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Topological and Regulatory Aspects of Dolichyl Phosphate Mediated Glycosylation of Proteins

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Phil. Trans. R. Soc. Lond. B 1982 **300**, 129-144
doi: 10.1098/rstb.1982.0161

Phil. Trans. R. Soc. Lond. B **300**, 129–144 (1982)
Printed in Great Britain

Topological and regulatory aspects of dolichyl phosphate mediated glycosylation of proteins

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From the time of their synthesis in the rough endoplasmic reticulum until they are secreted, packaged in lysosomes, or appear as membrane components at the cell surface, the polypeptide chains of *N*- and *O*-linked glycoproteins remain associated with intracellular membranes that are components of the secretory pathway. The various co-translational and post-translational modifications of the carbohydrate moieties of glycoproteins have been shown to occur within morphologically and functionally distinct regions of this complex membrane system. However, the sugar nucleotides, which serve as precursors to the oligosaccharide moieties of these glycoproteins, are synthesized almost exclusively in the cytoplasm. These findings raise a number of questions about the mechanisms involved in the transmembrane assembly of membrane and secretory glycoproteins. In this paper these questions are reviewed and recent studies directed towards providing answers to them are summarized. In addition, information related to the possible role of dolichyl phosphate in regulating the glycosylation of proteins is presented.

Introduction

Although many of the basic steps involved in the synthesis of the oligosaccharide chains of *N*-linked glycoproteins are now fairly well understood, a good deal remains to be learned about the topological aspects of this assembly process and the basic mechanisms that serve to regulate it. In figure 1, a number of the key steps involved in synthesis of an *N*- and *O*-linked glycoprotein are outlined. In addition, current views of the spatial and temporal aspects of this process that will be discussed in this paper are depicted. As shown, the *N*- and *O*-glycosylation of a newly synthesized polypeptide chain is a topologically asymmetric process. The process is initiated by the asymmetric synthesis of $(GlcNAc)_2$-PP-dolichol (chitobiosyl-lipid) with its carbohydrate chain facing the luminal side of the rough endoplasmic reticulum (r.e.r.). Subsequent elongation by addition of Man and Glc units leads to a complete oligosaccharide chain, still attached to dolichyl pyrophosphate and still facing the lumen of the r.e.r. Transfer of the oligosaccharide chain to a nascent polypeptide is believed to occur when the growing polypeptide containing a tripeptide, -Asn-X-Ser/Thr-, has transited the membrane so that the Asn site is accessible to the oligosaccharide-lipid. After transfer, the oligosaccharide chain undergoes the initial stages of processing, which involve the removal of glucose residues. After movement of this partly processed chain from the r.e.r. to the Golgi complex, further processing occurs if it is destined to become a complex-type chain. If it is destined to be a mature polymannose-type chain, relatively little additional processing occurs. For the complex chains, the removal of Man, followed by the addition of GlcNAc, Gal, Fuc and sialic acid residues results in the formation of a mature complex chain. Most of the available evidence also indicates

that it is here in the Golgi complex that the assembly of *O*-linked chains occurs. As shown with this hypothetical protein containing both *N*- and *O*-linked chains, the assembly of the *O*-linked chain involves sequential addition of GalNAc, Gal and sialic acid residues.

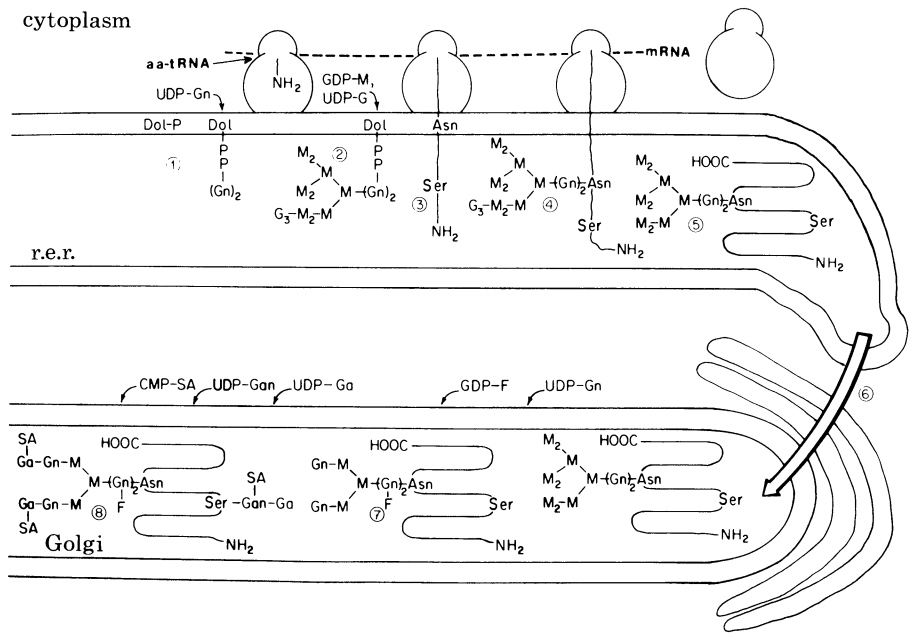

FIGURE 1. Model depicting events in the *N*- and *O*-glycosylation of a hypothetical secretory protein containing sites for both types of chains. The abbreviations are: Gn, *N*-acetylglucosamine; M, mannose; G, glucose; Ga, galactose; F, fucose; Gan, *N*-acetylgalactosamine; SA, sialic acid.

TOPOLOGICAL ORIENTATION OF SACCHARIDE-LIPIDS

A priori, there is no reason to postulate the topological orientation of the dolichol-linked saccharide shown in figure 1. An equally reasonable possibility is that assembly of these saccharide-lipids occurs at the cytoplasmic face of the r.e.r., where sugar nucleotides would be readily available. *N*-glycosylation of the growing polypeptide at this face, followed by transit of the already glycosylated protein through the membrane as translation proceeded, would result in the same end product. What, then, is the evidence that the topology is that shown in figure 1? In approaching this question, we first studied the topology of the chitobiosyl-lipid. Earlier it was observed that purified chitobiosyl-lipid was an excellent substrate for galactosyl transferase. Thus when purified chitobiosyl-lipid suspended in detergent was incubated with galactosyl transferase and UDP–Gal it was quantitatively converted from the disaccharide-lipid to a Gal-containing trisaccharide-lipid. This finding suggested the possibility that galactosyl transferase might be useful as a probe for the topological distribution of the disaccharide units of chitobiosyl-lipid in the membrane of the r.e.r. In a series of preliminary experiments, phospholipid liposomes containing small amounts of labelled chitobiosyl-lipid were prepared. These unilamellar liposomes, prepared in the two sizes shown in figure 2, were then incubated with UDP–Gal and galactosyl transferase, and the proportion of the disaccharide-lipid that was converted to trisaccharide-lipid was assessed. As shown, by using either large or small liposomes of known diameters, the extent of galactosylation was in excellent agreement with that calcu-

lated on the basis of the surface areas of the inner and outer faces of the liposome. When a detergent was added to disrupt the liposomes, all of the disaccharide units were converted to trisaccharide. Further, when this galactosylation process with liposomes was studied kinetically, with higher levels of both galactosyl transferase and UDP–Gal, it became clear that the values observed in this experiment were kinetic endpoints. That is, no further galactosylation of

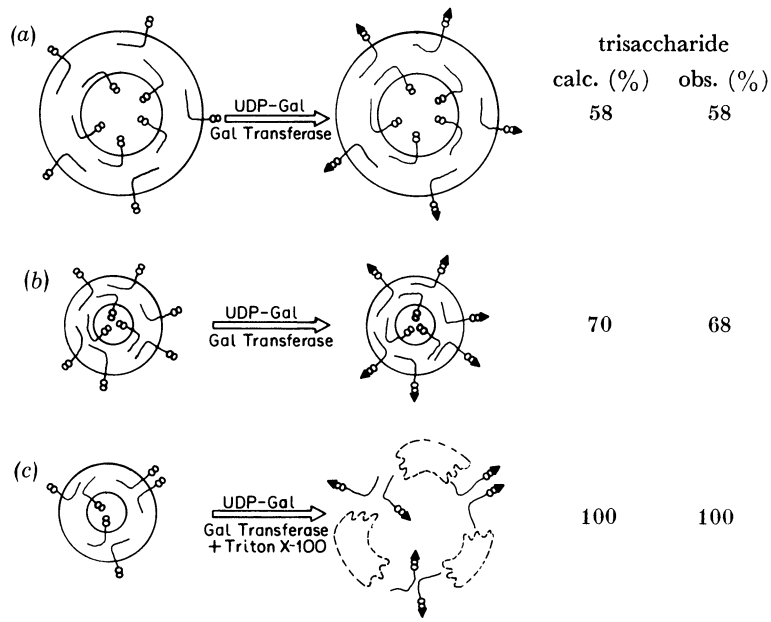

FIGURE 2. Use of galactosyl transferase as a probe for the topological distribution of chitobiosyl-lipid in phospholipid liposomes: (*a*) large liposomes (60 nm); (*b*) small liposomes (22 nm); (*c*) detergent-disrupted liposomes.

chitobiosyl-lipid could be observed under conditions of prolonged incubation. This strongly suggests that, at least in liposomes, the disaccharide-lipid on the inner face is unable to undergo significant flip-flop to the outer face (Hanover & Lennarz 1980).

With this information in hand, we turned to microsomes prepared from hen oviduct, and generated chitobiosyl-lipid by incubation with UDP-GlcNAc (Hanover & Lennarz 1980). Subsequently we assessed the accessibility of the preformed chitobiosyl-lipid to galactosyl transferase, using both total microsomes and purified r.e.r. As shown in figure 3(*a*, *b*), in the absence of detergent less than 10% of the disaccharide-lipid was accessible to galactosyl transferase, suggesting that it was present primarily at the luminal face of the membranes. However, when various amounts of detergent were added to disrupt the membranes, the disaccharide-lipid became increasingly accessible to the galactosyl transferase. At levels of detergent above 0.15% all of the previously synthesized disaccharide units were accessible. As shown in figure 3(*c*, *d*), in parallel experiments assessing the intactness of the membranes by measuring the latency of two known luminal enzymes, a reciprocal relation between latency and accessibility of chitobiosyl-lipid was observed. Thus in the absence of detergent, mannose-6-phosphate phosphatase and β-glucuronidase were almost completely latent. Only when the membrane was disrupted did they lose their latency and become accessible to exogenous substrates.

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It was important to rule out several alternative explanations for the apparent inaccessibility of the chitobiosyl-lipid. One obvious possibility was that the disaccharide-lipid was indeed synthesized at the cytoplasmic face but was not accessible to galactosyl transferase because of the presence of proteins or ribosomes on the surface of the membranes. To test this possibility, the accessibility of chitobiosyl-lipid was measured in microsomes pretreated with proteases, such as trypsin or chymotrypsin, to remove surface proteins, or with puromycin–KCl to discharge the ribosomes. It was found that neither treatment enhanced the accessibility of the disaccharide-

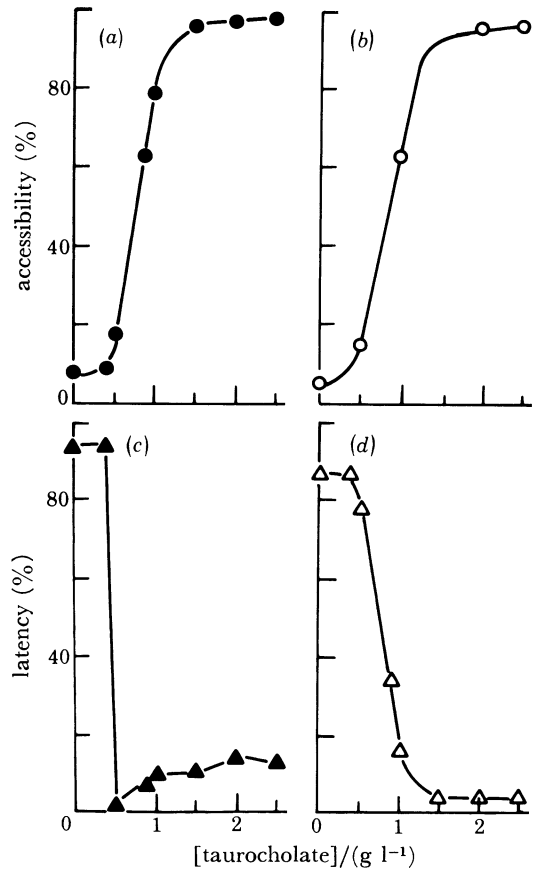

FIGURE 3. Accessibility of chitobiosyl-lipid in microsomes to galactosyl transferase in the absence or the presence of detergent: (*a*) total microsomes; (*b*) r.e.r. Also shown is the latency of two microsomal enzymes: mannose-6 phosphate phosphatase (*c*) and β-glucuronidase (*d*).

lipid to the probe. Another possibility was that the effect of detergent was selective extraction of the disaccharide-lipid from the membranes, and only after it was extracted and present in solution was it accessible to the galactosyl transferase. However, direct measurements of the release of disaccharide-lipid from the membrane as a function of the concentration of detergent excluded this possibility; no extraction of the disaccharide-lipid was observed by using the detergent concentrations employed in these experiments.

Three possible mechanisms to explain the apparently asymmetric synthesis of chitobiosyl at the luminal face of the microsomes were considered, as shown in figure 4. In A, assembly of the

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chitobiosyl-lipid occurs at the cytoplasmic face, but is followed by rapid, unidirectional flip-flop to the inner face. In B, assembly occurs at the luminal face but is preceded by the transport of the sugar nucleotide into the lumen. In C, the intact sugar nucleotide is not transported into the lumen. Rather, the GlcNAc and GlcNAc-1-PO_4 units that participate in synthesis of chitobiosyl-lipid are transferred from the sugar nucleotide to an enzyme complex containing

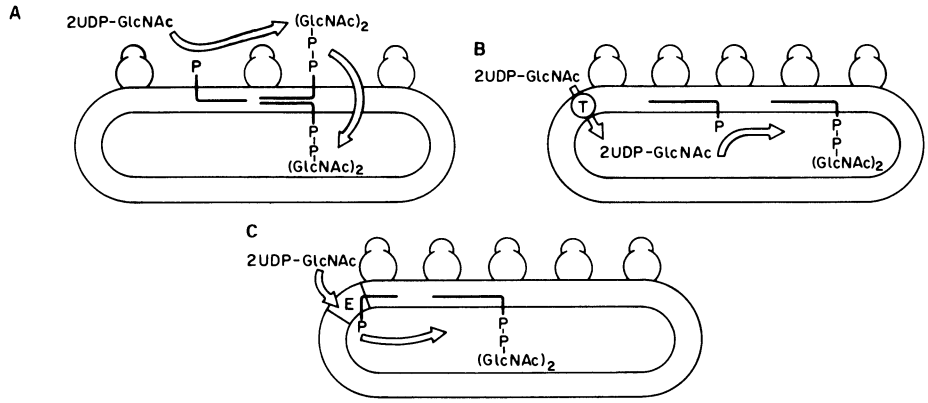

FIGURE 4. Alternative models for the asymmetric synthesis of chitobiosyl-lipid at the luminal face of microsomes.

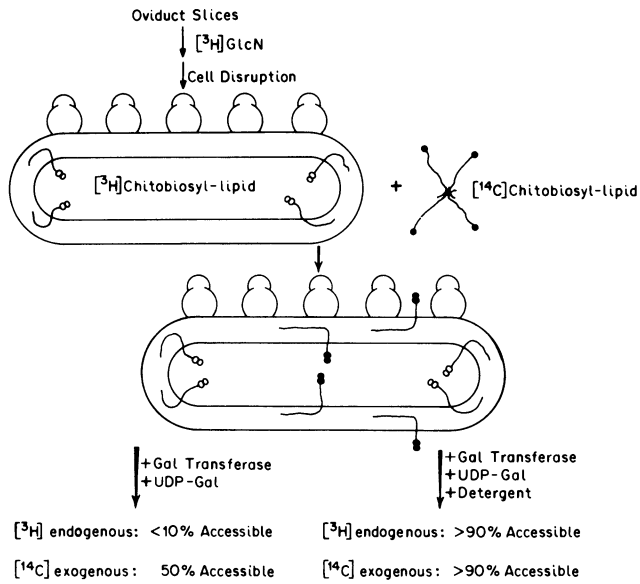

FIGURE 5. Comparison of the accessibility of chitobiosyl-lipid generated *in vivo* with the accessibility of chitobiosyl-lipid introduced into isolated microsomes.

the transferases and dolichylphosphate; subsequent transfer of the activated sugars from the putative enzyme intermediate to dolichyl phosphate results in the formation of chitobiosyl-lipid at the luminal face. In an attempt to differentiate between these possible models, and to provide further support for the conclusion that chitobiosyl-lipid is orientated luminally, the experiment outlined in figure 5 was carried out (Hanover & Lennarz 1982). Oviduct tissue slices were incubated with [^{3}H]GlcN under conditions that lead to formation of chitobiosyl-lipid. Microsomes were then prepared from these oviduct tissue slices. These microsomes, containing

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^{3}H-labelled chitobiosyl-lipid synthesized *in vivo*, were then incubated with a highly purified preparation of ^{14}C-labelled chitobiosyl-lipid. After centrifugation and washing of the microsomes to remove any [^{14}C]chitobiosyl-lipid not tightly associated with the microsomes, the preparation was incubated with UDP–Gal and galactosyl transferase to study the topological distribution of both the ^{3}H- and ^{14}C-labelled chitobiosyl-lipid. As shown, in the absence of detergent, virtually all of the [^{3}H]chitobiosyl-lipid synthesized *in vivo* was inaccessible to the probe until detergent was added. This observation provides strong support for the validity of

TABLE 1. UTILIZATION OF [^{3}H]UDP–[^{14}C]GlcNAc IN CHITOBIOSYL-LIPID SYNTHESIS IN OVIDUCT MICROSOMES

incubation time	[^{14}C]GlcNAc-PP-dolichol	[^{14}C](GlcNAc)$_2$-PP-dolichol	[^{3}H]UMP	[^{3}H]UDP
min	pmol	pmol	pmol	pmol
0	< 0.06	< 0.06	0.25	< 0.05
30	9.7	9.45	1.6	< 0.05
60	11.7	15.9	2.0	< 0.05

the previous studies on the topology of chitobiosyl-lipid synthesized in isolated microsomes. When the topological distribution of the externally introduced [^{14}C]chitobiosyl-lipid was examined, the results were quite different. As shown, 50% of the chitobiosyl-lipid could be galactosylated, indicating a cytoplasmic orientation. When detergent was added, all of the [^{14}C]chitobiosyl-lipid was galactosylated. Furthermore, when galactosylation of the [^{14}C]-chitobiosyl-lipid was studied kinetically, it was found that there was no increase in the extent of galactosylation with time, indicating that both ^{14}C- and ^{3}H-labelled chitobiosyl-lipid are static in the membrane, and do not undergo any significant flip-flop.

To further test the possibility that synthesis occurred on the outer face, followed by flip-flop to the inner face, we modified our original *in vitro* experiments by adding galactosyl transferase and UDP–Gal during, rather than after, synthesis of chitobiosyl-lipid in microsomes (Hanover & Lennarz 1982). Under these conditions, no significant increase in the conversion of disaccharide-lipid to trisaccharide-lipid was observed in the absence of detergent. Thus the results of both this and the previous experiment indicate that it is unlikely that synthesis occurs at the outer face as shown in model A.

In model B the sugar nucleotide is first transferred into the microsome where it reacts at the luminal face with dolichyl phosphate. However, using the techniques of Ballas & Arion (1977) to study the uptake of solutes into microsomes, we found that UDP-GlcNAc and GDP-Man behaved more like dextran, an impermeant molecule, than like glucose, a readily permeant molecule (Hanover & Lennarz 1982). In addition, it was found that under the same conditions CMP-sialic acid and UMP were readily permeable, in agreement with earlier findings by others. If the UDP-GlcNAc was taken up into the microsomes, and the transfer reactions occurred in the lumen, one would expect to find UMP and UDP, the other products of transferases, present in amounts equivalent to the chitobiosyl-lipid formed. However, as shown in table 1, this is not so: the level of the two nucleotides detected was only 10% that of the chitobiosyl-lipid formed (Hanover & Lennarz 1982).

In addition to thse findings *in vitro* arguing against model B, we carried out studies *in vivo* to measure the level of sugar nucleotides found inside the endoplasmic reticulum (Hanover &

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Lennarz 1982). By using the procedure of Carey & Hirschberg (1981), it was found that little or no UDP-GlcNAc and GDP-Man could be detected in microsomes isolated from tissue slices that had synthesized the sugar nucleotides *in vivo*. Thus all of these studies are inconsistent with model B, leaving us with model C, which postulates concerted transfer of the activated sugar units, mediated by an enzyme complex interacting with dolichyl phosphate. As a direct consequence of the enzymatic transfer reaction, the observed asymmetry is introduced. Clearly, further studies will be necessary to refine and test this model.

As shown in figure 1, further elongation of the chitobiosyl-lipid leads to the assembly of the completed oligosaccharide still attached to the dolichyl pyrophosphate. Although we have not been able to develop topology probes to study the orientation of the oligosaccharide-lipid, we took advantage of the finding that under certain conditions oviduct microsomes rapidly hydrolyse a significant portion of the oligosaccharide attached to dolichyl pyrophosphate. With this information, and with the expectation that oligosaccharides this size would be impermeable to the membrane of the r.e.r., we undertook to determine where the oligosaccharide was after hydrolysis. The results of this experiment indicate that the hydrolytically released oligosaccharide remains in the lumen and is only released from the microsomes when the membrane is disrupted with detergent (Hanover & Lennarz 1982). Thus it appears that not only the synthesis of chitobiosyl-lipid but also its elongation occurs inside the r.e.r.

Temporal aspects of translation and glycosylation

If the model postulated in figure 1 is correct, the polypeptide chain at the time that the Asn residue is glycosylated must be sufficiently long to transit both the ribosomal cleft and at least most of the membrane bilayer. Using oviduct tissue slices and studying ovalbumin, we undertook to determine how long the ovalbumin polypeptide chain must grow after addition of the Asn residue before this residue is glycosylated (Glabe *et al.* 1980). This question is outlined schematically in figure 6, showing the growing ovalbumin chain, the addition of Asn-293, and the presence of the carbohydrate chain attached to this residue on the mature polypeptide. Recently the nucleotide sequence of the ovalbumin gene and, independently, the amino acid sequence of the protein have been reported (Nesbit *et al.* 1981). As shown in figure 7, examination of the sequence around the glycosylation site indicates that there are two tryptic cleavage sites present, which should enable one to excise a glycopeptide containing 32 amino acid residues. To ascertain that this was feasible, we subjected mature ovalbumin to trypsin digestion, and recovered a glycopeptide by gel filtration and Concanavalin A affinity chromatography. Sequence analysis of the purified glycopeptide revealed that the first 10 residues (shown underlined) had the expected sequence. With this reference glycopeptide in hand we proceeded to study the nascent chains of ovalbumin. Oviduct tissue slices were incubated with [^{35}S]methionine and with [^{3}H]mannose, and the ovalbumin nascent chains were isolated. As outlined in figure 8, the total nascent chain mixture was subjected to trypsin digestion and the glycopeptides were isolated. If no glycopeptide smaller than 32 residues were detectable (figure 8*a*), one would conclude the chains must grow a *minimum* of 32 residues in length after addition of the Asn residue before the carbohydrate is added. However, if smaller glycopeptides were obtained (figure 8*b*), one would conclude that carbohydrate attachment occurred before the addition of 32 more residues. The isolated glycopeptides from the nascent chains were mixed with authentic glycopeptide isolated from the mature chain, and analysed by both gel filtration

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and sodium dodecyl sulphate polyacrylamide gel electrophoresis. As shown in figure 9, the glycopeptide from the nascent chains was identical to that from the mature chain, indicating that at least 32 residues must be added before glycosylation occurs.

In addition to asking this question, in other experiments we determined the length of the ovalbumin nascent chains that is protected in the ribosomal cleft from proteolysis by trypsin

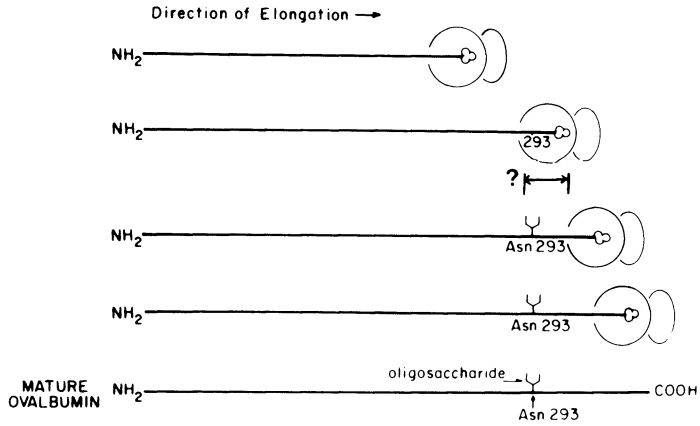

FIGURE 6. Intermediate stages in the biosynthesis of ovalbumin.

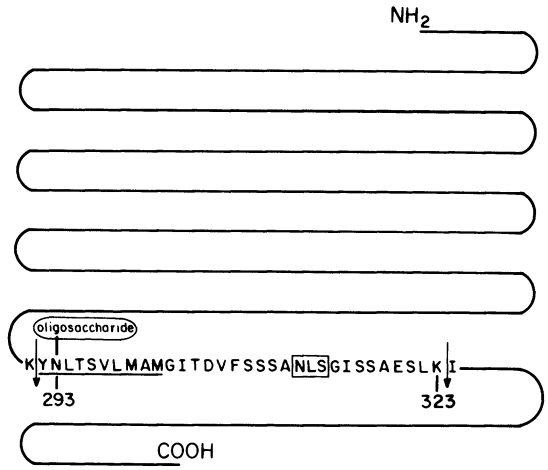

FIGURE 7. Partial primary structure of ovalbumin.

(Glabe *et al.* 1980). These experiments yielded values for a peptide approximately 20–25 residues in length, in reasonable agreement with earlier studies (Blobel & Sabatini 1970). Thus the overall minimum length of the polypeptide chain at the time of glycosylation appears to be 52 residues, i.e. 32 plus 20. This length is clearly sufficient for glycosylation of the Asn residue to occur at the luminal face of the endoplasmic reticulum, as postulated in figure 1.

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O-GLYCOSYLATION

The hypothetical protein in figure 1 is depicted as having a site for *O*- as well as *N*-glycosylation. Evidence for and against the idea that *O*-glycosylation is a co-translational process has been reported (Strous 1979; Jokinen *et al.* 1979, 1981; Ruddon *et al.* 1980; Ruddon *et al.* 1981;

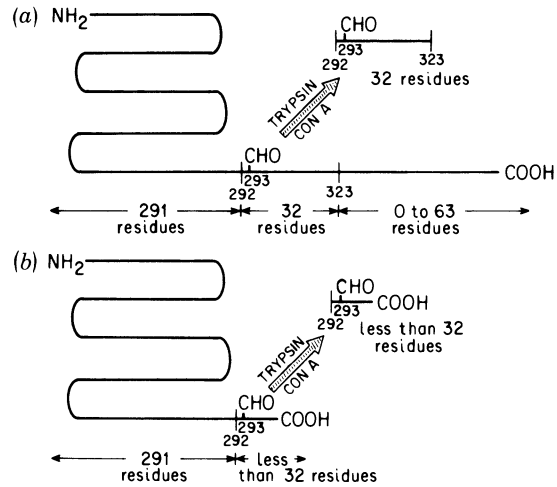

FIGURE 8. Strategy used for the isolation of glycopeptides from ovalbumin nascent chains.

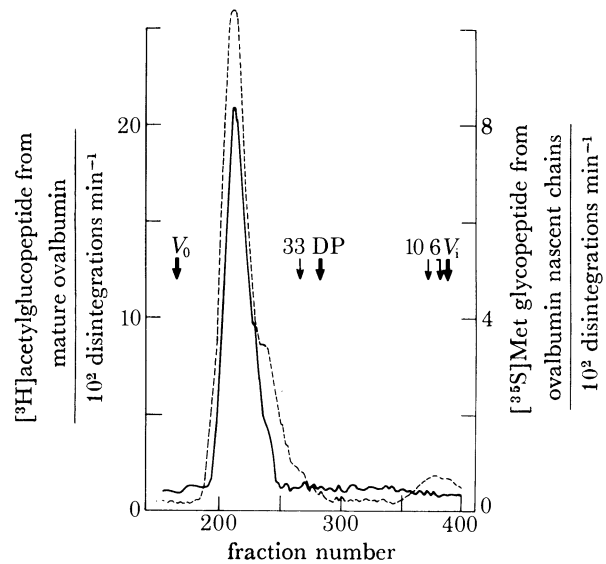

FIGURE 9. Comparison by gel filtration of the glycopeptide isolated from nascent chains (—) and from mature ovalbumin (– – –). Numbers against arrows are molecular masses in hectodaltons.

Kim *et al.* 1971; Ko & Raghupathy 1972; White & Speake 1980). Studies with oviduct membrane preparations and synthetic peptides that act as acceptors of a GalNAc unit from UDP–GalNAc indicated that the processes of *N*- and *O*-glycosylation were quite different in several respects (Hanover *et al.* 1980). First, *N*-glycosylation was stimulated by dolichyl phosphate and inhibited by tunicamycin, whereas *O*-glycosylation was unaffected by either compound.

Second, and more important in the context of whether or not *O*-glycosylation is a co-translational process, was the finding that the transferase catalysing the transfer of GalNAc to synthetic peptides was highly enriched in a smooth membrane fraction consisting of the Golgi complex and smooth endoplasmic reticulum. In contrast the enzymes involved in *N*-linked glycosylation were enriched in the r.e.r. Clearly these observations strongly suggest that unlike *N*-glycosylation, *O*-glycosylation is a post-translational process. In this context it should be noted that unlike the r.e.r., the Golgi complex appears to be permeable to nucleotides (Carey & Hirschberg 1981; Fleischer 1981).

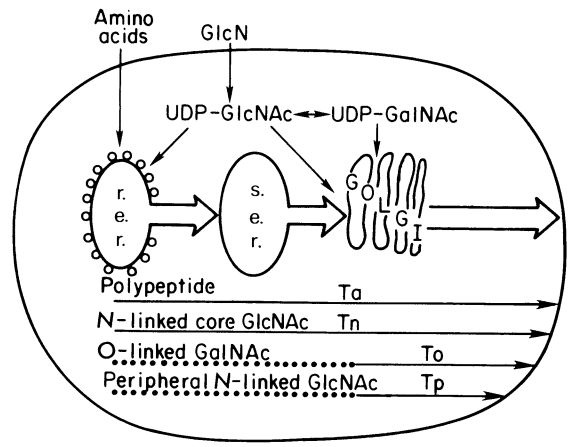

FIGURE 10. Approach for studying the synthesis and the *N*- and *O*-glycosylation of hCG in Be Wo cells.

An alternative method to determine if *N*- and *O*-glycosylation processes are temporally and spatially distinct processes would be to study the kinetics of the *N*- and *O*-glycosylation of a single polypeptide containing both types of chains. Human chorionic gonadotropin (hCG) contains both *O*- and *N*-linked chains in its β subunit. Therefore, studies with the use of the choriocarcinoma cell line Be Wo were undertaken and the synthesis and glycosylation of hCG were examined (Hanover *et al.* 1982). As shown in figure 10, the approach was to use amino acids to label the polypeptide backbone, and GlcN to label the precursors to both types of hexosamine units, namely UDP–GlcNAc and UDP–GalNAc. In preliminary experiments it was shown that GlcN was an effective precursor of both types of sugar nucleotides and that the uptake, activation and epimerization of GlcNAc to GalNAc units were rapid processes. When the kinetics of the appearance of amino acid- and hexosamine-labelled hCG in the culture medium were examined a very different profile was observed for the two precursors (figure 11*a*). No newly synthesized polypeptide chains were observed in the culture medium before 1.5 h. In contrast, hexosamine label was rapidly incorporated into the secreted hCG, well before newly synthesized, amino acid-labelled hCG was secreted. In the case of intracellular hCG (figure 11*b*), both the hexosamine and amino acid labels enter the polypeptide at similar rates.

Next we sought to compare the kinetics of incorporation of each of the three types of amino sugars in hCG, i.e. GlcNAc in the core and in peripheral positions on the *N*-linked chains, and GalNAc in the *O*-linked chains. To accomplish this the sequential degradation procedure shown in figure 12, followed by h.p.l.c. analysis of the amino sugars released after acid hydro-

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lysis, was used. When this procedure was applied to secreted hCG isolated from the culture medium at various time intervals, the results in figure 13 were obtained. It is clear that at the earliest time interval, before any newly synthesized polypeptide chains of hCG have been secreted, virtually all of the hexosamine label is found in GalNAc units in the *O*-linked chain and in peripheral GlcNAc units in *N*-linked chains. Previous studies have shown that addition

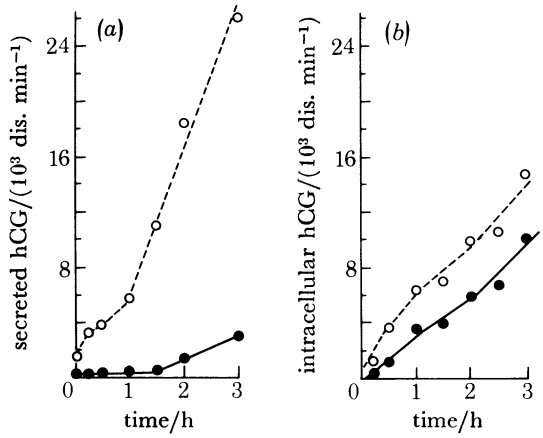

FIGURE 11. Kinetics of appearance of [^{35}S]methionine-labelled (closed circles) and hexosamine-labelled (open circles) hCG in the culture medium (*a*) and in the cells (*b*).

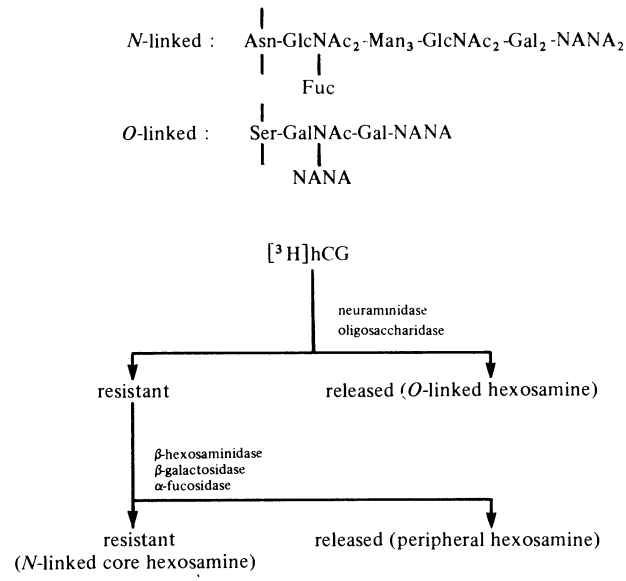

FIGURE 12. Procedure used to determine the distribution of the hexosamine units in the *N*- and *O*-linked chains of hCG.

of peripheral sugars, i.e. GlcNAc, Gal and NANA, occur in the Golgi complex. The fact that the timing of addition of GalNAc units to *O*-linked chains and of peripheral GlcNAc units to *N*-linked chains is identical suggests that, indeed, *O*-linked glycosylation also occurs in the Golgi complex, and is therefore not a co-translational process. At later time intervals the distribution pattern radically changes, as expected, because now newly synthesized hCG chains are appearing in the culture medium. Under these circumstances most of the label is

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found in GlcNAc units in the *N*-linked core. In fact, the distribution of the three types of labelled amino sugars is in agreement with the theoretical values for mature hCG. These results, as well as the experiments carried out in oviduct tissue slices *in vitro*, strongly support the idea that *O*-glycosylation is a post-translational process.

With reference to the scheme outlined in figure 1, it is of interest to consider why radically different mechanisms are used for the addition of *N*- and *O*-linked chains. Why does the attachment of *N*-linked chains involve the preassembly of the completed oligosaccharide on

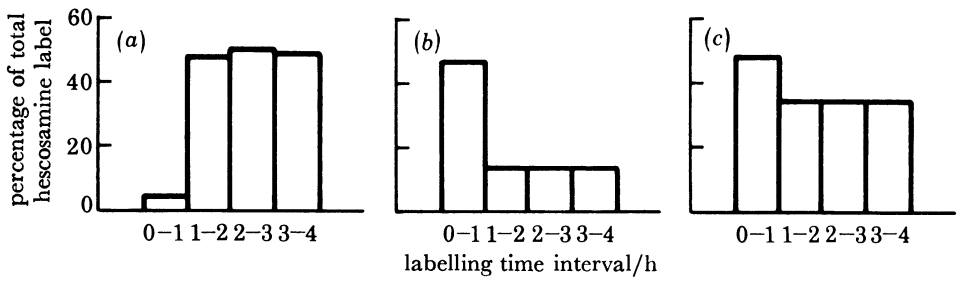

FIGURE 13. Distribution of GlcNAc and GalNAc units in secreted hCG: (*a*) *N*-core GlcNAc; (*b*) *O*-GalNAc; (*c*) peripheral GlcNAc.

dolichyl pyrophosphate, whereas the synthesis of the *O*-linked chains involves the direct transfer of the sugars from sugar nucleotides without any preassembly process? One possible explanation is that both the assembly of an oligosaccharide for a *N*-linked chain containing 14 saccharide units and its transfer to an Asn residue on a growing polypeptide must be rapid processes that presumably are accomplished with fidelity. Prepackaging of the oligosaccharide on a dolichyl phosphate 'anchor' would facilitate such rapid transfer. The need for such a preassembly process is not obvious when one considers *O*-glycosylation, as well as the addition of peripheral sugars to *N*-linked chains. In these cases the polypeptides are complete and are being stored, at least temporarily, in the Golgi complex. In addition, these late steps involve the addition a relatively smaller number of sugar units. Under these circumstances the need for a preassembly process to ensure rapid transfer would be unnecessary. If this idea is correct, the function of dolichyl phosphate may merely be to serve as a hydrophobic anchor for the preassembly of the oligosaccharide chains, and not as a 'sugar carrier' as earlier postulated for lipid intermediates in the synthesis of bacterial glycans.

REGULATION OF GLYCOSYLATION

Finally, it is of interest to consider possible mechanisms for the regulation of protein glycosylation. Three possible factors that could serve a regulatory role in the glycosylation of proteins would be the availability of (*a*) sugar nucleotides, (*b*) dolichyl phosphate, and (*c*) nascent polypeptide chains with glycosylatable Asn sites. One indication that dolichyl phosphate might, at least in some systems, be a rate-limiting factor in glycosylation came from studies on the relation between glycoprotein synthesis and embryonic development in sea urchins (Carson & Lennarz 1979). In these studies we had observed that compactin, an inhibitor of HMG–CoA reductase, blocked dolichol synthesis and caused an arrest in development. However, when

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embryos were cultivated in the presence of compactin plus dolichol, glycosylation activities, as assessed by synthesis of Man-P-dolichol, oligosaccharide-PP-dolichol and *N*-linked glycoproteins, were not only restored but significantly exceeded control values. This finding, suggesting that under normal circumstances dolichyl phosphate may be rate-limiting, prompted us to examine other biological systems. As shown in table 2, experiments with the fibroblastic LM

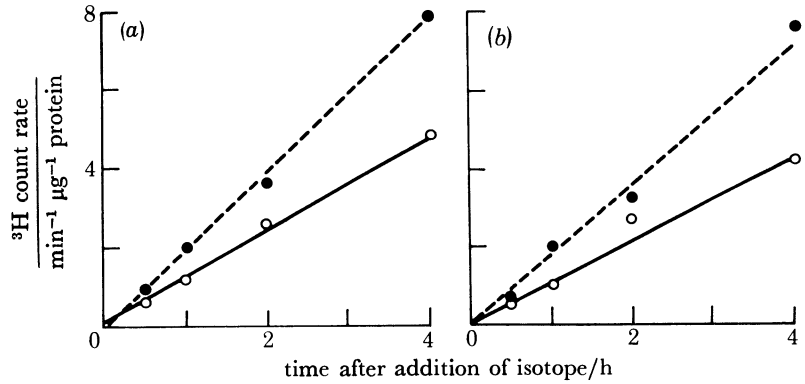

FIGURE 14. Stimulation of glycosylation of proteins in oviduct tissue slices by dolichyl phosphate: (*a*) [^{3}H]Man; (*b*) [^{3}H]GlcNAc; ●, with Dol-P; ○, without Dol-P.

TABLE 2. EFFECT OF EXOGENOUS DOLICHYL PHOSPHATE ON OLIGOSACCHARIDE-LIPID SYNTHESIS IN LM CELLS

addition to culture medium	radioactive count in oligosaccharide-lipid / min^{-1}	
	10 min	20 min
none	6500	14000
Dol-P	17000	38000

TABLE 3. DOLICHYL PHOSPHATE SUPPLEMENTATION DOES NOT STIMULATE OVALBUMIN TRANSLATION OR GLYCOSYLATION

addition to culture medium† ...	radioactive count in anti-ovalbumin precipitate / min^{-1} mg^{-1} protein		
	[^{3}H]mannose	[^{35}S]methionine	[^{3}H]/[^{35}S]
control	70305	13730	5.1
plus dolichyl phosphate	68243	12406	5.5

† Oviduct tissue slices (50 mg wet mass) were preincubated with or without dolichyl phosphate (20 µg ml^{-1}).

cell line indicated that the synthesis of oligosaccharide-lipid *in vivo* was doubled by supplementation of the culture medium with dolichyl phosphate (Grant & Lennarz 1982). Similar findings were observed in a very different system, namely oviduct tissue slices (Carson *et al.* 1981). Synthesis of *N*-linked glycoproteins in such slices was measured by using radioactive Man or GlcNAc as precursors. As shown in figure 14, it was found that the presence of dolichyl phosphate in the culture medium consistently doubled or trebled the total *N*-linked glycoprotein synthesis. However, when we specifically measured the effect of added dolichyl phosphate on

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the synthesis of the major glycoprotein produced by oviduct, ovalbumin, it was found that neither its synthesis nor its glycosylation was stimulated by added dolichyl phosphate (table 3). Further study revealed that the stimulation observed was the result of increased glycosylation of a variety of as yet unidentified glycoproteins. For this reason, we turned to another tissue slice system, bovine pancreas, which synthesizes and secretes ribonuclease (Carson *et al.* 1981). Ribonuclease secreted from bovine pancreas under normal conditions is largely unglycosylated;

TABLE 4. DOLICHYL PHOSPHATE SUPPLEMENTATION STIMULATES RIBONUCLEASE GLYCOSYLATION IN BOVINE PANCREAS TISSUE SLICES

	radioactive count in ribonuclease min^{-1}		
addition to culture medium†...	[^{3}H]mannose	[^{14}C]amino acids	[^{3}H]/[^{14}C]
−dolichyl phosphate	78097	5168	15.1
+dolichyl phosphate	409050	6650	61.5

† Bovine pancreas tissue slices (50 mg wet mass) in 5 ml medium were preincubated with or without dolichyl phosphate (20 μg ml^{-1}).

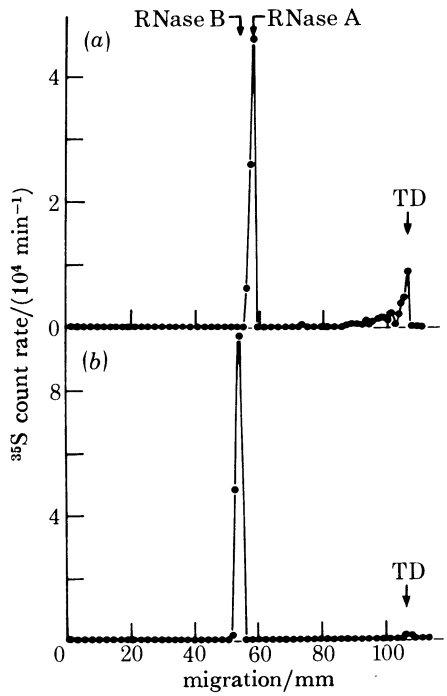

FIGURE 15. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of ribonuclease secreted by pancreatic tissue slices incubated in the absence (*a*) or presence (*b*) of dolichyl phosphate.

only 10% of the secreted chains are ribonuclease B, the glycosylated form of the enzyme. Using an affinity column to isolate ribonuclease secreted from the tissue slices, we assessed the affect of dolichyl phosphate in the culture medium on both mannose and amino acid incorporation. As shown in table 4, amino acid incorporation into secreted ribonuclease was unaffected by the presence of dolichyl phosphate, whereas incorporation of mannose was very markedly stimulated. To verify that enhanced glycosylation of the ribonuclease occurred in the presence of dolichyl phosphate, the product formed under these two conditions was examined by

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polyacrylamide gel electrophoresis. As shown in figure 15, it is clear that virtually all of the ribonuclease synthesized and secreted by the slices in the absence of dolichyl phosphate is ribonuclease A, the unglycosylated form. In contrast, in tissue sizes supplemented with dolichyl phosphate, most of the product is ribonuclease B, the glycosylated form. Final confirmation of this finding was accomplished by assessing the extent of glycosylation of ribonuclease as a function of the concentration of dolichyl phosphate in the culture medium. As shown in figure

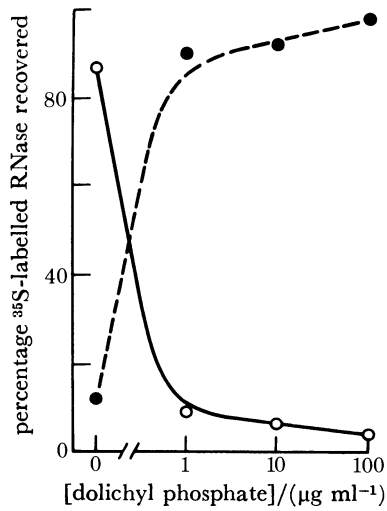

FIGURE 16. Effect of exogenous dolichyl phosphate on the synthesis of glycosylated ribonuclease in pancreatic tissue slices. ●, RNase bound to Con A–Sepharose; ○, RNase not bound.

16, in the absence of dolichyl phosphate most of the ribonuclease, as assessed by its ability to bind to Concanavalin A, is unglycosylated. In contrast, upon addition of even low levels of exogenous dolichyl phosphate most of the secreted ribonuclease is found to be glycosylated. Thus it is clear that, at least in the systems investigated so far, dolichyl phosphate can be a rate-limiting factor in glycoprotein synthesis. Whether or not sugar nucleotides and availability of glycosylatable polypeptide chains also serve as regulatory factors remains to be established.

The work reported from the author's laboratory was supported by grants from the National Institutes of Health (nos GM 21451 and GM 24821). It is a pleasure to acknowledge the efforts of my associates in these studies: D. D. Carson, B. J. Earles, J. Elting, C. G. Glabe, S. Grant, J. A. Hanover and G. R. Mintz.

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