 2000). In contrast to the lipid phosphatases encoded by *LPP1* and *DPP1*, the *CWH8* phosphatase is labile in Triton X-100 but can be readily assayed in 0.4% octylglucoside. Under these conditions Cwh8p actively hydrolyzes Dol-P-P, and to a lesser extent Dol-P. It is noteworthy that the phosphatase encoded by *CWH8* does not dephosphorylate phosphatidate or diacylglycerol diphosphate. Consistent with a role in the recycling model, it appears to have a chymotrypsin-sensitive site that is protected in sealed ER vesicles.

The recycling model described above is a variation of the model proposed by Wolf *et al.* (1991) in which Dol-P-P/Dol-P, which was transported to the Golgi compartment by bulk flow, would be dephosphorylated there and then rephosphorylated by dolichol kinase after being retrieved by the ER. This process could serve as a recovery mechanism for Dol-P-P/Dol-P that escapes the ER by vesicular transport.

## Potential recycling of Undec-P in prokaryotic cells by similar mechanisms

As noted above, the biosynthesis of numerous cell envelope components, including peptidoglycan, O-antigens, teichoic acids, and mannan in bacterial cells, involves polyisoprenoid glycosyl carrier lipids (Lennarz and Scher, 1972). In the assembly of peptidoglycan, Undec-P-P is discharged on the outer leaflet of the cytoplasmic membrane. Theoretically, the carrier lipid could be reutilized for another round of lipid intermediate biosynthesis after returning to the inner face of the cytoplasmic membrane as Undec-P-P, Undec-P, or the free polyprenol. As outlined above for the dolichol pathway, Undec-P-P could be completely dephosphorylated on the exterior face of the cytoplasmic membrane, allowing undecaprenol to diffuse transversely in an unassisted translocation event to the inner leaflet where it could be rephosphorylated by undecaprenol kinase (Sandermann and Strominger, 1971).

It is also formally possible that Undec-P-P could be translocated by a flippase-assisted mechanism, and then converted to Undec-P, the "active" form of the carrier lipid on the inner face of the cytoplasmic membrane by a specific pyrophosphate phosphatase. An Undec-P-P pyrophosphatase with a cytoplasmically oriented active site would also be necessary to complete the *de novo* synthesis of Undec-P from Undec-P-P in bacterial cells. Because premature cleavage of the nascent Poly-P-P intermediate would abort further chain elongation by the *cis*-IPTase, the recognition of the chain length of Undec-P-P by the pyrophosphate phosphatase is critical for the biosynthesis of the bacterial carrier lipid. Although all three of the required enzyme activities for these models have been documented (Sandermann and Strominger, 1971; Goldman and Strominger, 1972; Willoughby *et al.*, 1972), the specificity and topological orientation of the active sites have not been established definitively.

Similarly, there is evidence that approximately 48 Undec-Ps are formed on the exterior face of the cytoplasmic membrane in *M. luteus* as the membrane-associated mannan is elongated with Man-P-Undec serving as the mannosyl donor (Lennarz and Scher, 1972). In a slight variation of the mechanism for the recycling of Undec-P-P described above, Undec-P could be translocated by a flippase. Alternatively, Undec-P could be dephosphorylated on the exterior face of the cytoplasmic membrane and undecaprenol could diffuse without the assistance of a membrane protein to the inner leaflet and be rephosphorylated by undecaprenol kinase. In either case, the recycled Undec-P would be available to participate in another round of Man-P-Undec or peptidoglycan lipid intermediate synthesis.

The bacterial systems still offer great potential for learning more about fundamental molecular mechanisms for the recycling of Undec-P, providing new insight into similar processes in mammalian cells. With regard to bacterial cell envelope assembly, it will be interesting to see if the Wzx gene encodes the O-antigen translocase in *E. coli* as proposed by Feldman *et al.* (1999).

## Important goals for future studies

An attempt has been made to emphasize important questions remaining about the enzymology, regulation, and topology of Dol-P and lipid intermediate biosynthesis. Why the chain length of the polyisoprenoid glycosyl carrer lipids increased and reduction of the α-isoprene units occurred during evolution from prokaryotes to lower eukaryotes and finally to mammals remain intriguing questions. Determining how the gradual changes in chain length and appearance of saturated α-isoprene units of the polyisoprenols were required to achieve the biophysical properties required to fulfill the biochemical functions of the glycosyl carrier lipids will be challenging goals for future studies. A prospectus for work in this field should also include further investigation into the very interesting recent observation that mannosylphosphorylpolyisoprenols in mycobacterial cells are recognized by CD1c in T cells (Moody *et al.*, 2000).

It is almost shocking to realize that the dolichol pathway has been studied for over 30 years, and there is virtually nothing known about the hypothetical "flippases," or if they actually exist, that could facilitate the transbilayer movement of Man-P-Dol, Glc-P-Dol, and $Man_5GlcNAc_2$-P-P-Dol from the cytoplasmic leaflet to the lumenal monolayer in the ER. The possible mechanisms facilitating the transbilayer movement of lipid intermediates and the potential recycling of Dol-P to be

reutilized for additional rounds of lipid intermediate biosynthesis on the cytoplasmic leaflet of the ER certainly warrant intensive further investigation. It is very likely that answers to these difficult questions will require the application of a combination of biochemical, molecular biological and biophysical approaches.

Finally, we conclude with a cautionary note. The isolation and cloning of the corresponding cDNAs of the family of *alg* mutants in yeast has produced an enormous amount of important information about the structure of many enzymes in the lipid intermediate pathway (Huffaker and Robbins, 1983; Herscovics and Orlean, 1993; Burda and Aebi, 1999). The yeast mutants have also provided important structural information and a valuable system for identifying mammalian homologues and related mutants in CDG patients (Korner *et al.*, 1998; Tomita *et al.*, 1998; Imbach *et al.*, 1999, 2000; Takahashi *et al.*, 2000;). However, the structure of Man-P-Dol synthase in mammalian cells (Tomita *et al.*, 1998; Maeda *et al.*, 1998, 2000) is more complex than in yeast, and the Lec35 gene product, which has no apparent homologue in yeast, plays a key role in the utilization of Man-P-Dol and Glc-P-Dol in mammalian cells (Anand *et al.*, 2001). Thus, these and perhaps other more elaborate mechanisms may have evolved that will require further studies in mammalian systems to be elucidated.

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## Abbreviations

CHO, Chinese hamster ovary; Dol-P, dolichyl monophosphate; ER, endoplasmic reticulum; F-P-P, farnesyl pyrophosphate; GPI, glycosylphosphatidylinositol; GPT, UDP-GlcNAc:Dol-P N-acetylglucosaminyl 1-P transferase; I-P-P, isopentenyl pyrophosphate; Poly-P-P, polyprenyl pyrophosphate; Undec-P, undecaprenyl monophosphate; UPR, unfolded protein response; cis-IPTase, cis-isoprenyltransferase.

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