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:Glcα2Man9GlcNAc2-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:Glcα2Man9GlcNAc2-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 [3H]Glc-labeled Glc-P-Dol10, Glc-P-(α,β)Dol15, Glc-P-(α,β,β)Dol20, and Glc-P-(α,β,β,β)Dol20 as glucosyl donors with [3H]Glcα3Man9GlcNAc2-P-P-Dol as the major product labeled in vitro. A preference was exhibited for C15–20 substrates containing an internal cis-isoprene unit in the β-position. In addition, the water-soluble analog, Glc-P-Dol10, 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-Dol14 was not transported into or adsorbed by synthetic PC-liposomes or bovine erythrocytes. The results of these studies indicate that (1) the internal 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”

Introduction

Glucosylphosphoryldolichol (Glc-P-Dol) functions as the direct glucosyl donor for the synthesis of the triglucosyl cap of Glc1Man0GlcNAc2-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 polyisoprenol-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 Man9GlcNAc2-P-P-Dol (Filippase I), Man-P-Dol (Filippase II), and Glc-P-Dol (Filippase 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 mannosylphosphorylcitronellol (Man-P-Dol10), 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:Glcα2Man9GlcNAc2-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 cis-isoprene unit in the β-position, as well as the saturated α-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 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 flipase (III(522,204),(932,284)) proposed in Figure 1. To address this question, the transport of Glc-P-Dol into 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 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 מס锰GlcNAc 2 3-P-dolichol (Flipase I), Man-P-dolichol (Flipase II), and Glc-P-dolichol (Flipase III).

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 "flipfaxes," [3 H]Glc-citronellol (Glc-P-Dol 10) and other short-chain stereoisomers of Glc-P-Dol have been enzymatically synthesized enzymatically by incubating hen oviduct microsomes with UDP-[3 H]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 anomeric 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 [DolPO(1-CH(2)OH)HPO(4-)]. The major fragment ion at m/z 235 arises from a fragmentation pathway characteristic of glycosyl-P-polysoprenoids with trans-hydroxyls at the 1 and 2 carbons of the glycosyl residue (Wolucka et al., 1996, 1998). Hexosyl-P-dolichols with cis-hydroxyls at the 1 and 2 positions yield prominent fragment ions corresponding to [DolPO(1-CH(2)OH)2] generated by cleavage across the hexose ring and to [M - H2O - H] dehydration products. The absence of the [DolPO(2-CH(2)OH)2] (m/z 277) and of the [M - H2O - H] (relative intensity 0.5%) dehydration product (m/z 379) (Figure 2, Table I), confirms the structure of the enzymatic product as β-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-([α,α,c]Dol 15 and m/z 533 for Glc-P-([α,α,t,c]Dol 20 and Glc-P-([α,β,t,c]Dol 20 (Table I). The only prominent product ions observed were m/z 303 (relative intensity, 100%) for Glc-P-([α,α,c]Dol 15 and m/z 371 for Glc-P-([α,α,t]Dol 20 and Glc-P-([α,α,c]Dol 20 (relative intensity, 100%), and corresponded to the respective dolichyl phosphates. The relatively minor product ions arising from the respective [DolPO(1-CH(2)OH)2] (relative intensities ≤ 5%) and the absence of the [M - H2O - H] dehydration products in the tandem mass spectrometry of the water-soluble Glc-P-Dols, confirm that the compounds are β-stereoisomers.

The structural relationships between Glc-P-Dol 95 and the water-soluble analogs, Glc-P-Dol 10, Glc-P-([α,α]Dol 15, Glc-P-([α,α,c]Dol 20 and Glc-P-([α,α,t,c]Dol 20, are illustrated in Figure 3. The water-soluble analogs contain an α-isoprene unit and the reduced α-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>
<thead>
<tr>
<th>Table I</th>
<th>Relative Intensity %</th>
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<tbody>
<tr>
<td>90</td>
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<td>80</td>
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<td>10</td>
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Fig. 1. Hypothetical ER proteins proposed to mediate the transbilayer movement of Man-GlcNAc 2 3-P-Dolichol (Flipase I), Man-P-Dolichol (Flipase II), and Glc-P-Dolichol (Flipase III).

Fig. 2. Negative-ion electrospray-ionization tandem mass spectrometry of synthetic Glc-P-Dol 10. The product ion spectrum of the [M - H] deprotonated molecule.
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>[PO4]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc-P-Dol10</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-Dol15</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,ω)Glc-P-Dol20</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-Dol20</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, %

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-Dol10, Glc-P-(ω,c)Dol15, Glc-P-(ω,t,c)Dol20, and Glc-P-(ω,t,t)Dol20 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 identical to the properties observed for the activities utilizing the natural long-chain (C95) glucosyl donor (Waechter 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 isoprenyl 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)Dol15 and Glc-P-(ω,t,c)Dol20 (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,t)Dol20 (Figure 4, open squares).

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. 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

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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-Dol10 (A), Glc-P-(ω, c)Dol15 (B), Glc-P-(ω, t, c)Dol20 (C), or Glc-P-(ω, t, t)Dol20 (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).

GlcTase reactions using water-soluble Glc-P-Dols as substrates coeluted with Glc3Man9GlcNAc2 by high pH anion-exchange chromatography on a PA1 strong anion exchange column (Figure 5 a–d). Minor amounts of oligosaccharide products with 1–2 glucosyl residues were also present. These results show that at least the Glc-P-Dol-mediated GlcTase adding the third glucosyl unit, and probably all three, utilize the water-soluble analogs as glucosyl donors.

Sealed microsomal vesicles from rat liver and pig brain contain a transport system that facilitates the uptake of Glc-P-Dol10

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-Dol10 (Rush and Waechter, 1995) was followed as illustrated in Figure 6. As seen in Figure 7, β-[3H]Glc-P-Dol10 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-Dol10 internalized. Assuming that Glc-P-Dol10 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-Dol10 uptake system were found to be similar to the Man-P-Dol10 transporter system (Rush and Waechter, 1995). First, Glc-P-Dol10 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-Dol10 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-Dol10 concentration (Figure 9) with transport reaching a half-maximal rate at ∼1 mM Glc-P-Dol10. Vesicular integrity was unaffected by this concentration of Glc-P-Dol10.

Uptake of the water-soluble analog is also apparently reversible since, approximately 85% of the internalized Glc-P-Dol10 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-Dol10 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-Dol10 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-Dol10.

The specificity of the Glc-P-Dol10 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, and glucose 1,6-diphosphate had no appreciable effect on Glc-P-Dol10 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-Dol10 is not.

Fig. 6. Experimental strategy for assaying the transport of Glc-P-Dol10 in sealed ER vesicles.

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Fig. 7. Time course for the uptake of $\beta$-[3H]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 $\mu$g of membrane protein) and 0.1 mM $\beta$-[3H]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 $\beta$-[3H]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 $\mu$g membrane protein), 3 $\mu$M [3H]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 [3H]Glc-P-Dol$_{10}$ was replaced by 1 mM [3H] 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 (Jandel Scientific).

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 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. $\beta$-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.

Glc-P-Dol$_{10}$ transport system is protein-mediated and enriched in ER vesicles

To determine if Glc-P-Dol$_{10}$ uptake by rat liver ER microsomes involves a membrane protein, the effect of trypsin treatment on transport activity was evaluated. Glc-P-Dol$_{10}$ transport activity decreased in a time-dependent manner during incubation with trypsin added at 2.5 mg/ml (Figure 10, solid circles). Glc-P-Dol$_{10}$ uptake was not affected during control incubations in the absence of trypsin (open circles) or in which protease activity was blocked by a 10-fold excess of trypsin inhibitor (soy bean) (open triangles), excluding the possibility that the loss of uptake was due to nonspecific inactivation of the transport system. In addition, Man 6-P phosphatase latency (solid triangles) was unaffected during the incubation period at 19°C, indicating that the vesicular permeability barrier remained intact during the experiment.

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Fig. 10. Transport of $\beta$-[3H]Glc-P-Dol$_{10}$ into sealed microsomes is inactivated by exposure to trypsin. Glc-P-Dol$_{10}$ transport assays contained 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, rat liver ER vesicles (170 $\mu$g membrane protein), 3 $\mu$M $[^{3}$H]Glc-P-Dol$_{10}$ (962 c.p.m./pmol) in a total volume of 0.02 ml. Microsomal vesicles were preincubated without (open circles) or with trypsin (2.5 mg/ml) (solid circles) or the combination of trypsin and soybean trypsin inhibitor (25 mg/ml) (open triangles) for the indicated periods of time at 19°C. Following the preincubation period, Glc-P-Dol$_{10}$ uptake was assayed for 30 s at 19°C as described in Materials and methods. Vesicular integrity was unaffected by incubation with trypsin for up to 240 min (solid triangles).

Fig. 11. $\beta$-[3H]Glc-P-Dol$_{10}$ does not freely enter PC-liposomes or bovine red blood cells. Glc-P-Cit transport assay mixtures (A) contained 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 $\mu$M $[^{3}$H]Glc-P-Dol$_{10}$ (20 c.p.m./pmol), and the indicated amount of either rat liver microsomes (solid circles), bovine erythrocytes (open circles) or synthetic PC-liposomes (open triangles) in a total volume of 0.05 ml. Glucose transport assay mixtures (B) were identical except that 80 $\mu$M $[^{14}$C]glucose (52 c.p.m./pmol) was added instead of $[^{3}$H]Glc-P-Dol$_{10}$. Following incubation at 19°C for 1 min, the uptake of $[^{3}$H]Glc-P-Dol$_{10}$ or glucose was assayed as described in Materials and methods.

erythrocytes (Figure 11). 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$_{10}$ 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$_{10}$, 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$_{10}$ from the outside to the luminal compartment of sealed ER vesicles.

Transfer of $[^{3}$H]glucose from $\beta$-[3H]Glc-P-Dol$_{20}$ to $[^{3}$H]Glc$_3$Man$_9$GlcNAc$_2$-P-P-Dol in sealed microsomal vesicles from pig brain

To prove more conclusively that $\beta$-[3H]Glc-P-Dol$_{20}$ 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 $[^{3}$H]glucose was incorporated into $[^{3}$H]Glc$_3$Man$_9$GlcNAc$_2$-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 $[^{3}$H]glucose was incorporated into $[^{3}$H]Glc$_3$Man$_9$GlcNAc$_2$-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 $\beta$-[3H]Glc-P-Dol$_{20}$. 1200
Table II. \( \beta^4\)\([\text{H}]\text{Glc-P-Dol}_{10} \) transport system is enriched in ER membrane fractions

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Man 6-Pase activity (nmol/min/mg)</th>
<th>Gal-Tase activity (nmol/min/mg)</th>
<th>SDH activity (nmol/min/mg)</th>
<th>Glc-P-Dol(_{10} ) uptake (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<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>
</tr>
</tbody>
</table>

The details of the isolation of each fraction and the marker enzyme assays are presented in Materials and methods.

Table III. Incorporation of \([\text{H}]\text{glucose into} \ [\text{H}]\text{GlcMan}_{3}\text{GlcNAc}_{2}-\text{P-P-Dol when} \ [\text{H}]\text{Glc-P-Dol}_{20} \) is incubated with sealed vesicles from pig brain

<table>
<thead>
<tr>
<th>Additions</th>
<th>([\text{H}]\text{Glc incorporated into} \ [\text{H}]\text{oligo-P-P-Dol} ) (pmol/mg)</th>
<th>Glucosidase I/II activity (% of total activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.8</td>
<td>0.03</td>
</tr>
<tr>
<td>0.05% Triton X-100</td>
<td>2.8</td>
<td>85.4</td>
</tr>
</tbody>
</table>

Note: GlcTase and glucosidase I/II activities were measured in intact and Triton X-100 disrupted pig brain microsomes as described in Materials and methods. Glucosidase I/II activity is expressed as a percentage of the activity detected in pig brain vesicles in which the permeability barrier has been completely disrupted by the addition of 0.2% Triton X-100.

When 0.05% Triton X-100 was added, over 80% of the vesicles were unsealed, as judged by the loss of latent glucosidase I/II activity, but there was virtually no increase in the labeling of \([\text{H}]\text{GlcMan}_{3}\text{GlcNAc}_{2}-\text{P-P-Dol}, \) as would be expected if the labeling in the control experiment were due to the reaction of the water-soluble substrate with enzymes on the luminal surface exposed in a small fraction of disrupted vesicles. Although the absolute rates of incorporation were slightly lower, very similar results were obtained with \([\text{H}]\text{Glc-P-Dol}_{10} \). These results provide additional evidence that the water-soluble analogs are, indeed, transported into the luminal compartment of sealed microsomal vesicles.

Discussion

This article describes the enzymatic synthesis of water-soluble analogs of Glc-P-Dol containing short chain, stereochemically defined isoprenyl moieties. The structures of the water-soluble analogs have been thoroughly characterized by electrospray-ionization tandem mass spectrometry and analyses of their chemical and chromatographic properties. When \([\text{H}]\text{Glc-P-Dol}_{10}, \ [\text{H}]\text{Glc-P-}((\alpha\text{c})\text{Dol}_{15}, \ [\text{H}]\text{Glc-P-}((\omega\text{t})\text{Dol}_{20}, \) and \([\text{H}]\text{Glc-P-}((\omega\text{t},\text{c})\text{Dol}_{20}) \) were tested as substrates for the three GlcTases catalyzing the addition of the three glucosyl units in the triglucosyl cap of GlcMan_{3}GlcNAc_{2}-P-P-Dol to evaluate the recognition of the stereoconfiguration of the \( \beta \)-isoprene unit, the pig brain GlcTases actively transferred glucosyl units from each of the water-soluble analogs to endogenous Glc_{0,2}Man_{3}GlcNAc_{2}-P-P-Dol acceptors. Even considering the limitations of this pseudokinetic comparison of the three glucosyltransferase activities with the stereochemically defined substrates, it appears that the GlcTases recognize and prefer glucosyl donors containing a cis-isoprene unit in the \( \beta \)-position of the isoprenyl moiety as found in the natural substrate.

Discrimination between polisoprenyl moieties containing saturated (eukaryotes) or unsaturated (prokaryotes) \( \alpha \)-isoprene units by enzymes synthesizing glycosyl-P-polyisoprenols 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). The preference for Glc-P-Dols containing \( \beta \)-isoprene units of the cis-stereochemical configuration by the GlcTases extends the information on the structural features of the dolichyl moieties recognized by enzymes participating in polysaccharidoid lipid intermediate pathways. In a related study, all trans Dol-P was found to be inactive as a substrate for rat liver Glc-P-Dol synthase, whereas (\( \omega_3\text{t}_2\text{c}_2\))Dol-P and (\( \omega_2\text{t}_2\text{c}_2\))Dol-P are both efficiently glucosylated (Jaenicke et al., 1991). Conversely, other studies report that rat liver Glc-P-Dol synthase glucosylates \( \alpha \)-dihydro undecaprenyl-P (\( \omega_2\text{c}_2\text{t}_2\text{c}_2\)) and \( \alpha \)-dihydro solanosyl-P (\( \omega_2\text{c}_2\text{t}_2\text{c}_2\)) at virtually equal rates (Mankowski et al., 1975, 1977). In yeast, Man-P-Dol synthase utilizes \( \alpha \)-dihydro undecaprenyl-P (\( \omega_2\text{c}_2\text{t}_2\text{c}_2\)) and \( \alpha \)-dihydro solanosyl-P (\( \omega_2\text{t}_2\text{c}_2\text{t}_2\)) as substrates and the mannolipid products serve as mannolipid carriers for PMT1 (Pless and Palamarczyk, 1978; Palamarczyk et al., 1980). However, GPT1 exhibited a strong preference for substrates with a cis-isoprene unit in the \( \beta \)-position. The enzymes that catalyze the synthesis of the pyrophosphoryl-polyprenyl-linked trisaccharide of the S.\textit{anatum} O-specific polysaccharide also strongly prefer a polisoprenyl phosphate acceptor with a cis-isoprene unit in the \( \beta \)-position (Danilov et al., 1989).

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 glycosyl donor for the synthesis of GlcMan_{3}GlcNAc_{2}-P-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 “flipfase” as proposed for phosphatidylincholine, 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 flipases 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 “flipfase” 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
analog is transported by a mechanism with the properties expected for a protein-mediated process. Transport of the water-soluble analog 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-Dol\textsubscript{10} was utilized as a glucosyl donor by the microsomal GlcTase(s) that transfer glucosyl residues from Glc-P-Dol to endogenous Glc\textsubscript{0},2\textsubscript{Man}GlcNAc\textsubscript{2}-P-P-Dol acceptors in sealed pig brain vesicles, indicating that the water-soluble analog is transported into the lumenal compartment and is not simply adsorbed to the outer leaflet of the microsomal vesicle.

Although the protein-mediated transport systems for the water-soluble analogs of Man-P-Dol and Glc-P-Dol are enriched in the ER where the “flippases” 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 “flippases” 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 lumenal compartment are in progress.

Materials and methods
Materials
S-Citronellol, DE-52 cellulose, monensin, valinomycin, ionomycin, carbonylcyanide p-trifluoromethoxy-phenyldihydrazide (FCCP), carbonylcyanide m-chlorophenylhydrazide (CCCP), ATP, and γ-S-ATP were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphorus trichloride oxide was obtained from Sigma Chemical Co. (Mount Prospect, IL). All other chemicals and reagents were purchased from commercial sources. Analysis of Glc-P-Dol was performed as described previously (Jaenicke and Siegmund, 1989) using geranyl-4-tolyl sulfone and either 8-chloro-(6-t,t)-Dol\textsubscript{20} and (ω-t,t)-Dol\textsubscript{20}, or 8-chloro-(6-c,c)-Dol\textsubscript{20} and (ω-c,c)-Dol\textsubscript{20}.

Synthesis of (ω-c,c)-Dol\textsubscript{15}, (ω-t,t)-Dol\textsubscript{20}, and (ω-t,c)-Dol\textsubscript{20}
(ω-t,t)-Dol\textsubscript{20} and (ω-t,c)-Dol\textsubscript{20} were prepared as described previously (Jaenicke and Siegmund, 1989) using geranyl-4-tolyl sulfone and either 8-chloro-(6-c,c) citronellylbenzyl ether or 8-chloro-(6-t,t) citronellylbenzyl ether as building blocks for the synthesis. For the synthesis of (ω-c,c)-Dol\textsubscript{15}, 1 g of (6-benzyloxy-4-methyl-(c,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 PtO\textsubscript{2}. 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-benzyloxy-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 MgSO\textsubscript{4} 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 (ω-c,c)-Dol\textsubscript{15} (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 NH\textsubscript{3} at -78°C. After 30 min at -33°C (refluxing NH\textsubscript{3}), 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 \textsuperscript{1}H-NMR and the \textsuperscript{13}C-NMR of the double bonds were established by integration of the carbonyl signals. Citronellol phosphate was synthesized by phosphorylation of citronellol using trichloroacetonitrile and phosphorus trichloride oxide (Danilov and Chojnacki, 1981) and purified as described previously (Rush et al., 1993). Enzymatic reactions for the synthesis of Glc-P-Dol\textsubscript{10} contained 10 mM MgCl\textsubscript{2}, 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 2 mM CrP, 5 mM 2-mercaptoethanol, 1 mM sodium orthovanadate, 20–80 \mu M UDP-[\textsuperscript{3}H]Glc (1–500 c.p.m./pmol), and hen oviduct microsomes (0.75 mg protein/mg) in a total volume of 0.5 ml. Following incubation for 1 h at 37°C, the reaction was centrifuged (100,000 \times 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-Dol\textsubscript{10} was eluted with a 50 ml gradient (0–0.5 M) of NH\textsubscript{4}HCO\textsubscript{3}. The fractions containing Glc-P-Dol\textsubscript{10} 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 \times 40 cm) eluted with distilled water. The fractions containing Glc-P-Dol\textsubscript{10} were again combined, concentrated and stored at -20°C until use.

Enzymatic reactions for the synthesis of (ω-c,c)-Glc-P-Dol\textsubscript{15}, (ω-t,t)-Glc-P-Dol\textsubscript{20}, and (ω-t,c)-Glc-P-Dol\textsubscript{20} contained 10 mM MgCl\textsubscript{2}, 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 1 mM sodium orthovanadate, 20–80 \mu M UDP-[\textsuperscript{3}H]Glc (5–500 c.p.m./pmol), and hen oviduct microsomes (J.S. Rush et al.).

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(1–10 mg protein) and the appropriate isoprenyl 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 CH₂OH. 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/CH₂OH/H₂O (10:10:3) and recentrifuged, and the supernatants were combined with the CH₂OH layer. The organic extract was then supplemented with additional CHCl₃ to give a final composition of CHCl₃/CH₂OH/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/CH₂OH/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 decylation at 0°C, 30 min in toluene/CH₂OH (1:1) containing 0.1 M KOH. The reaction mixture was neutralized with glacial acetic acid and dried under a stream of N₂. The decylated 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 chromatoigraphy, 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/CH₂OH/H₂O/NH₄OH (pH 7.4), 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 Ecosafe 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 mM 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 Ecosafe liquid scintillation cocktail.
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 reduction dependent reaction of potassium ferricyanide to inhibition by antimycin A as described by Klingenborg (1979).

Preparation of synthetic PC-liposomes

Synthetic liposomes were prepared using liposome kit No. L-4012 (Sigma Chemical Co.) containing egg phosphatidylcholine, diethylphosphate, 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 $[^{3}H]$Glc-P-Cit by synthetic PC-liposomes

Uptake of $[^{3}H]$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-Dol$_{10}$:Glc$_{2}$Man$_{2}$GlcNAc$_{2}$-P-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 $[^{3}H]$Glc-P-Dol$_{10}$-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 $[^{3}H]$glucose into Glc$_{1-3}$Man$_{3}$GlcNAc$_{2}$-P-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 mM $[^{3}H]$Glc-P-Dol$_{20}$ (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 $[^{3}H]$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 N$_{2}$, 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 oligosaccharide standards and an exogenously added internal standard with the composition Man$_{n}$GlcNAc$_{1}$.

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, mannosyl-phosphoryldolichol; Man-P-Dol$_{10}$, mannosylphosphorylcitronellol; PBS, phosphate-buffered saline; GlcTase, Glc-P-Dol:Glc$_{2}$Man$_{3}$GlcNAc$_{2}$-P-P-Dol glucosyltransferase; ER, endoplasmic reticulum; Dol-P, dolichyl phosphate; FCCP, carbonylcyanide m-trifluoromethoxyphenoxylhydrazone; CCCP, carbonyl cyanide m-chlorophenoxylhydrazone.

References


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