Dolichol is not a necessary moiety for lipid-linked oligosaccharide substrates of the mannosyltransferases involved in *in vitro* N-linked-oligosaccharide assembly

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Dolichol is utilized *in vivo* as an unusually large anchor on which the precursor for N-linked oligosaccharides is assembled by a series of glycosyltransferases. The role of dolichol in enzyme substrate recognition is investigated. Thus the biosynthetic intermediate *NN'*-diacetylchitobiose was chemically linked to either dolichol or the much shorter fully saturated tetraisoprenoid phytanol. Both lipids were used as substrates by a recombinant, soluble β -1,4-mannosyltransferase. β -[3 H]Mannosylated lipids from this reaction were then used as substrates for the subsequent

mannosyltransferases from yeast or rat liver microsomes. It was found that both the dolichyl- and phytanyl-linked substrates were easily mannosylated to form $\text{Man}_5\text{GlcNAc}_2$, with some further mannosylation to $\text{Man}_6\text{GlcNAc}_2$ and $\text{Man}_7\text{GlcNAc}_2$ at low concentrations of lipid-linked substrate. It is concluded that dolichol is not necessary *in vitro* as part of the substrate for the mannosyltransferases in the biosynthetic pathway for N-glycosylation.

INTRODUCTION

During the biosynthesis of N-linked oligosaccharides, the tetradecasaccharide intermediate $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ (Figure 1) is assembled whilst attached to the polyisoprenoid dolichol (1, Figure 2) through a pyrophosphate bridge and then transferred to the nascent polypeptide chain on the ribosome before further processing. The oligosaccharide assembly begins at the reducing end of the oligosaccharide, to which monosaccharide units are added in a stepwise fashion. The formation of the glycosidic linkages are catalysed by specific glycosyltransferases associated with the endoplasmic-reticulum (ER) membrane. Of the nine mannose residues, the first five are transferred on the cytoplasmic

face of the ER membrane, with GDP-Man as the donor, whereas the final four mannose residues and all three glucose residues are transferred on the luminal face from dolichyl-linked donors [1]. Thus, before the transfer of the sixth mannose residue, the precursor must be translocated across the membrane while still attached to its dolichyl anchor.

The unusual size and structure of dolichol (1), which consists of up to 20 isoprene units, have raised questions as to its function. It has been proposed that the majority of the length of the dolichol chains is located between the leaflets of the lipid bilayer [2]. Thus its role might just be that of a hydrophobic anchor for oligosaccharide precursors. On the other hand, it might have additional, more specific roles in facilitating the

Figure 1 Structure of the oligosaccharide precursor to N-glycosylation

The $\text{Man}_5\text{GlcNAc}_2$ structure is boxed.

Abbreviations used: *ALG1*, asparagine-linked glycosylation gene 1 (yeast β -1,4-mannosyltransferase gene); *Alg1ΔTM*, β -1,4-mannosyltransferase gene product with residues 2–35 deleted; *Dol-P-Man*, dolichyl phosphate mannose; *DPGn2* or *PPGn2*, dolichyl- or phytanyl-pyrophospho- α -*NN'*-diacetylchitobioside; *DPGn2(M)** or *PPGn2(M)**, partially β -mannosylated forms of *DPGn2* or *PPGn2* respectively, carrying a radiolabel (^3H) at the 2-position in the mannose residue; *HPAEC*, high-pressure anion-exchange chromatography; *g.u.*, glucose units; *PDRS*, putative dolichol recognition sequence; *ER*, endoplasmic reticulum.

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Figure 2 Structures of dolichol (1) and phytanol (2)

action of any translocating protein, or in recognition or activity of some or all of the glycosyltransferases in the pathway.

Indeed, a dolichol recognition sequence was proposed on the basis of sequence alignments [3] of glycosyltransferases. Some recent experimental evidence would suggest that this region of at least some of the enzymes utilizing dolichyl-linked substrates [β -1,4-mannosyltransferase and Dol-*P*-Man (dolichyl phosphate mannose) synthetase] is either not required or not crucial for enzyme activity [4,5], although there is some evidence to suggest some necessary recognition in the case of *N*-acetylglucosamine-1-phosphate transferase [6]. Previous studies have demonstrated that the dolichyl moiety of the substrates for Dol-*P*-Man synthetase and β -1,4-mannosyltransferase can be substituted by phytanol (2, Figure 2), a shorter, fully saturated and, in terms of chain length, homogeneous polyisoprenol [7–9]. Also, deletion of the entire transmembrane domain of β -1,4-mannosyltransferase appears to have no effect on enzyme activity with a phytanyl-linked substrate [4], whereas other data suggest that the enzyme cannot be inhibited by phytanyl-*NN'*-diacetylchitobioside (lacking the pyrophosphate bridge), *NN'*-diacetylchitobiose or *NN'*-diacetylchitobiose phosphate [10]. The experiments reported here have been directed at examining whether other mannosyltransferases can accept phytanyl-linked substrates and whether the deletion of the transmembrane domain of β -1,4-mannosyltransferase still allows transfer to the natural dolichyl-linked substrate. The data could be interpreted as suggesting that dolichol may be important not so much as a necessary component of the substrate for endoplasmic reticulum glycosyltransferases, but as a flexible, highly hydrophobic anchor with an, as yet, undefined rôle.

EXPERIMENTAL

Reagents

Plasmid pRA106 [11] was kindly given by Professor P. W. Robbins (Massachusetts Institute of Technology, Cambridge, MA, U.S.A.). GDP-[2-³H]mannose was purchased from du Pont–New England Nuclear (Stevenage, Herts., U.K.); exoglycosidases were from Oxford GlycoSystems (Abingdon, Oxfordshire, U.K.); dolichol, used in the synthesis of dolichylpyrophospho- α -*NN'*-diacetylchitobioside (DPGn2), was purified from porcine liver [12] and phosphorylated with phosphorus oxychloride in the presence of triethylamine, to form the phosphate dichloride, which was subsequently hydrolysed to give dolichyl phosphate [13]; the mannosyltransferase substrates phytanylpyrophospho- α -*NN'*-diacetylchitobioside (PPGn2) and

DPGn2 were synthesized according to previously published procedures [4,14] (spectroscopic data were in agreement with those quoted in the literature); anhydrous hydrazine, used to release standard oligosaccharides, was from Pierce and Warriner (Chester, Cheshire, U.K.); Ultima Gold scintillation fluid from Packard (Pangbourne, Berkshire, UK); His·bind resin from AMS Biotechnology (Witney, Oxfordshire, U.K.); all other chemicals and proteins were from Sigma, Aldrich or Fluka. Oligomannose standards were prepared by hydrazinolysis of egg white (Man₃₋₇GlcNAc₂; Man₁GlcNAc₂ was subsequently prepared by jack-bean (*Canavalia ensiformis*) α -mannosidase treatment of Man₃GlcNAc₂), ribonuclease (Man₂₋₉GlcNAc₂) and soybean agglutinin (Man₆GlcNAc₂) based on the procedure described by Kobata and co-workers ($\approx$ 25 mg of protein/ml of anhydrous hydrazine; final temperature 85 °C, 18 h; with purification by chromatography on Bio-Rad AG50WX8, Merck microcrystalline cellulose and, finally, Bio-Gel P-4) [15].

Sources of glycosyltransferase activities

Recombinant *Escherichia coli* strain TG1 or strain BL21(DE3)-pLysS transformed with, respectively, plasmid pLR23 or pLR36 and expressing modified forms of yeast β -1,4-mannosyltransferase (Alg1 Δ TM or His10-myc-Alg1 Δ TM) were grown as previously described for pLR23 [4]. The generation of the transmembrane-deleted asparagine-linked glycosylation gene 1 (ALG1) mutant, Alg1 Δ TM (lacking codons 2–35), from the pRA106 template by PCR, and the subsequent construction of pLR23 have been previously described [4]; details of the construction of the pET16b-derived plasmid pLR36 encoding a transmembrane-deleted form of β -1,4-mannosyltransferase with N-terminal His-tag and Myc sequences can be obtained from S. L. F. on request. Microsomal fractions were prepared from *Saccharomyces cerevisiae* (strain X2180-A, A.T.C.C. 26786) with a French press [8]. Rat liver microsomes were prepared by a method based on that of Forsee and Schutzbach [16]. Briefly, four male Wistar rats ($\approx$ 150 g, starved overnight) were killed by cranial fracture and the livers excised and washed in 50 mM Tris/acetate (pH 7.0)/0.25 M sucrose/1 mM EDTA/1 mM GSH/5 mM MgCl₂. The livers were then homogenized in the same buffer using an Atomix blender for 150 s and debris was removed by centrifugation at 10000 *g* (4 °C, 20 min). The microsomes were recovered as a pellet after centrifugation at 100000 *g* (4 °C, 60 min), homogenized in a minimal amount of buffer and stored at –80 °C.

Preparation of [³H]mannosylated lipid-linked oligosaccharides

Phytanylpyrophospho- α -*NN'*-diacetylchitobiosyl β -1,4-[³H]-mannoside (PPGn2M*) or the corresponding dolichyl-linked compound (DPGn2M*) was prepared by incubation of 50 nmol of PPGn2 and 150 nmol (1.5 μ Ci) of GDP-[2-³H]mannose with homogenate of *Escherichia coli* expressing the modified yeast β -1,4-mannosyltransferase (ALG1 Δ TM) gene [1% (v/v) Triton X-100, 37 °C, 15 min] or an immobilized form of β -1,4-mannosyltransferase (His10-myc-Alg1 Δ TM) bound to Ni(II)-charged His·bind resin (0.1% Triton X-100, 37 °C, 15 min). The reaction was terminated by addition of an equal volume of chloroform/methanol (1:1, v/v), centrifuged, and the particulate material washed twice with 1 vol. of water and 0.5 vol. of chloroform/methanol. Then, 1.8 vol. of methanol/water (5:1, v/v) was added to the remaining organic phase and particulate material at the interface. The particulate material was pelleted and the chloroform/methanol/water phase retained. This phase

Scheme 1 Formation of PPGn2M* using recombinant *E. coli* harbouring plasmid pLR23

contained the majority of the transferred radioactivity. The pellet was washed with chloroform/methanol/water (10:10:3, by vol.) and the washings were combined. Typically, a 35% transformation of PPGn2 into PPGn2M (60% for DPGn2 into DPGn2M) was attained [hence the designations for the incompletely mannosylated compounds as PPGn2(M) and DPGn2(M)]. For further mannosylation, typically 2–5 nmol (40 000–100 000 d.p.m.) of *E. coli*-generated DPGn2(M)* or PPGn2(M)* were incubated with a 10-fold molar excess of unlabelled GDP-mannose, 1 ml of yeast microsomes (12 mg of protein) or rat liver microsomes and 0.25% Triton (37 °C, 2 h). The reaction was terminated and the DPGn2M5 or PPGn2M5 extracted as described above for PPGn2M. The conditions for low-concentration reactions are given in the Results and discussion section.

Analysis of lipid-linked oligosaccharides

The saccharide components of lipid-linked oligosaccharides were hydrolysed and prepared for the Bio-Gel P-4 column [8]. Aliquots of released oligosaccharides were also analysed by high-performance anion-exchange chromatography (HPAEC) using a Dionex DX300 system fitted with a CarboPak PA100 column and controlled by Dionex AI450 chromatography software. The analysis of oligosaccharides was performed by pulsed electrochemical detection, using the electrode potentiometric waveform supplied by the manufacturer. The standard conditions for analysis of oligomannosidic oligosaccharides were: 0–10 min, 0–30 mM sodium acetate in 50 mM NaOH; 10–40 min, 30 mM sodium acetate in 50 mM NaOH; 40–41 min, 30–0 mM sodium acetate in 50 mM NaOH. Oligosaccharides were digested with mannosidases as follows: *Aspergillus saitoi* α -1,2-mannosidase, 0.2 units/ml; *Canavalia ensiformis* α -mannosidase, 50 units/ml. Mannosidase digestions were performed for 16 h at 37 °C in a toluene atmosphere using the supplier's buffer. Digestion products were passed through resins as previously described [8] prior to analysis by HPAEC or chromatography on Bio-Gel P4.

Glucose oligomers, prepared by the partial acid hydrolysis of dextran [8], were used as an internal standard for Bio-Gel P4 chromatography. For HPAEC, oligomannose oligosaccharides, isolated from glycoproteins as described above, were used as an internal standard. Owing to some irreproducibility in the retention times of oligosaccharides over a period of time on HPAEC, a mixture composed of these oligomannose oligosaccharides (Man₁₋₅GlcNAc₂) was co-injected with each sample.

RESULTS AND DISCUSSION

Previous studies have indicated that phytanyl phosphate and PPGn2 can act as substrates for yeast Dol-P-Man synthetase and yeast β -1,4-mannosyltransferase respectively [8,9]. In order to study other mannosyltransferases involved in the biosynthesis of N-linked oligosaccharides, PPGn2(M)* was generated as described in the Experimental section using *E. coli* transformed with pLR23, expressing Alg1ATM (Scheme 1). The integrity of the product was checked by analysis of the oligosaccharide portion, following release from the lipid anchor, by Bio-Gel P-4 chromatography and HPAEC. The oligosaccharide was found to have a hydrodynamic volume corresponding to five glucose units (g.u.) (results not shown) and to be co-eluted with a Man₁GlcNAc₂ standard prepared by exoglycosidase treatment of Man₃GlcNAc₂ isolated from egg white, by HPAEC (Figure 3a), as expected. Incubation of yeast microsomes with PPGn2M* and a 10-fold molar excess of GDP-mannose resulted in a single radiolabelled oligosaccharide after mild acid hydrolysis. Bio-Gel P-4 chromatography indicated that the oligosaccharide product was of the correct hydrodynamic volume (8.5 g.u.) and had a Man₅GlcNAc₂ structure (results not shown). It was also co-eluted on HPAEC (Figure 3b) with a Man₅GlcNAc₂ standard generated from bovine ribonuclease B. The isomer of Man₅GlcNAc₂ isolated from ribonuclease B is the 'catabolic' isomer generated after the action of ER mannosidase and Golgi

Figure 3 HPAEC analysis and enzyme digestion of lipid-linked oligosaccharides

Oligosaccharides were released from their lipid anchor and passed through resins prior to their analysis. Fractions (0.5 min each) were collected and aliquots subjected to liquid-scintillation counting. Identification of the products was performed by co-injection of a mixture of oligomannose oligosaccharides as an internal standard. The chromatograms corresponding to the elution positions of the radiolabelled components are shown: (a) Algl Δ TM-generated Man₁GlcNAc₂; (b) incubation of 4.5 nmol of Algl Δ TM-generated PPGn2(M)* with yeast microsomes and 45 nmol of unlabelled GDP-mannose (total incubation volume 1 ml); (c) product from (b) (Man₆GlcNAc₂) incubated with *Aspergillus saitoi* mannosidase; (d) result of digestion of product from (c) with *Canavalia* mannosidase; (e) Incubation of yeast microsomes with 10 nmol of PPGn2 and 30 nmol of radiolabelled GDP-[2-³H]mannose (final volume 1 ml); (f) incubation of rat liver microsomes with 10 nmol of PPGn2 and 20 nmol of labelled GDP-[2-³H]mannose (final reaction volume 1 ml).

mannosidase I and has no α 1,2-linked mannose residues [17], whereas the 'anabolic' isomer generated during the biosynthesis of the lipid-linked precursor has two α 1,2-linked mannose residues (the structure of the 'anabolic' isomer is shown boxed in Figure 1) [18,19]. Digestion of the yeast-generated putative Man₆GlcNAc₂ with *Aspergillus saitoi* mannosidase, which is specific for α 1,2-linked mannose linkages [20], reduced retention time by HPAEC to that of Man₃GlcNAc₂ derived from egg white (Figure 3c). Similarly, P-4 gel filtration chromatography indicated a shift from a structure corresponding to 8.5 g.u. to 6.7 g.u. (results not shown). Digestion with *Canavalia ensiformis* mannosidase, which has a broad specificity [21] and at 50 units/ml should cleave all structurally unhindered α -linked mannose residues, results in a structure that is co-eluted with Man₁GlcNAc₂ on both chromatography systems (see Figure 3d for the Dionex profile).

These results indicate that the expected anabolic isomer of Man₆GlcNAc₂ is generated. However, there was no indication of synthesis of Man₆₋₉GlcNAc₂ at the substrate concentrations used. This could be for one, or a combination, of the following

reasons: (i) the phytanyl-linked substrate is not translocated across the ER membrane; (ii) the phytanyl-linked substrate is translocated but is not a substrate for the mannosyltransferases which synthesize Man₆₋₉GlcNAc₂; (iii) there is an insufficiently large pool of Dol-*P*-Man to act as donor for the subsequent mannosylation steps; (iv) some or all of the mannosyltransferases which synthesize Man₆₋₉GlcNAc₂ are not active in these preparations; (v) the transferases responsible for the synthesis of Man₆₋₉GlcNAc₂ are present at too low concentrations for products to be detected at the levels of substrate specific radioactivity used. To address these possibilities, the natural dolichyl-linked substrate was chemically synthesized as a control, and Dol-*P*-Man was generated by enzymic mannosylation.

The immobilized form of β -1,4-mannosyltransferase (His10-myc-Algl Δ TM) was used to generate DPGn2(M)* as well as PPGn2(M)*. Higher percentage yields (based on the concentrations of substrate as estimated by NMR) after 30 min incubation were found when DPGn2 (60% transformation) was used as the β -1,4-mannosyltransferase substrate rather than PPGn2 (35% transformation; data not shown). The analysis of

Figure 4 Comparison of DPGn2 and PPGn2 as substrates

Reactions were carried out as described in the Experimental section. Oligosaccharides were released from the lipid prior to analysis by Bio-Gel P4 gel-filtration chromatography. Fractions were taken at 6 min intervals and aliquots subjected to liquid-scintillation counting. The position of glucose oligomers (internal standard) are indicated by the bars above the chromatographic traces: (a) His10-myc-Alg1 Δ TM-generated DPGn2(M)*; (b) His10-myc-Alg1 Δ TM-generated PPGn2(M)*; (c) incubation of 1.5 nmol of DPGn2(M)* with yeast microsomes in the presence of 200 nmol of unlabelled GDP-mannose (final volume 1 ml); (d) incubation of 1.5 nmol of PPGn2(M)* with yeast microsomes and 200 nmol of unlabelled GDP-mannose (reaction volume 1 ml); (e) incubation of 1.5 nmol of DPGn2(M)* with rat liver microsomes and 200 nmol of unlabelled GDP-mannose (final volume 1 ml); (f) incubation of 1.5 nmol of PPGn2(M)* with rat liver microsomes and 200 nmol of unlabelled GDP-mannose (final volume 1 ml).

the products after mild acid hydrolysis indicated products of 5 g.u. with both DPGn2 and PPGn2 as determined by Bio-Gel P-4 chromatography (Figures 4a and 4b). The anomeric specificity of the reaction was confirmed by digestion of the oligosaccharide with snail (*Helix pomatia*) β -mannosidase (results not shown). Thus, even with the dolichyl-linked substrate, the previously published results showing that the transmembrane domain of β -1,4-mannosyltransferase is not required for enzymic activity [4] are verified with the novel His10-myc-Alg1 Δ TM form of the enzyme. The convenience of use of the immobilized enzyme is being examined with larger-scale syntheses of PPGn2M. PPGn2 was also demonstrated to be a substrate for the native microsomal form of β -1,4-mannosyltransferase from both yeast and rat liver. Incubation of yeast microsomes with 10 nmol of PPGn2 and 30 nmol of GDP-[2- 3 H]mannose resulted in a range of structures from Man₅GlcNAc₂ down to Man₁GlcNAc₂ (Figure 3e). The Man₂₋₅GlcNAc₂ structures are in addition to those found by Flitsch et al. [9] when using the same PPGn2 substrate, since an excess of GDP-mannose was used. With rat liver, similar incubations indicated that the main structure was eluted at the

position expected for Man₁GlcNAc₂ on both HPAEC (Figure 3f) and gel-filtration systems.

At the level of 5 nmol, DPGn2(M)* and PPGn2(M)*, generated using His10-myc-Alg1 Δ TM, were incubated in parallel, in the presence of unlabelled GDP-mannose with both yeast and rat microsomes. In the absence of dolichyl phosphate (Dol-P) or Dol-P-Man, mainly Man₅GlcNAc₂ was formed by both sources of microsomes as judged by Bio-Gel P-4 chromatography of the products of mild acid hydrolysis (Figures 4c–4f). Again, at this concentration, no evidence for the synthesis of more extensively mannosylated forms was found for either dolichyl- or phytanyl-linked substrates. Addition of Dol-P-[2- 3 H]Man to the incubations of PPGn2(M)* and DPGn2(M)* with GDP-mannose and yeast microsomes indicated no significant elongation of Man₅GlcNAc₂ to higher structures (results not shown). A point of interest that we have observed during the acid hydrolysis of lipid-linked oligosaccharides is the formation of 1-O-ethers. Using the method of Chambers et al. [22] for the release of oligosaccharides from their lipid anchors, we found some evidence for the formation of 1-O-propyl ethers at the reducing

Figure 5 Comparison of DPGn2 and PPGn2 at low substrate concentrations

Experiments were performed as described in the Results and discussion section. Oligosaccharides were released from their lipid anchor prior to analysis by Bio-Gel P4 chromatography. Fractions (6 min each) were collected and subjected to liquid-scintillation counting. Glucose oligomers were used as an internal standard and their positions are indicated: (a) His10-Myc-Alg1 Δ TM-generated DPGn2(M)* (—); subsequently, 50% of pooled oligosaccharides were treated with β -mannosidase (---); (b) His10-Myc-Alg1 Δ TM-generated PPGn2(M)* (—), 50% of pooled oligosaccharides were treated with β -mannosidase (---); (c) Incubation of 0.05 nmol of DPGn2(M)* with yeast microsomes and 5 nmol of unlabelled GDP-mannose (total volume 1.05 ml); (d) Incubation of 0.05 nmol of PPGn2(M)* with yeast microsomes in the presence of 5 nmol of unlabelled GDP-mannose (total volume 1.05 ml); (e) digestion of product of (c) with *Canavalia* α -mannosidase; (f) digestion of product of (d) with *Canavalia* α -mannosidase.

terminus of the oligosaccharides when propan-1-ol was used as the co-solvent in these reactions. This observation does not seem to have been previously reported. The generation of these ethers adds about 1 g.u. to the hydrodynamic volume of the oligosaccharides (the minor peak at 6 g.u. seen in Figures 4a and 4b, and at 10 g.u. in Figures 4c–4f). These ‘artefactual’ peaks hampered our determination of the extent of elongation of DPGn2(M)* and PPGn2(M)*. Subsequently, it was found that the release of oligosaccharides was as efficient in the absence of any co-solvent and hence, for further hydrolyses, no co-solvent was added. Thus, acid hydrolysis of lipid-linked oligosaccharides generated by the action of His10-Myc-Alg1 Δ TM on DPGn2 and PPGn2 respectively in water only prevented the formation of ethers at the anomeric centre and hence led to the disappearance of peaks at 1 g.u. larger than the released oligosaccharides (see, e.g., Figures 5a and 5b).

In the final set of experiments, much lower concentrations (but higher specific radioactivities) of DPGn2(M)* and PPGn2(M)* were used. The integrity of the His10-Myc-Alg1 Δ TM-generated

products was confirmed following release of the oligosaccharides, by acid hydrolysis in the absence of co-solvent, by P4 chromatography (Figures 5a and 5b respectively; —). A single radioactive peak, corresponding to the Man₁GlcNAc₂ structure was observed. Treatment of these oligosaccharides with β -mannosidase from *H. pomatia* resulted in a shift of the radioactivity to 0.9 g.u., consistent with the release of a terminal-linked β -mannose residue (Figures 5a and 5b respectively; ---). With incubations of 0.05 nmol of DPGn2(M)* or PPGn2(M)*, 5 nmol of GDP-mannose and 1 ml of yeast microsomes, Man₆GlcNAc₂ and Man₉GlcNAc₂ could be detected by Bio-Gel P4 chromatography, after mild acid hydrolysis of the products, with both dolichyl- and phytanyl-linked substrates. However, the major peak still corresponded to Man₆GlcNAc₂ (Figures 5c and 5d). Since activity of the transferases responsible for the synthesis of Man₆GlcNAc₂ could be detected, the explanation for the lack of these oligosaccharides in the previous analyses would suggest that these transferases are of far lower activity in our microsomal preparations than the transferases responsible for the synthesis

of $\text{Man}_{1-5}\text{GlcNAc}_2$. Digestion of both the formerly dolichyl- and phytanyl-linked oligosaccharides with *Canavalia* mannosidase resulted in a single peak of 5 g.u. (Figures 5e and 5f), thus offering evidence for the addition of up to eight α -linked mannose residues. About 10% of the radioactivity from these incubations of either DPGn2(M)* or PPGn2(M)*, appeared to be associated with the insoluble pellet (after extraction of the incubation mixture as described in the Experimental section), thus suggesting possible transfer to protein. However, the levels of radioactivity would have been too low in order to verify efficiently whether N-linked glycosylation had occurred and which structures had, if any, been transferred. Further studies will be required in order to check which conditions are necessary for complete conversion of both DPGn2M and PPGn2M into lipid-linked $\text{Man}_6\text{GlcNAc}_2$ and, also, if any transfer to protein occurs. The possibility that lack of Dol-P-Man was responsible for the low amounts of $\text{Man}_{6-9}\text{GlcNAc}_2$ was discounted by experiments in which 0.05 nmol of DPGn2 or PPGn2 (or no exogenous lipid) was incubated with yeast microsomes and labelled GDP-[2- ^3H]mannose (the resulting oligomannose structure now has the radiolabel uniformly distributed throughout the added mannose residues). By far the major product after mild acid hydrolysis was of just under 1 g.u. (typical for a single mannose residue). This indicated that most of the mannose label had been incorporated into Dol-P at the concentration of exogenous DPGn2 or PPGn2 used (Figures 6a–6c). This peak, corresponding to labelled mannose, is notably absent in incubations of DPGn2 or PPGn2 with *E. coli* homogenates (Figures 4a, 4b, 5a and 5b) and in incubations of PPGn2 with yeast or rat liver microsomes and higher concentrations of GDP-[2- ^3H]mannose (Figures 3e and 3f), confirming that the [2- ^3H]mannose had been derived from Dol-P-Man and not from acid hydrolysis of GDP-[2- ^3H]mannose that remained 'unwashed' from the chloroform/methanol/water extract. It also demonstrates the value of using the recombinant system to generate radiolabelled DPGn2(M)* or PPGn2(M)* as probes for the N-glycosylation pathway.

The results of the present study would suggest that there is no requirement for the Dol moiety of the substrates for the first five (and possibly all nine) mannosyltransferases of the ER. No difference could be discerned between the use of PPGn2(M) and DPGn2(M) as substrates for the α -mannosyltransferases. Also, the previous results, indicating that the putative transmembrane domain is not required for enzymic activity [4], have been extended to indicate that the natural dolichol-linked substrate for β -1,4-mannosyltransferase is an acceptor even in the absence of the putative dolichol recognition sequence. This would suggest that the rôle of dolichol in the biosynthesis of N-linked oligosaccharides is not so much a matter of enzyme–substrate recognition, but suggests that its rôle is less defined. For instance, since phytanyl-linked substrates are more readily solubilized in water, it is possible that dolichyl-linked oligosaccharides have a much more clearly defined amphipathicity, thus indicating a better anchoring function for dolichol. Also, even though a saturated α -isoprene unit appears important for some of the glycosyltransferases ([9] and references cited therein), the presence of subsequent *cis* and *trans* unsaturated units may confer flexibility which may play a rôle, *in vivo*, in translocation ([2] and references cited therein). It is conceivable, in a detergent-solubilized system, that translocation may not be necessary for mannosylation to occur, although any impairment of translocation may affect the overall flux through the pathway. In comparison with studies on the Lec35 Chinese-hamster ovary cell line mutant which can synthesize Dol-P-Man, but is defective in the synthesis, *in vivo*, of $\text{Man}_6\text{GlcNAc}_2$ [23] and in glycosylphosphatidylinositol biosynthesis [24], proteins analogous in

Figure 6 Comparison of incubation of DPGn2 or PPGn2 as substrates with 5 nmol of labelled GDP-[2- ^3H]mannose in the presence of yeast microsomes (final reaction volume 1 ml)

Experiments were performed as described in the Results and discussion section. Uniformly labelled oligosaccharides in the mannose residues were hydrolysed and analysed by Bio-Gel P4 chromatography. Fractions (6 min each) were taken, and aliquots were further analysed by liquid-scintillation counting. Glucose oligomer positions are marked: (a) 0.05 nmol of DPGn2; (b) 0.05 nmol of PPGn2; (c) blank (radiolabelled GDP-[2- ^3H]mannose incubated with yeast microsomes in the absence of any exogenous lipid-linked substrate). The peak at 1 g.u. is derived from the formation of Dol-P-Man from endogenous Dol-P.

function to a Lec 35 gene product may be necessary for translocation of dolichyl-linked $\text{Man}_6\text{GlcNAc}_2$ [25] and of glycosylphosphatidylinositol intermediates [26] which are formed on the cytoplasmic face, but are substrates for enzymes and are transferred subsequently to protein on the luminal side of the ER. Another possible explanation, not addressed in the present study, for the requirement for dolichol as a 'cofactor' in oligosaccharide biosynthesis, is that the substrate requirements of the three glucosyl- and two *N*-acetylglucosaminyl-transferases (for a review, see [27] and references cited therein) and of oligosaccharyltransferase may differ from those of the mannosyltransferases. Thus overall, as a result of a variety of factors, evolution may have favoured dolichol, over any other candidate, as the optimal lipid anchor for assembly of N-linked oligosaccharides.

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