Topography of Initiation of N-Glycosylation Reactions*

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Previous studies on the topography of the reactions leading to the formation of dolichol-P-P-GlcNAc2Man5Glc3 have shown that these occur on both sides of the endoplasmic reticulum membrane (Hirschberg, C. B., and Snider, M. D. (1987) Annu. Rev. Biochem. 56, 63–87). Dolichol-P-P-GlcNAc2Man5 has been detected on the cytoplasmic side of the endoplasmic reticulum membrane while the subsequent dolichol-oligosaccharide intermediates face the lumen. Less clear is the side of the membrane where dolichol-P-P-GlcNAc2 is assembled. We now present evidence strongly suggesting that the active sites of the enzymes catalyzing the synthesis of this latter intermediate are on the cytoplasmic side of the endoplasmic reticulum membrane. In addition, dolichol-P-P-GlcNAc2 has also been detected on this side.

Incubations of sealed, "right side out" rat liver endoplasmic reticulum-derived vesicles with [3-32P]UDP-GlcNAc in the presence of 5-Br-UMP resulted in the formation of radiolabeled dolichol-P-P-GlcNAc and dolichol-P-P-GlcNAc2 under conditions where there was complete inhibition of transport of the nucleotide sugar. In other experiments with the above radiolabeled nucleotide sugar and sealed vesicles, it was demonstrated that EDTA (a membrane-impermeable reagent) inhibited the N-acetylglucosamine-1-phosphate transferase under conditions where transport of the nucleotide sugar into the lumen was unaffected.

Finally, sealed vesicles were first incubated with [32P]UDP-GlcNAc and subsequently with UDP-Gal and soluble galactosyltransferase. This resulted in galactosylation of dolichol-P-P-GlcNAc2. The above results, together with the previous observations, strongly suggest that all reactions leading to this latter dolichol intermediate occur on the cytosolic side of the endoplasmic reticulum membrane.

The biosynthesis of precursors of protein N-glycosylation is initiated by the formation of dolichol-P-P-GlcNAc2. Subsequently, five mannoses are added yielding dolichol-P-P-GlcNAc2Man5. The immediate precursor sugars in these reactions are the corresponding nucleotide sugars. Thereafter, four mannoses and three glucoses are added to form dolichol-P-P-GlcNAc2Man5Glc5, the glycosylation donor to nascent polypeptide chains in the lumen of the rough endoplasmic reticulum (RER). The immediate sugar donors in these latter reactions are the corresponding dolichol monosaccharide derivatives; the sugars, in turn, are derived from the respective nucleotide sugars.

Studies on the topography of the previous reactions have shown that the biosynthesis of these dolichol derivatives occurs on both sides of the endoplasmic reticulum (ER) membrane. Dolichol-P-P-GlcNAcMan5 has been detected on the cytoplasmic side of the endoplasmic reticulum membrane while the subsequent dolichol intermediates, with additional mannoses and glucoses, face the luminal side (3, 4). Consistent with this has been the observation that the ER membrane is not permeable to GDP-mannose (5–7).

Less clear is the site of the endoplasmic reticulum membrane on which dolichol-P-P-GlcNAc and dolichol-P-P-GlcNAc2 are assembled. While there is no information on the topography of biosynthesis of dolichol-P-P-GlcNAc, proteolysis experiments have suggested that the active site of the enzyme leading to the formation of the subsequent intermediate, namely dolichol-P-P-GlcNAc2, faces the cytosolic side of the ER membrane (6, 8, 9). Attempts to localize this reaction product on the ER membrane, using a soluble galactosyltransferase and UDP-Gal as probes, showed that this could only be accomplished after the addition of small amounts of detergent to the membrane, suggesting that the acceptor was not facing the cytosolic side of the membrane (6, 10). Studies from our laboratory have shown that the UDP-GlcNAc, the immediate sugar precursor of dolichol-P-P-GlcNAc and dolichol-P-P-GlcNAc2, is transported across the endoplasmic reticulum membrane in a saturable manner suggesting a membrane carrier protein (6, 11). However, this observation does not answer whether this transport is related to the formation of these dolichol derivatives.

We now show that transport of UDP-GlcNAc into the lumen of the rough endoplasmic reticulum is unrelated to the transfer of the sugar phosphate and sugar alone to dolichol phosphate and dolichol-P-P-GlcNAc. In addition, the N-acetylglucosamine-1-phosphate transferase can be inhibited on the cytosolic side of the ER membrane while transport of UDP-GlcNAc into the lumen still occurs; this strongly suggests that the formation of dolichol-P-P-GlcNAc occurs on the cytosolic side of the endoplasmic reticulum membrane. Finally, we have also detected a significant portion of dolichol-P-P-GlcNAc2 on the cytoplasmic side of the RER membrane.

Materials and Methods

Radioactive Materials—The following radioactive materials (purchased from Du Pont–New England Nuclear) were used: UDP-[6–3H]GlcNAc (27 Ci/mmol), [3H]acetic acid, sodium salt (100 mCi/mmol), [3-32P]UDP-GlcNAc (6 Ci/mmol) was synthesized as previously described by Lang and Kornfeld (12).

Isolation of Rat Liver Rough Endoplasmic Reticulum-derived Vesicles—Rat liver RER vesicles were isolated as described previously by Carey and Hirschberg (13) using a modification of the method originally described by Fleischer and Kervina (14). The vesicles were enriched 4.6-fold over the homogenate in glucose-6-phosphatase spe-
Glycosylation in Rat Liver Rough Endoplasmic Reticulum

Theoretical basis for the assays to determine translocation of different nucleotide derivatives into RER and Golgi-derived vesicles has been previously described in detail (18). Briefly, it consists of (i) determining the total radioactive solutes associated with the RER pellet (S,) following centrifugation of the vesicles and (ii) subtracting from this amount the total radioactive solutes trapped in between the vesicles in the RER pellet (S,). This latter value is obtained by multiplying the volume per mg of protein outside the vesicles in the RER pellet (V,) by the concentration of radioactive substrates in the incubation medium. V, measured with a standard nonpenetrator such as \(^{3}H\)acetate and calculated to be 2.4 \(\mu\)l/mg of protein (18).

The pellet was surface-washed as described under Incubation Conditions and was resuspended in 0.5 ml of water by vigorous vortexing. Incubation was performed as described previously (18), 1-butanol, saturated with water (0.6 ml), followed by water, saturated with butanol (0.4 ml), were then added, and the suspension was centrifuged in a Microfuge for 2 min. The lower, aqueous phase was subjected to liquid scintillation spectrometry to determine the total solutes in the incubation medium. V, was measured with a standard nonpenetrator such as \(^{3}H\)acetate and calculated to be 2.4 \(\mu\)l/mg of protein (18).

Incubation Conditions—RER-derived vesicles (sealed or permeabilized) were incubated at a concentration of 1 mg of protein in 250 \(\mu\)l of buffer A with \(^{32}P\)-UDP-GlcNAc (8 Ci/mmol, 0.5 \(\mu\)M, final concentration) or \(^{3}H\)-UDP-GlcNAc (8 Ci/mmol, 1 \(\mu\)M, final concentration) for 2 min at 20\(^\circ\)C. When indicated, the incubation medium also contained 1 mM 5-BrdUMP or 5 mM EDTA and no MnCl\(_2\) and MgCl\(_2\), or 5 \(\mu\)l of tunicamycin (Calbiochem). The conditions and temperature and time were chosen such as to be in the linear range for transfer of GlcNAc phosphate and GlcNAc to endogenous dolichol phosphate. Following incubations, reactions were stopped by dilution with 1 ml of cold buffer A and centrifugation at 45,000 rpm for 30 min in a Ti 50 rotor (Beckman). The pellet was surface-washed as described previously (18), 1-butanol, saturated with water (0.6 ml), followed by water, saturated with butanol (0.4 ml), were then added, and the suspension was centrifuged in a Microfuge for 2 min. The lower, aqueous phase was subjected to liquid scintillation spectrometry to determine the total solutes in the incubation medium. V, was measured with a standard nonpenetrator such as \(^{3}H\)acetate and calculated to be 2.4 \(\mu\)l/mg of protein (18).

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Incubations of RER vesicles with \(^{3}H\)-UDP-GlcNAc

In control incubations as described previously (18), 1-butanol, saturated with water (0.6 ml), followed by water, saturated with butanol (0.4 ml), were then added, and the suspension was centrifuged in a Microfuge for 2 min. The lower, aqueous phase was subjected to liquid scintillation spectrometry to determine the total solutes in the incubation medium. V, was measured with a standard nonpenetrator such as \(^{3}H\)acetate and calculated to be 2.4 \(\mu\)l/mg of protein (18).

Table I

<table>
<thead>
<tr>
<th>Additions</th>
<th>UDP-GlcNAc transport</th>
<th>GlcNAc transferred to endogenous dolichol-P</th>
<th>pmol/mg protein (\times 10^4)</th>
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<tr>
<td>Closed vesicles</td>
<td>Permeabilized vesicles</td>
<td>Closed vesicles</td>
<td>Permeabilized vesicles</td>
</tr>
<tr>
<td>None</td>
<td>1240</td>
<td>0</td>
<td>121</td>
</tr>
<tr>
<td>5-BrdUMP</td>
<td>10</td>
<td>50</td>
<td>120</td>
</tr>
<tr>
<td>EDTA</td>
<td>1260</td>
<td>20</td>
<td>6</td>
</tr>
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</table>
molecule. This latter result in consistent with the extremely low level of contamination of the RER vesicles by Golgi membranes as determined by sialyltransferase specific activity and the fact that the GlcNAc-1-P transferase, which is responsible for the biosynthesis of the lysosomal enzyme recognition marker, is a Golgi enzyme (21, 22).

Table II shows that virtually the same results were obtained when incubations were done with UDP-[3H]GlcNAc instead of [β-32P]UDP-GlcNAc. With the former radiolabeled nucleotide sugar one can also determine the amount of radiolabeled acceptors. The presence of 5-BrdUMP resulted in approximately 75% inhibition of transfer of sugar to endogenous protein acceptors; the residual transfer to endogenous protein acceptors was very similar to the value obtained when permeabilized vesicles were incubated with UDP-[3H]GlcNAc in the absence or presence of 5-BrdUMP. These results suggest that the majority of the GlcNAc transfer to protein is dependent on UDP-GlcNAc transport.

The apparent $K_a$ values for UDP-GlcNAc of the GlcNAc-1-P and the second GlcNAc transferase are between 4.5 and 62 μM (23-25). Dolichol-P has been reported to be a limiting intermediate in N-glycosylation (26, 27). It is therefore highly unlikely that the 1 to 8% residual transport of 0.5 and 1 μM UDP-GlcNAc observed in Tables I and II could account for luminal transfer of GlcNAc-1-P and GlcNAc to endogenous dolichol.

A Blocker of Transfer of N-Acetylgalcosamine Phosphate to Endogenous Dolichol Does Not Inhibit Transport of UDP-GlcNAc into the Lumen of RER-Derived Vesicles—The previous experiments suggested that the transfer of GlcNAc-1-P to endogenous dolichol occurred on the cytosolic side of the RER membrane. To obtain independent proof for this hypothesis, we determined whether one could inhibit this reaction without affecting transport of UDP-GlcNAc into the lumen of RER vesicles. For this, it was important to use a membrane-impermeable inhibitor so that if transfer on the inside of the vesicle membrane would occur such an inhibitor would not be accessible to the active site of the transferase in intact vesicles.

It had been previously demonstrated that the enzymes leading to the formation of dolichol-P-P-GlcNAc require magnesium and that these reactions are inhibited by EDTA (1). Indeed, as shown in Table I, when permeabilized vesicles were incubated with [β-32P]UDP-GlcNAc in the presence of EDTA, a virtual complete inhibition of transfer of the sugar phosphate to endogenous dolichol occurred. This inhibition of transfer was also seen with closed vesicles (95% latent), although transport of the nucleotide sugar into the lumen of these vesicles was not affected. Because of this latter observation and previous studies demonstrating that EDTA was impermeable to membranes of Golgi and RER vesicles (28), these experiments strongly suggest that the transfer of GlcNAc-1-P to endogenous dolichol occurs on the cytosolic side of the rough endoplasmic reticulum membrane. Similar

![Figure 1](image)

**FIG. 1. Thin layer chromatogram autoradiography of 32P-labeled dolichol derivatives.** Intact (lanes 1 and 2) or permeabilized (lanes 3 and 4) RER vesicles were incubated with [β-32P]UDP-GlcNAc in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 5-BrdUMP. Incubation conditions and analyses of the samples have been described under "Materials and Methods." 15,000 dpm were applied to lanes 1 and 2 and 20,000 dpm to lanes 3 and 4. Exposure time, 2 days. o, origin; f, front; a is dolichol-P-P-GlcNAc; b is dolichol-P-P-GlcNAc2, based on published $R_f$ values (19).

### TABLE II

**Incubations of RER vesicles with UDP-[3H]GlcNAc**

<table>
<thead>
<tr>
<th>Additions</th>
<th>UDP-GlcNAc transport</th>
<th>GlcNAc Transferred to Endogenous dolichol-P Protein</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Closed vesicles</td>
<td>Permeabilized vesicles</td>
</tr>
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<td>520</td>
<td>10</td>
</tr>
<tr>
<td>5-BrdUMP</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>EDTA</td>
<td>680</td>
<td>15</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>440</td>
<td>10</td>
</tr>
</tbody>
</table>

RER-derived vesicles (1 mg of protein) were incubated with UDP-[3H]GlcNAc (0.5 μM; 2.2 × 10⁶ dpm) in a total volume of 0.25 ml for 2 min at 20°C. The final concentration of inhibitors was: 1 mM 5-BrdUMP, 5 mM EDTA, 0.5 μg/ml tunicamycin. Solute transport into the vesicles and GlcNAc transferred to endogenous dolichol and protein were determined as described under "Materials and Methods." The following is an example of how the various parameters were measured for closed vesicles with no additions: following centrifugation, it was determined that there were $8.8 \times 10^{10}$ dpm/ml in the incubation medium. Knowing the specific activity of the nucleotide sugar ($17.7 \times 10^{8}$ dpm/nmol), the concentration of solutes in the incubation medium ($S_o$) was calculated to be 0.5 μM (16). The total soluble radioactivity associated with the pellet ($S_p$) was 113,280 dpm/mg of protein or 6.4 pmol/ml protein (based on the specific activity of the nucleotide sugar). The total radioactive solutes in between or outside the vesicles in the pellet ($S_o$) was calculated to be 1.2 pmol/ml of protein. See below calculations for the volume outside the vesicles in the pellet $V_o$. The total radioactive solutes within the vesicles, $S_v$, was the difference between $S_p$ and $S_o$ and was calculated to be 5.2 pmol/mg of protein. Using the same RER preparation, the nonpenetrator ([3H]acetate (0.1 μM; 3.5 × 10⁶ dpm)) was incubated under conditions described above. The incubations resulted in 1080 dpm/mg of protein in the pellet and 450 dpm/μl in the supernatant. From these values, the volume outside the vesicles in the pellet $V_o$ was calculated to be 2.4 ml of protein (16). Latency of the vesicles following incubations with no addition, 5-BrdUMP, EDTA, and tunicamycin were, respectively, 96, 97, and 95%. Results are the average of two independent determinations.
results were obtained with UDP-[3H]GlcNAc (Table II). In this case, the transfer of radiolabeled GlcNAc to proteins was completely inhibited when permeabilized vesicles were incubated in the presence of EDTA while no effect was seen with sealed vesicles. We will provide evidence below that most of this sugar transferred to protein is not N-linked.

Tunicamycin is an antibiotic known to inhibit the transfer of GlcNAc-1-P to dolichol-P (29). When closed RER-derived vesicles were incubated with UDP-[3H]GlcNAc in the presence of tunicamycin (5 μg/ml), the transfer of sugar phosphate to dolichol was virtually abolished while there was only an 8–16% inhibition of UDP-GlcNAc transport into the lumen of the RER vesicles. Although interpretations of these results must be done with caution since it is not clear whether or not this antibiotic can penetrate the membrane, the results are consistent with transfer of sugar phosphate to endogenous dolichol on the cytosolic side of the ER membrane; the results also suggest that the majority of sugar transferred to protein in closed vesicles is dolichol-P-GlcNAc$_2$ independent and most likely represents O-linked GlcNAc. This is a recently described post-translational modification of proteins occurring in many organelles including both sides of the ER membrane (30).

Dolichol-P-P-GlcNAc$_2$ Occurs on the Cytoplasmic Side of the RER Membrane—In view of the above results, it was important to determine whether dolichol-P-P-GlcNAc and dolichol-P-P-GlcNAc$_2$ could be detected facing the cytoplasmic side of the RER membrane. For this purpose, intact sealed, RER-derived vesicles were incubated with [β-32P]UDP-GlcNAc to yield radiolabeled dolichol-P-P-GlcNAc and dolichol-P-P-GlcNAc$_2$. The sealed vesicles were then incubated with a partially purified, soluble galactosyltransferase and UDP-Gal. The RER membrane is impermeable to these reagents, which are known, in conjunction, to specifically add galactose, in a β1-4 linkage, to the terminal N-acetylgalactosamine of dolichol-P-P-GlcNAc$_2$ (2, 10). At the same time, a control incubation was performed in which the radiolabeled dolichol mono and disaccharide-containing vesicles had been permeabilized prior to their incubation with galactosyltransferase and UDP-Gal.

As can be seen in Fig. 2, lane 1, approximately 65% of radiolabeled dolichol-P-P-GlcNAc had been converted to a radioactive derivative which had an $R_F$ of 0.23. Longer incubation resulted in approximately 90% conversion to this derivative (not shown). This derivative had a $R_F$ similar to the one previously reported for a dolichol-P-P-trisaccharide (19), and its formation was dependent on the addition of both galactosyltransferase and UDP-Gal. As shown in Fig. 2, lane 4, a radiolabeled dolichol derivative with the same $R_F$ as in lane 1 was seen when intact RER vesicles were incubated with galactosyltransferase and UDP-Gal. In this latter case, approximately 65% of dolichol-P-P-GlcNAc$_2$ had been converted to the trisaccharide derivative as compared to the control incubation (lane 1). Quantitation was done as described under "Materials and Methods," and absolute values are given in the legend of Fig. 2. The failure of dolichol-P-P-GlcNAc to be galactosylated had also been previously observed by Hanover and Lennarz (6).

**DISCUSSION**

The above studies have provided strong evidence that the active site of the enzymes leading to the formation of the dolichol-P-P-GlcNAc$_2$ and this latter reaction product itself face the cytosolic side of the RER membrane. Previously, Snider and Rogers (3) made the important observation that dolichol-P-P-GlcNAc$_2$Man$_3$, faces the same side of the RER membrane, and we and others found this membrane to be impermeable toward GDP-Man (5–7). In addition, dolichol kinase has also been reported to be on the cytosolic face of the RER membrane (31). Together, these studies demonstrate that all the reactions leading to the synthesis of the latter dolichol derivative occur on the cytosolic side of the RER membrane (Fig. 3). These results are also consistent with studies which had suggested that the enzyme catalyzing the synthesis of dolichol-P-P-GlcNAc$_2$ has either its active site or an important activity domain facing the cytosolic side of the RER membrane (6, 8, 9). While previous studies had yielded no information on the topography of the dolichol N-acetylgalactosamine phosphate transferase, because no conditions had been found under which the activity could be inhibited by proteolysis, a very recent study by Kean (32) suggests that the catalytic site of the dolichol-P-GlcNAc-1-P

![Fig. 2. Thin layer chromatogram autoradiography of galactosylated, $^{32}$P-labeled dolichol derivatives. RER vesicles were incubated with [β-32P]UDP-GlcNAc as described under "Materials and Methods." Subsequently, closed vesicles (lane 4) or permeabilized ones (lanes 1, 2, and 3) were incubated with UDP-Gal plus bovine milk galactosyltransferase (lanes 1 and 4), UDP-Gal (lane 2), or with galactosyltransferase (lane 3). Analyses of the samples were done as described under "Materials and Methods," and the amount of radioactivity detected was for lane 1: a, 5100 dpm; b, 630 dpm; c, 780 dpm; and for lane 4: a, 4900 dpm; b, 1040 dpm; c, 500 dpm. Exposure time, 6 days. o, origin; f, front; a, dolichol-P-P-GlcNAc; b, dolichol-P-P-GlcNAc$_2$; c, dolichol-P-P-GlcNAc$_2$ Gal, based on published $R_F$ values (19).

![Fig. 3. Model of the topography of initiation of N-glycosylation reactions is the rough endoplasmic reticulum. α-1, dolichol-P; Gn, N-acetylgalactosamine; M, mannos.](image-url)
and dolichol-P-P-GlcNAc:GlcNAc transferase face the cytosolic side of the ER membrane. Our results shown in Tables I and II and Fig. 1 strongly suggest that the synthesis of this dolichol derivative also occurs on the cytosolic side of the RER membrane.

Previous attempts to localize dolichol-P-P-GlcNAc, on the cytosolic side of the RER membrane using UDP galactose and galactosyltransferase as probes were negative unless the membrane was first permeabilized with detergents (6, 10). Although it is difficult to determine how our studies with rat liver RER-derived vesicles differ from those previously described with hen oviduct, one possibility may be the higher substrate specificity of $[^32P]UDP$-GlcNAc for specifically labeling dolichol-P-P-GlcNAc and not glycoproteins. Another reason may be the higher detection sensitivity as a result of using radiolabeled phosphorus.

The alg-1 mutant of Saccharomyces cerevisiae, where dolichol-P-P-GlcNAc$_2$ is accumulated, provides indirect evidence that dolichol-P-P-GlcNAc$_2$ can be translocated across the ER membrane into the lumen where it can serve as sugar donor in N-glycosylation reactions (33). Alg-1 produces invertase with large portions of its oligosaccharides being endo-H-resistant, indicating that the transfer of GlcNAc$_2$ to protein occurs in vivo. One might speculate that the above dolichol derivatives may be subjected to two competing reactions; one, occurring on the cytosolic side of the RER membrane, results in the addition of either the second N-acetylglucosamine or mannose; at the same time the above derivatives may also undergo translocation into the lumen. In the alg mutant, where mannosylation on the cytosolic side is blocked, the translocation reaction prevails. Future studies need to provide direct evidence for this dolichol oligosaccharide translocation across the RER membrane and establish what factors regulate the different reaction rates.

Why is there transport of UDP-GlcNAc into the lumen of the RER? We believe that one reason is to serve as a substrate for synthesis of O-GlcNAc-linked glycoproteins which recently were found to face the lumen of the RER membrane, in addition to their unexpected occurrence on the cytosolic side of this membrane (30). Preliminary, unpublished results suggest that the synthesis of this linkage is catalyzed by different enzymes on both sides of the RER membrane arguing against the possibility that proteins on one side of the membrane arise via their translocation from the opposite side (34). Another, more speculative, possibility for UDP-GlcNAc transport is for this nucleotide sugar to serve as substrate in the biosynthesis of the phosphatidylinositol-anchoered glycoproteins although at this time it is not clear on which side of the membrane this anchor is synthesized (35).

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**REFERENCES**