Transbilayer movement of Glc-P-dolichol and its function as a glucosyl donor: protein-mediated transport of a water-soluble analog into sealed ER vesicles from pig brain

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The results described in the accompanying article support the model in which glucosylphosphoryldolichol (Glc-P-Dol) is synthesized on the cytoplasmic face of the ER, and functions as a glucosyl donor for three Glc-P-Dol:Glc\(\beta\)Man\(\beta\)-GlcNAc\(\beta\)-P-P-Dol glucosyltransferases (GlcTases) in the luminal compartment. In this study, the enzymatic synthesis and structural characterization by NMR and electrospray-ionization tandem mass spectrometry of a series of water-soluble β-Glc-P-Dol analogs containing 2–4 isoprene units with either the cis- or trans-stereoconfiguration in the β-position are described. The water-soluble analogs were (1) used to examine the stereospecificity of the Glc-P-Dol:Glc\(\beta\)Man\(\beta\)-GlcNAc\(\beta\)-P-P-Dol glucosyltransferases (GlcTases) and (2) tested as potential substrates for a membrane protein(s) mediating the transbilayer movement of Glc-P-Dol in sealed ER vesicles from rat liver and pig brain. The Glc-P-Dol–mediated GlcTases in pig brain microsomes utilized \([\beta^3\text{H}]\text{Glc}-\)labeled Glc-P-Dol\(_{10}\), Glc-P-(\(\alpha\),\(c\))Dol\(_{15}\), Glc-P-(\(\alpha\),\(t\))Dol\(_{20}\), and Glc-P-(\(\alpha\),\(c\))Dol\(_{20}\) as glucosyl donors with \([\beta^3\text{H}]\text{Glc}\)\(\beta\)Man\(\beta\)-GlcNAc\(\beta\)-P-P-Dol as the major product labeled \textit{in vitro}. A preference was exhibited for C15–20 substrates containing an internal \textit{cis}-isoprene unit in the β-position. In addition, the water-soluble analog, Glc-P-Dol\(_{10}\), was shown to enter the luminal compartment of sealed microsomal vesicles from rat liver and pig brain via a protein-mediated transport system enriched in the ER. The properties of the ER transport system have been characterized. Glc-P-Dol\(_{10}\) was not transported into or adsorbed by synthetic PC-liposomes or bovine erythrocytes. The results of these studies indicate that (1) the internal \textit{cis}-isoprene units are important for the utilization of Glc-P-Dol as a glucosyl donor and (2) the transport of the water-soluble analog may provide an experimental approach to assay the hypothetical “flippase” proposed to mediate the transbilayer movement of Glc-P-Dol from the cytoplasmic face of the ER to the luminal monolayer.

**Key words:** Glc-P-Dol synthesis/ER/“flippase”

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**Introduction**

Glucosylphosphoryldolichol (Glc-P-Dol) functions as the direct glucosyl donor for the synthesis of the triglucosyl cap of Glc\(\beta\)Man\(\beta\)GlcNAc\(\beta\)-P-P-Dol, the precursor oligosaccharide donor in the eukaryotic N-glycosylation pathway (Kornfeld and Kornfeld, 1985; Hirschberg and Snider, 1987; Waechter, 1989; Cummings, 1992). The results of the preceding study (Rush and Waechter, 1998) support the topological model proposed by Hirschberg and Snider (1987) in which Glc-P-Dol is synthesized on the cytosolic face of the ER and then functions as the glucosyl donor for three lipid-mediated glycosylation reactions forming the terminal triglucosyl cap of the dolichol-bound precursor oligosaccharide on the luminal side of the ER in pig brain.

In view of evidence obtained from different experimental approaches indicating that the unassisted transbilayer movement of polysisoprenol-linked sugars in synthetic liposomes is extremely slow (Hanover and Lemmarz, 1978; McCloskey and Troy, 1980), it is quite possible that the transverse diffusion of Man\(\beta\)GlcNAc\(\beta\)-P-P-Dol (Flippase I), Man-P-P-Dol (Flippase II), and Glc-P-P-Dol (Flippase III) from the site of synthesis on the cytoplasmic face to the luminal leaflet is mediated by ER membrane proteins (Figure 1). We have previously used mannosphosphorylcitronellol (Man-P-P-Dol\(_{10}\)), a water-soluble analog of mannosylphosphoryldolichol (Man-P-Dol), to demonstrate the presence of a stereoselective ER protein(s) that facilitated the transbilayer movement of the analog in sealed microsomal vesicles from liver (Rush and Waechter, 1995).

To extend this approach to investigate membrane proteins that might play a role in the transbilayer movement of Glc-P-Dol in the ER, we have enzymatically synthesized a series of water-soluble analogs of Glc-P-Dol, by incubating hen oviduct microsomes with UDP-glucose and stereochemically defined, short-chain dolichyl monophosphates (Dol-P). The short chain Dol-Ps were synthesized chemically by the procedures developed by Jaenicke and colleagues (Jaenicke and Siegmund, 1986, 1989; Jaenicke et al., 1991; Berendes and Jaenicke, 1992).

To evaluate their potential to be recognized by a membrane protein facilitating the transverse diffusion of Glc-P-Dol, the water-soluble analogs were tested as substrates for the pig brain Glc-P-Dol:Glc\(\beta\)Man\(\beta\)-GlcNAc\(\beta\)-P-P-Dol glucosyltransferases (GlcTases). These reactions are believed to be catalyzed by three separate glucosyltransferases (Runge et al., 1984; Runge and Robbins, 1986; D’Souza-Schorey and Elbein, 1993; Stagljar et al., 1994; Zufferey et al., 1995). This enzymological comparison was conducted to determine if they were effective glucosyl donors, and to determine if the \textit{cis}-isoprene unit in the β-position, as well as the saturated \(\alpha\)-isoprene unit (D’Souza-Schorey et al., 1994), was recognized by the GlcTases. All of the water-soluble Glc-P-Dol analogs were found to be utilized as substrates by the Glc-P-Dol-mediated GlcTases with a slight preference shown for the presence of a \textit{cis}-isoprene unit in the β-position.
Since the water-soluble analogs were utilized as glucosyl donors by the GlcTases, it was plausible that their structural resemblance to Glc-P-Dol may also be sufficient for recognition by the hypothetical Glc-P-Dol flippase (III) proposed in Figure 1. To address this question, the transport of Glc-P-Dol$_{10}$ by sealed microsomal vesicles from rat liver and pig brain was assessed. The results of the transport studies document the presence of a membrane protein(s) that mediates the transbilayer movement of Glc-P-Dol$_{10}$ into sealed microsomal vesicles from rat liver and pig brain. The potential use of this transport system as an in vitro assay for a membrane protein involved in the "flip-flopping" of Glc-P-Dol in the ER is discussed. Parts of this study were reported in preliminary form (Rush et al., 1997a).

**Results**

**Enzymatic synthesis and structural characterization of short-chain, water-soluble analogs of Glc-P-Dol**

The results in the preceding article (Rush and Waechter, 1998) are consistent with the topological model of Hirschberg and Snider (1987) in which Glc-P-Dol functions as a glucosyl donor for the synthesis of Glc$_2$Man$_2$GlcNAc$_2$-P-Dol in the ER after diffusing transversely ("flip-flopping") from the site of synthesis on the cytoplasmic face. It is therefore possible that the transbilayer movement of the polar headgroup of the glucolipid is mediated by an ER protein (Flippase III, Figure 1).

In a previous study, Man-P-Dol$_{10}$ was used as a substrate for transport studies to detect ER protein(s) mediating the transmembrane movement of Man-P-Dol (Rush and Waechter, 1995). To extend the use of water-soluble analogs as a potential approach to assay the hypothetical "flippases," $[^{3}$H]$\text{Glc-P-citronellol}$ (Glc-P-Dol$_{10}$) and other short-chain stereoisomers of Glc-P-Dol have been synthesized enzymatically by incubating hen oviduct microsomes with UDP-$[^{3}$H]$\text{glucose}$ and the appropriate Dol-P were liberated by mild acid hydrolysis (0.05 N HCl in 50% isopropanol, 50°C, 60 min).

Finally, the predicted molecular weights of the analogs and anomic configuration of the glucosyl 1-phosphate bonds were confirmed by electrospray-ionization tandem mass spectrometry (Figure 2, Table I). The product ion spectrum of the deprotonated [M - H]$^-$ molecule at m/z 397 for Glc-P-Dol$_{10}$ contained a prominent fragment ion at m/z 235 (relative intensity, 64%) corresponding to [Dol$_{10}$HPO$_4$$^-$$^-$]. The major fragment ion at m/z 235 arises from a fragmentation pathway characteristic of glycosyl-P-polyspirooligos with trans-hydroxyls at the 1 and 2 carbons of the glucosyl residue (Wolucka et al., 1996, 1998). Hexosyl-P-dolichols with cis-hydroxyls at the 1 and 2 positions yield prominent fragment ions corresponding to [Dol$_{10}$PO$_4$(C$_2$H$_3$O)]$^-$ generated by cleavage across the hexose ring and to [M - H$_2$O - H]$^-$ dehydration products. The absence of the [Dol$_{10}$PO$_4$(C$_2$H$_3$O)]$^-$ ion (m/z 277) and of the [M - H$_2$O - H]$^-$ (relative intensity 0.5%), dehydration product (m/z 379) (Figure 2, Table I), confirms the structure of the enzymatic product as $\beta$-Glc-P-Dol$_{10}$.

The deprotonated [M - H]$^-$ molecules observed in the electrospray-ionization spectra of the water-soluble Glc-P-dolichols were m/z 465 for Glc-P-(t,c)$\text{Dol}_{15}$ and m/z 533 for Glc-P-(t,t)$\text{Dol}_{20}$ and Glc-P-(c,t)$\text{Dol}_{20}$ (Table I). The only prominent product ions observed were m/z 303 (relative intensity, 100%) for Glc-P-(t,c)$\text{Dol}_{15}$ and m/z 371 for Glc-P-(t,t)$\text{Dol}_{20}$ and Glc-P-(c,t)$\text{Dol}_{20}$ (relative intensity, 100%), and corresponded to the respective dolichyl phosphates. The relatively minor product ions arising from the respective [Dol$_{10}$PO$_4$(C$_2$H$_3$O)]$^-$ (relative intensities $\leq$ 5%) and the absence of the [M - H$_2$O - H]$^-$ dehydration products in the tandem mass spectrometry of the water-soluble Glc-P-Dols, confirm that the compounds are $\beta$-stereoisomers.

The structural relationships between Glc-P-Dol$_{95}$ and the water-soluble analogs, Glc-P-Dol$_{10}$, Glc-P-(t,c)$\text{Dol}_{15}$, Glc-P-(t,t)$\text{Dol}_{20}$ and Glc-P-(c,t)$\text{Dol}_{20}$, are illustrated in Figure 3. The water-soluble analogs contain an $\alpha$-isoprene unit and the reduced $\alpha$-isoprene, characteristic of dolichols, but are considerably less hydrophobic due to lacking 15–17 of the intervening isoprene units found in mammalian C95-dolichol.
Table I. Negative-ion electrospray-ionization tandem mass spectrometry of water-soluble Glc-P-dolichols

<table>
<thead>
<tr>
<th>Compound (MW)</th>
<th>[M - H2O - H]</th>
<th>[Dol-HPO4]</th>
<th>[Dol-PO4-(C2H3O)]</th>
<th>[PO3]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc-P-Dol 10</td>
<td>–</td>
<td>64</td>
<td>–</td>
<td>7</td>
</tr>
<tr>
<td>(398)</td>
<td>(379)</td>
<td>(235)</td>
<td>(277)</td>
<td>(79)</td>
</tr>
<tr>
<td>(ω,c)Glc-P-Dol 15</td>
<td>–</td>
<td>100</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>(466)</td>
<td>(447)</td>
<td>(303)</td>
<td>(345)</td>
<td>(79)</td>
</tr>
<tr>
<td>(ω,t,t)Glc-P-Dol 20</td>
<td>–</td>
<td>100</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>(534)</td>
<td>(515)</td>
<td>(371)</td>
<td>(413)</td>
<td>(79)</td>
</tr>
<tr>
<td>(ω,t,c)Glc-P-Dol 20</td>
<td>–</td>
<td>100</td>
<td>4</td>
<td>13</td>
</tr>
</tbody>
</table>

Negative-ion electrospray-ionization tandem mass spectra were collected as described in Materials and methods. Only signals of relative abundances greater than 2% are given. MW, Molecular weight of each analog is given in parentheses.

*Relative intensities of fragments (m/z) obtained from the [M - H] – deprotonated molecule at 20 V collision-offset voltage, %

Fig. 3. Structural relationship between short-chain water-soluble analogs and Glc-P-Dol95.

**Enzymatic transfer of [3H]glucose from the water-soluble analogs of Glc-P-Dol into [3H]Glc3Man9GlcNAc2-P-P-Dol in pig brain microsomes**

To verify that the structural features of the water-soluble analogs were recognized by proteins or enzymes interacting with Glc-P-Dol, their ability to serve as glucosyl donors for the pig brain microsomal GlcTase(s) catalyzing the addition of the three terminal glucose units in Glc3Man9GlcNAc2-P-P-Dol synthesis was tested. When [3H]Glc-labeled Glc-P-Dol 10, Glc-P-(ω,c)Dol 15, Glc-P-(ω,t,t)Dol 20, and Glc-P-(ω,t,c)Dol 20 were incubated with pig brain microsomes, [3H]glucose was transferred to endogenous Glc0–2 Man9GlcNAc2-P-P-Dol acceptor substrates (Figure 4). The enzymatic properties of the GlcTase(s) utilizing the water-soluble Glc-P-Dols as substrates were similar to the properties observed for the activities utilizing the natural long-chain (C95) glucosyl donor (Waechtert and Scher, 1978).

A comparison using the various Glc-P-Dols as substrates indicated that the rate of glucosylation and the affinity of the Glc-P-Dol–mediated GlcTases for the glucosyl donors increased when the isoprenoid chain was increased in length from 2 to 3 isoprene units (Figure 4). Increasing the chain length of the isoprenoid moiety by the addition of a fourth isoprene unit had only a minor effect on both the maximal velocity and the affinity of the GlcTases for the glucosyl donors. Furthermore, Glc-P-(ω,c)Dol 15 and Glc-P-(ω,t,t)Dol 20 (Figure 4, solid circles and open triangles), which contain internal cis-isoprene units in the β-position, were slightly better substrates than Glc-P-(ω,t,c)Dol 20 (Figure 4, open squares).

Fig. 4. Comparison of short chain, water-soluble analogs of Glc-P-Dol as substrates for Glc-P-Dol:Glc0–2 Man9GlcNAc2-P-P-Dol GlcTase activity in pig brain microsomes. Reaction mixtures for the assay of the lipid-mediated GlcTases contained 50 mM Tris–HCl (pH 7.4), 0.075 mM sucrose, 10 mM EDTA, 0.75 mg of pig brain microsomal protein and the indicated concentration of [3H]Glc-P-Dol 10 (open circles), [3H]Glc-P-(ω,c)Dol 15 (solid circles), [3H]Glc-P-(ω,t,t)Dol 20 (open squares), or [3H]Glc-P-(ω,t,c)Dol 20 (open triangles). Initial enzymatic rates were determined as described in Materials and methods and the data were analyzed by linear regression using the Sigma Plot Scientific Graph System 4.1 (Jandel Scientific, Corte Madera, CA).

The lipid-bound oligosaccharides synthesized by pig brain microsomes during incubation with the water-soluble [3H]Glc-P-Dols were sensitive to mild acid hydrolysis (Lucas et al., 1975) releasing [3H]oligosaccharides with chromatographic properties identical to authentic Glc1–3Man9GlcNAc2. The major enzymatic product formed in the Glc-P-Dol:Glc0–2Man9GlcNAc2-P-P-Dol
Fig. 5. Characterization of lipid-linked [3H-Glc]oligosaccharides synthesized in GlcTase reactions. The free [3H]oligosaccharides were released from the enzymatic products formed with either Glc-P-Dol 10 (A), Glc-P-(ω,c)Dol 15 (B), Glc-P-(ω,t,c)Dol 20 (C), or Glc-P-(ω,t,t)Dol 20 (D) serving as the glucosyl donor and analyzed by high pH anion-exchange chromatography as described in Materials and methods. The sodium acetate gradient is depicted with the dotted trace in (A).

Sealed microsomal vesicles from rat liver and pig brain contain a transport system that facilitates the uptake of Glc-P-Dol_{10}

Since the water-soluble analogs were utilized as substrates by the brain GlcTase(s), it seemed reasonable that the same structural features might be recognized by a protein involved in the translocation of the Glc-P headgroup from the cytosolic face of the ER to the luminal compartment. Basically, the same experimental strategy reported for Man-P-Dol_{10} (Rush and Waechter, 1995) was followed as illustrated in Figure 6. As seen in Figure 7, β-[3H]Glc-P-Dol_{10} was transported into sealed microsomal vesicles from rat liver and pig brain. Uptake of the water-soluble analog reached a maximum value within 2 min at 19°C with ~3% of the radiolabeled Glc-P-Dol_{10} internalized. Assuming that Glc-P-Dol_{10} transport occurs by facilitated diffusion, an intravesicular volume of ~3 µl/mg membrane protein for rat liver microsomes can be calculated. This estimate is similar to a previous value calculated by Bishop and Bell (1985) based on diC4PC transport.

Due to the relatively higher rate of uptake observed with rat liver microsomes, these preparations were used to characterize the transport system more extensively. Several properties of the Glc-P-Dol_{10} uptake system were found to be similar to the Man-P-Dol_{10} transporter system (Rush and Waechter, 1995). First, Glc-P-Dol_{10} uptake requires an intact permeability barrier. The transport of the water-soluble analog (Figure 8, solid circles) is lost in close parallel with Man 6-P phosphatase latency (Figure 8, open circles). Approximately 50% of Glc-P-Dol_{10} transport activity was lost when intact rat liver vesicles were incubated with 0.1% (wt/vol) Triton X-100. In addition, uptake was saturable with respect to Glc-P-Dol_{10} concentration (Figure 9) with transport reaching a half-maximal rate at ~1 mM Glc-P-Dol_{10}. Vesicular integrity was unaffected by this concentration of Glc-P-Dol_{10}.

Uptake of the water-soluble analog is also apparently reversible since, approximately 85% of the internalized Glc-P-Dol_{10} is lost in a time-dependent manner when preloaded rat liver vesicles were diluted with 25 volumes of isotonic buffer (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose) at 20°C (data not included).

The effects of various ionophores were tested to determine if Glc-P-Dol_{10} transport required an electrochemical gradient, but no effects on the uptake system were seen when valinomycin, monensin, FCCP or CCCP were present. The transport of Glc-P-Dol_{10} was stimulated significantly (~2–3 fold) by the addition of 5–10 mM ATP, but not by the addition of the nonhydrolyzable analog, γ-S-ATP (data not included). Other nucleoside triphosphates as well as AMP and ADP were significantly less effective in stimulating Glc-P-Dol_{10}.

The specificity of the Glc-P-Dol_{10} transport system was investigated by testing a variety of structurally related compounds as potential competitive inhibitors of uptake. β-Methyl glucose, p-nitrophenyl-β-glucoside, UDP-glucose, glucose 1-P, glucose 6-P, glucose, and glucose 1,6-diphosphate had no appreciable effect on Glc-P-Dol_{10} uptake when added in a 100-fold molar excess. The failure of an excess of UDP-Glc and Glc 6-P to compete for transport is good evidence that Glc-P-Dol_{10} is not
Transbilayer movement of Glc-P-dolichol

**Fig. 7.** Time course for the uptake of $[^{3}H]$Glc-P-Dol$_{10}$ by sealed microsomal vesicles from rat liver and pig brain. Transport assay mixtures contained 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, liver, or brain microsomal vesicles (93 µg of membrane protein) and 0.1 mM $[^{3}H]$Glc-P-Dol$_{10}$ (25 c.p.m./pmol) in a total volume of 0.01 ml. Following incubation at 19°C for the indicated periods of time, the amount of radiolabeled Glc-P-Dol$_{10}$ transported into either rat liver (solid circles) or pig brain (open circles) microsomal vesicles was determined as described in Materials and methods.

**Fig. 8.** Uptake of $[^{3}H]$Glc-P-Dol$_{10}$ by sealed microsomes requires an intact permeability barrier. Transport assay mixtures contained 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, rat liver ER vesicles (290 µg membrane protein), 3 mM $[^{3}H]$Glc-P-Dol$_{10}$ (962 c.p.m./pmol), and the indicated concentration of Triton X-100 in a total volume of 0.02 ml. Man 6-P phosphatase reaction mixtures were identical to the transport reactions except that $[^{3}H]$Glc-P-Dol$_{10}$ was replaced by 1 mM $[^{3}H]$ Man 6-P. The fraction of latent Man 6-P phosphatase activity (open circles) at each detergent concentration, and the amount of radiolabeled Glc-P-Dol$_{10}$ (solid circles) transported into microsomal vesicles during a 30 s incubation at 23°C was determined as described in Materials and methods. The data were analyzed by nonlinear regression using the Marquardt-Levenberg algorithm provided by the Sigmaplot Scientific Graph System 4.1 (Jandel Scientific).

being nonspecifically transported by the transporters for these glucose-containing compounds. When the other short-chain Glc-P-Dol analogs used in the enzymological study described above were surveyed in the transport assay, only very modest differences were found in the rate of uptake. $[^{3}H]$Man-P-Dol$_{10}$ when added at a 100-fold excess reduced Glc-P-Dol$_{10}$ uptake by less than 30%, suggesting that Flippases II and III (Figure 1) could be separate ER proteins.

The requirement for a membrane protein(s) in Glc-P-Dol$_{10}$ uptake was examined further by testing the ability of the analog to associate with other bilayer systems. Under conditions that were optimal for uptake by liver microsomes, Glc-P-Dol$_{10}$ was actively transported by microsomal vesicles, but not by bovine ER vesicles.

**Fig. 9.** $[^{3}H]$Glc-P-Dol$_{10}$ transport system in sealed microsomal vesicles is saturable. Glc-P-Dol$_{10}$ transport assays contained 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, rat liver ER vesicles (93 µg membrane protein), and the indicated concentration of $[^{3}H]$Glc-P-Dol$_{10}$ (5.2 c.p.m./pmol) in a total volume of 0.01 ml. Following incubation for 30 s at 19°C, the uptake of $[^{3}H]$Glc-P-Dol$_{10}$ was determined as described in Materials and methods. An apparent $K_{m}$ for uptake of 1 mM was calculated from the double-reciprocal plot in the inset. The data were analyzed by second order nonlinear regression using the Marquardt-Levenberg algorithm provided by the SigmaPlot Scientific Graph System 4.1 (Jandel Scientific). The data in the inset were analyzed by linear regression using the Sigma Plot Scientific Graph System 4.1 (Jandel Scientific).
The analog did not adsorb to or enter synthetic PC-liposomes, indicating that it did not simply adsorb to the outer leaflet of the microsomal vesicles. Glucose was actively transported under the same incubation conditions by bovine erythrocytes, but not microsomal vesicles or PC liposomes (Figure 11B), confirming the integrity of the RBC transport system.

When Glc-P-Dol\textsubscript{10} transport activity was compared in a limited number of rat liver subcellular fractions, transport activity was found to be enriched in the ER, the site of lipid intermediate synthesis, relative to Golgi vesicles and intact mitochondria (Table II). Although the Golgi and mitochondrial preparations take up a measurable amount of Glc-P-Dol\textsubscript{10}, the transport activity present in these fractions can be accounted for by cross-contamination with ER vesicles, assessed by the Man 6-P phosphatase activity. The ER-enriched fraction, on the other hand, contains very low amounts of the other two marker enzyme activities, indicating that it is relatively free of Golgi vesicles or mitochondria. All of these results provide evidence for the presence of a membrane protein that facilitates the transbilayer movement of Glc-P-Dol\textsubscript{10} from the outside to the luminal compartment of sealed ER vesicles.

Transfer of \textsuperscript{3}Hglucose from \textsuperscript{3}H\textsubscript{β}[\textsuperscript{3}H]Glc-P-Dol\textsubscript{20} to \textsuperscript{3}H\textsubscript{Glc}\textsubscript{3}\textsubscript{Man}\textsubscript{9}\textsubscript{GlcNAc}\textsubscript{2}-\textsubscript{P-P-Dol in sealed microsomal vesicles from pig brain}

To prove more conclusively that \textsuperscript{3}H\textsubscript{β}[\textsuperscript{3}H]Glc-P-Dol\textsubscript{20} was transported into the luminal compartment of brain microsomal vesicles and not simply adsorbed or intercalated into the outer leaflet, an experiment was conducted to see if \textsuperscript{3}Hglucose was incorporated into \textsuperscript{3}H\textsubscript{Glc}\textsubscript{3}\textsubscript{Man}\textsubscript{9}\textsubscript{GlcNAc}\textsubscript{2}-\textsubscript{P-P-Dol when sealed vesicles were incubated with the water-soluble analog. To establish that the pig brain microsomes remained intact during the experiment, latency of the deoxynojirimycin-sensitive, processing glucosidase I/II activities was monitored as described in the preceding report (Rush and Waechter, 1998).

The data in Table III show that a significant amount of \textsuperscript{3}Hglucose was incorporated into \textsuperscript{3}H\textsubscript{Glc}\textsubscript{3}\textsubscript{Man}\textsubscript{9}\textsubscript{GlcNAc}\textsubscript{2}-\textsubscript{P-P-Dol when at least 97% of the vesicles are sealed. The enzymatically labeled oligosaccharide-lipid represented ~5% of the total amount of radioactivity associated with the sealed vesicles after incubation with \textsuperscript{3}H\textsubscript{β}[\textsuperscript{3}H]Glc-P-Dol\textsubscript{20}.
The details of the isolation of each fraction and the marker enzyme assays are presented in Materials and methods.

**Table II. β-[3H]Glc-P-Dol_{10} transport system is enriched in ER membrane fractions**

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Man 6-Pase activity (pmol/min/mg)</th>
<th>Gal-Tase activity (pmol/min/mg)</th>
<th>SDH activity (nmol/min/mg)</th>
<th>Glc-P-Dol_{10} uptake (pmol/min/mg)</th>
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<tr>
<td>ER</td>
<td>22</td>
<td>0.1</td>
<td>19.2</td>
<td>16.8</td>
</tr>
<tr>
<td>Golgi</td>
<td>12.6</td>
<td>1.8</td>
<td>24.9</td>
<td>6.6</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>3.7</td>
<td>0.1</td>
<td>153</td>
<td>2.4</td>
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</table>

The results in the preceding article (Rush and Waechter, 1998) are consistent with the topological model of Hirschberg and Snider (1987), in which Glc-P-Dol functions as a glucosyl donor for the synthesis of Glc_{3}Man_{6}GlcNAc_{2}-P-Dol in the luminal compartment of the ER after diffusing transversely (“flip-flopping”) from the site of synthesis on the cytoplasmic face. Thus, the transbilayer movement of Glc-P-Dol could be mediated by a “flippase” as proposed for phosphatidylcholine, phosphatidylserine, glucosylceramide, glycosyl-phosphatidylinositol anchor precursors, and other dolichol-linked intermediates of the N-glycosylation pathway (Hirschberg and Snider, 1987; Trotter and Voelker, 1994; Menon, 1996). Prokaryotic flippases are also thought to play a role in the assembly of the bacterial cell wall (Bugg and Brandish, 1994) and many other bacterial exopolysaccharides (McGrath and Osborn, 1991; Liu et al., 1996).

The utilization of the water-soluble analogs as substrates by the Glc-P-Dol-mediated GlcTases suggested that their structural resemblance to Glc-P-Dol might also be recognized by the hypothetical membrane protein involved in facilitating the transverse diffusion of the glucolipid (Figure 1). In order to develop an assay for this putative “flippase” activity, the transport of Glc-P-Dol_{10} was investigated in sealed ER-enriched vesicles from rat liver and pig brain. The results of this study demonstrate that Glc-P-Dol_{10} is rapidly transported into the luminal compartment of intact rat microsomal vesicles from liver and pig brain. Extensive studies with rat liver vesicles indicate that the water-soluble units by enzymes synthesizing glycosyl-P-polysisoprenols or utilizing the glycolipids as glycosyl donors is well-documented (Rush et al., 1993; Deluca et al., 1994; D'Souza-Schorey et al., 1994; Keam et al., 1994; Dotson et al., 1995, Rush et al., 1997b, and numerous references cited therein).
analogue is transported by a mechanism with the properties expected for a protein-mediated process. Transport of the water-soluble analogue was found to: (1) be time-dependent, (2) require an intact permeability barrier, (3) be saturable, (4) be inactivated by trypsin, and (5) be enriched in ER vesicles relative to Golgi vesicles and intact mitochondria. In addition, Glc-P-Dol10 was utilized as a glucosyl donor by the microsomal GlcTase(s) that transfer glucosyl residues from Glc-P-Dol to endogenous Glc0, 2-ManGlcnAc2-P-P-Dol acceptors in sealed pig brain vesicles, indicating that the water-soluble analogue is transported into the luminal compartment, and is not simply adsorbed to the outer leaflet of the microsomal vesicle.

Although the protein-mediated transport systems for the water-soluble analogues of Man-P-Dol and Glc-P-Dol are enriched in the ER where the "flipases" would be expected to be located, considerably more work will be required to prove conclusively that the analogs are, in fact, being transported by the same proteins facilitating the transbilayer movement of the lipid intermediates. So far, attempts to identify a mutant in yeast or mammalian cells to provide a genetic correlation between the transport protein and the Man-P-Dol or Glc-P-Dol "flipases" have been unsuccessful. The results to date are nevertheless encouraging, and efforts to isolate a mutant and to define biochemical correlates between the transport systems and factors required for the movement of the dolichol-linked sugars from the cytoplasmic leaflet to the luminal compartment are in progress.

Materials and methods

**Materials**

S-Citronellol, DE-52 cellulose, monensin, valinomycin, ionomycin, carbonylcyanide p-trifluoromethoxy-phenylhydrazone (FCCP), carbonylcyanide m-chlorophenylhydrazone (CCCP), ATP, and γ-S-ATP were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphorus trichloride oxide was obtained from Alfa Products (Danvers, MA). Trichloroacetanilide was obtained from Aldrich Chemical Co. (Milwaukee, WI). [U-14C]Glucose (360 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). UDP-[3H]glucose was synthesized enzymatically as described previously (Rush and Waechter, 1998). [2-3H]Man 6-P was synthesized enzymatically and purified as described previously (Rush and Waechter, 1992). Millipore HA (0.45 μm) filter disks were purchased from Baxter Scientific Products (Obetz, OH). Whatman GFC glass microfiber filter disks were obtained from American Scientific Products (McGaw Park, IL). Econosafe Liquid Scintillation counting cocktail is a product of Research Products International Corp. (Mount Prospect, IL). All other chemicals and reagents were purchased from standard commercial sources.

**Synthesis of (αc)Dol15, (αt.t)Dol30, and (αt.c)Dol30**

(αt.t)Dol30 and (αt.c)Dol30 were prepared as described previously (Jaenicke and Siegmund, 1989) using geranyl-4-tolyl sulfone and either 8-chloro-(6-c)-citronellylbenzyl ether or 8-chloro-(6-t)-citronellylbenzyl ether as building blocks for the synthesis. For the synthesis of (αt.c) Dol15, 1 g of (6-benzoyloxy-4-methyl-(c)-4-hexen-1-yl)triphenylphosphonium iodide, prepared according to Sato et al. (1983), was dissolved in methanol and shaken overnight at room temperature under a 35 psi hydrogen atmosphere in the presence of 100 mg PtO2. After filtration over Celite, the filtrate was concentrated under reduced pressure to yield 1 g of a yellowish oil that was directly used for the following Wittig reaction. The reaction product, (6-benzoyloxy-4-methyl-1-hexyl)-triphenylphosphonium iodide was dissolved in 5 ml anhydrous tetrahydrofuran and stirred for 10 min at -78°C after the addition of 1.16 ml (1.85 mmol, 1.1 eq.) of 1.6 M n-butyl-lithium in hexane. After 10 min, 0.235 g (1.1 eq.) of 6-methyl-5-hepten-2-one (Aldrich) was added dropwise and the reaction was stirred overnight at room temperature. The reaction mixture turned light brown, and TLC analysis indicated that no starting material was left. After the addition of saturated ammonium chloride solution, the mixture was decanted and extracted twice with ether. The combined organic layers were washed once with brine, then dried briefly over MgSO4 and concentrated under reduced pressure to give an oily solid, which was purified on a short path silica gel column equilibrated in hexane and eluted stepwise with 2%, then 3% ethyl acetate to yield 0.27 g of the benzyl ether of (αt.c) Dol15 (51%, based on phosphonium salt). The benzyl group was removed by adding 0.3 g (0.95 mmol) of the benzyl ether in 5 ml anhydrous tetrahydrofuran to a solution of 110 mg (4.77 mmol, 5 eq.) Na in NH3 at -78°C. After 30 min at -33°C (refluxing NH3), solid ammonium chloride was added until the blue color disappeared and ammonia was allowed to evaporate over a period of ~45 min. The residue was evaporated under reduced pressure and purified over a short path silica gel column with ethyl acetate/hexane (1:3) to yield 177 mg (82.7%) of a colorless oil. Progress of reactions was monitored by thin layer chromatography on silica gel in either ethyl acetate/hexane (1:9) for the Wittig reaction or ethyl acetate/hexane (1:2) for the removal of the benzyl group. Reaction products were analyzed by 1H-NMR and the trans/cis ratios of the double bonds were established by integration of the vinyl methyl shifts at 1.6 ppm according to Jaenicke and Siegmund (1989). (αt.c)Dol15, (αt.t)Dol30, and (αt.c)Dol30 were chemically phosphorylated as described below for citronellol.

**Synthesis and purification of short chain, water-soluble analogs of Glc-P-Dol**

Citronellyl phosphate was synthesized by phosphorylation of citronellol using trichloroacetanilide and phosphoric trichloride oxide (Danilov and Chojnacki, 1981) and purified as described previously (Rush et al., 1993). Enzymatic reactions for the synthesis of Glc-P-Dol10 contained 10 mM MgCl2, 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 2 mM Cit-P, 5 mM 2-mercaptoethanol, 1 mM sodium orthovanadate, 20–80 µM UDP-[3H]Glc (1–500 c.p.m./pmol), and hen oviduct microsomes (0.75 mg protein) in a total volume of 0.5 ml. Following incubation for 1 h at 37°C, the reaction was centrifuged (100,000 × g, 10 min) in a Beckman TL-100 tabletop ultracentrifuge and the supernatant removed. The resulting membrane pellet was washed two times with 0.25 ml of water. The supernatants were combined and loaded onto an 8 ml column of DE-52 cellulose. After rinsing the ion-exchange column with 5 column volumes of distilled water, Glc-P-Dol10 was eluted with a 50 ml gradient (0–0.5 M) of NH4HCO3. The fractions containing Glc-P-Dol10 were combined, concentrated by rotary evaporation under reduced pressure at 30°C and desalted by gel-filtration chromatography on a Bio-Gel P-2 column (1.5 cm × 40 cm) eluted with distilled water. The fractions containing Glc-P-Dol10 were again combined, concentrated and stored at -20°C until use.

Enzymatic reactions for the synthesis of (αc)-Glc-P-Dol15, (αt.t)-Glc-P-Dol30, and (αt.c)-Glc-P-Dol30 contained 10 mM MgCl2, 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM sodium orthovanadate, 20–80 µM UDP-[3H]Glc (5–500 c.p.m./pmol), hen oviduct microsomes...
(1–10 mg protein) and the appropriate isopropyl monophosphate substrate (0.2 mM) in a total volume of 0.5–4 ml. Following incubation for 2 h at 37°C, the reaction was stopped by the addition of 5 ml of CH₃OH. The precipitated protein was sedimented by centrifugation (1000 × g, 5 min) and the supernatant removed and placed on ice. The pellet was then resuspended twice with 2 ml of CHCl₃/CH₃OH/H₂O (10:10:3) and recentrifuged, and the supernatants were combined with the CH₃OH layer. The organic extract was then supplemented with additional CHCl₃ to give a final composition of CHCl₃/CH₃OH/H₂O (3:2:1) and mixed vigorously. The two phases were separated by a brief centrifugation and the aqueous (upper) layer was removed. The organic (lower) layer was then reextracted with 5 ml of CHCl₃/CH₃OH/H₂O (3:48:47). The aqueous layers were combined, evaporated to dryness under reduced pressure at 35°C by rotary evaporation, redissolved in 1 ml water, and transferred with two rinses to a glass centrifuge tube. This solution was then mixed with 1 ml of water-saturated butanol and centrifuged briefly. The butanol phase was collected and the aqueous phase was re-extracted with 1 ml of water-saturated butanol. The butanol phases were combined, washed once with butanol-saturated water, and dried under a stream of N₂. Contaminating phospholipids were next destroyed by deacetylation at 0°C, 30 min in toluene/CH₃OH (1:1) containing 0.1 M KOH. The reaction mixture was neutralized with glacial acetic acid and dried under a stream of N₂. The deacylated mixture was desalted by partitioning with water-saturated butanol, as described previously, and the Glc-P-Dols were again desalted by butanol/water partitioning and centrifuged briefly. The butanol phase was collected and the aqueous phase was re-extracted with 1 ml of water-saturated butanol. The butanol phases were combined, washed once with butanol-saturated water, and dried under a stream of N₂. The deacylated mixture was desalted by partitioning with water-saturated butanol, as described previously, and the Glc-P-Dols were purified by ion-exchange chromatography on DE-52 cellulose as described by Waechter and Scher (1981). Following purification by DE-52 cellulose chromatography, Glc-P-Dols were again desalted by butanol/water partitioning and purified further by preparative thin layer chromatography on Silica Gel G developed in CHCl₃/CH₃OH/H₂O/NH₄OH (pH 7.4), and the amount of radioactivity retained on the filter determined by a filtration assay as described by Bishop and Bell (1985). The diluted assay mixtures were quickly transferred to a chilled filtration manifold equipped with a Millipore HA (0.45 μm) filter disk and suction-filtered. The disks were then washed with an additional 10 ml of ice-cold 0.25 M sucrose, 10 ml Tris-HCl (pH 7.4). The Millipore filter was then transferred to a 20 ml scintillation vial and the amount of radioactivity retained on the filter determined by scintillation spectrometry in a Packard 2100TR Scintillation Spectrometer after the addition of 1.0 ml of 1% SDS and 10 ml of Econosafe liquid scintillation cocktail.

Assay of Glc-P-Dol₁₀ transport by microsomal fractions from rat liver or pig brain

Assay mixtures for the measurement of [³H]Glc-P-Dol₁₀ uptake contained 10 mM Tris·Cl (pH 7.4), 0.25 M sucrose, the appropriate concentration of [³H]Glc-P-Dol₁₀ (5–500 c.p.m./pmol) and brain or liver microsomes (150–500 µg membrane protein) in a total volume of 10–20 µl. Following incubation at 19–23°C for 30 sec, [³H]Glc-P-Dol₁₀ transport was stopped by the addition of 0.5 ml of ice-cold 0.25 M sucrose, 10 mM Tris·HCl (pH 7.4), and the amount of [³H]Glc-P-Dol₁₀ transported was determined by a filtration assay as described by Bishop and Bell (1985). The diluted assay mixtures were quickly transferred to a chilled filtration manifold equipped with a Millipore HA (0.45 μm) filter disk and suction-filtered. The disks were then washed with an additional 10 ml of ice-cold 0.25 M sucrose, 10 ml Tris-HCl (pH 7.4). Filtration was usually completed in less than 30 sec. The Millipore filter was then transferred to a 20 ml scintillation vial and the amount of radioactivity retained on the filter determined by scintillation spectrometry in a Packard 2100TR Scintillation Spectrometer after the addition of 1.0 ml of 1% SDS and 10 ml of Econosafe liquid scintillation cocktail.

Measurement of glucose transport by erythrocytes and liver ER-enriched microsomes

Glucose transport assay mixtures contained 10 mM Tris·HCl (pH 7.4), 0.25 M sucrose, 0.5 mM [¹⁴C]glucose (52 c.p.m./pmol) and the indicated amount of either bovine erythrocytes or microsomal vesicles from rat liver (0–120 nmol membrane phospholipid) in a total volume of 50 µl. Following incubation at 19–23°C for 1 min, transport reactions were diluted with 0.5 ml of ice-cold 10 mM Tris·HCl (pH 7.4), 0.25 M sucrose. Glucose transport was determined exactly as described above for Glc-P-Dol₁₀ except that Whatman GF/C glass-fiber filter disks were used for the measurement of transport by human erythrocytes.

Assessment of the integrity of ER-enriched and Golgi-enriched vesicles and mitochondria

The integrity of liver ER microsomes was determined by measuring Man 6-P phosphatase latency using [³H]Man 6-P as substrate (Rush and Waechter, 1992). The intactness of pig brain
microsomes was assessed by assaying for glucosidase I/II latency (Rush and Waechter, 1998). The integrity of rat liver Golgi vesicles was determined by measuring the latency of rat liver galactosyltransferase to proteolytic digestion with trypsin. Mitochondrial integrity was estimated by measuring the sensitivity of succinate-dependent reduction of potassium ferricyanide to inhibition by antimycin A as described by Klingenber (1979).

**Preparation of synthetic PC-liposomes**

Synthetic liposomes were prepared using liposome kit No. L-4012 (Sigma Chemical Co.) containing egg phosphatidylicholine, dicetylphtohosphate, and cholesterol in a molar ratio of 7:2:1. Liposomes were formed by sonication in 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose and dialyzed against the same solution overnight before use as described previously (Rush and Waechter, 1995).

**Measurement of uptake of [3H]Glc-P-Cit by synthetic PC-liposomes.**

Uptake of [3H]Glc-P-Cit by synthetic PC-liposomes was determined by gel filtration on a column (0.5 x 20 cm) of Sephacryl S-300 equilibrated in 10 mM Tris-HCl pH 8.0, 0.25 M sucrose as described previously (Rush and Waechter, 1995).

**Assay of Glc-P-Dol10-20, Glc2-Man2GlcNAc2-P-Dol GlcTase activity in sealed pig brain vesicles**

Incubation mixtures contained 50 mM Tris-HCl (pH 8), 0.2 M sucrose, 10 mM EDTA, 2 mg/ml Triton X-100, 0.75 mg of pig brain microsomal protein, and the indicated concentration of [3H]Glc-P-Dol10-20 (500 c.p.m./pmol) in a total volume of 0.1 ml. Following incubation for 2 min at 37°C the incorporation of [3H]glucose into Glc1-3Man3GlcNAc2-P-Dol was determined by a multiple extraction procedure (Waechter and Scher, 1978). For the study described in Table III, reaction mixtures containing 50 mM Tris–HCl (pH 8), 0.2 M sucrose, 10 mM EDTA, 0.75 mg of pig brain microsomal protein, and 0.2 μM [3H]Glc-P-Dol10 (500 c.p.m./pmol) in a total volume of 0.1 ml were incubated for 3 min at 37°C in the presence or absence of Triton X-100 (0.5 mg/ml).

**Characterization of lipid-linked [3H]oligosaccharide products formed in Glc-P-Dol-mediated GlcTase reactions**

The lipid-bound oligosaccharide products of the enzymatic reactions were released by mild acid hydrolysis at 50°C, 30 min, in 80% tetrahydrofuran containing 0.1 M HCl (Lucas et al., 1975). The hydrolysate was neutralized by the addition of 1 N NaOH, dried under N2, and desalted by gel-filtration chromatography on a Sephadex G-10 column (1.5 X 30 cm) equilibrated in distilled water. The desalted oligosaccharides were characterized by high pH anion-exchange chromatography using a Dionex BioLC HPAEC system equipped with an analytical CarboPac PA1 column (0.4 x 25 cm) equilibrated in 0.25 M NaOH and eluted with a sodium acetate gradient (0–200 mM, 35 min) at a flow rate of 1 ml/min (Cooper and Rohrer, 1994). Fractions of 0.5 ml were collected, neutralized with glacial acetic acid and analyzed for radioactivity by scintillation spectrometry in a Packard Tri-Carb 2100TR liquid scintillation spectrometer. The chain lengths of the enzymatically labeled oligosaccharides were determined by comparing their elution positions with defined oligosaccharides standards and an exogenously added internal standard with the composition Man3GlcNAc1.

**Analytic methods**

Protein concentrations were determined by the method of Rodriguez-Vico et al. (1989) using a protein assay reagent (BCA, Pierce, Rockford, IL). Lipid-phosphorus was determined by the method of Bartlett (1959).

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**Abbreviations**

Glc-P-Dol, glucosylphosphoryldolichol; Man-P-Dol, mannosylphosphoryldolichol; Man-P-Dol10, mannosylphosphorylketonellol; PBS, phosphate-buffered saline; GlcTase, Glc-P-Dol/Man2GlcNAc2-P-Dol glucosyltransferase; ER, endoplasmic reticulum; Dol-P, dolichyl phosphate; FCCP, carbonyl cyanide m-trifluoro- methoxy-phenylhydrazide; CCCP, carbonyl cyanide m-chlorophenylhydrazine.

**References**


