The role of vitamin A in the glycosylation reactions of glycoprotein synthesis in an ‘*in vitro*’ system

Victor T. CHAN* and George WOLF†

Dept. of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, U.S.A.

Microsomal membrane preparations from rat livers, when incubated with labelled sugar-nucleotides, were shown to synthesize labelled oligosaccharide-lipids in the presence of excess exogenous dolichyl phosphate. Under the incubation conditions defined in the present study, dolichyl pyrophosphoryl(DolPP)GlcNAc₂-Man₅, DolPPGlcNAc₂Man₉ and DolPPGlcNAc₂Man₉Glc₃ were the principal oligosaccharide-lipids formed by both control and vitamin A-deficient membranes. However, deficient membranes synthesized 3.2 ± 0.8 times as much oligosaccharide-lipids and 2.6 ± 0.7 times as much dolichyl phosphate mannose (DolPMan) and dolichyl phosphate glucose (DolPGlc) as the controls. The transfer of the oligosaccharide chain from the dolichol carrier to the endogenous protein acceptors in vitamin A-deficient microsomes (microsomal fractions) was only $57.5 \pm 9.5\%$ of that of controls. After endo- β -N-acetylglucosaminidase treatment, only one oligosaccharide species was isolated from both control and vitamin A-deficient microsomal glycoproteins, and was characterized as GlcNAcMan₉Glc₃. We conclude that the decreased incorporation of labelled mannose and glucose from sugar-nucleotides into the glycoproteins must be due to decreased transfer of GlcNAc₂Man₉Glc₃ from the dolichol carrier to the protein acceptors. This conclusion was further substantiated by the finding that control membranes transferred 4–6 times as much labelled oligosaccharides from exogenously added dolichol-linked substrate (DolPPGlcNAc₂Man₉Glc₃) to endogenous microsomal protein acceptors as compared with the vitamin A-deficient membranes. Attempts to reverse this defect by addition of retinol or retinyl phosphate (a source of retinyl phosphate mannose) to the incubations were unsuccessful.

INTRODUCTION

Reports from several laboratories have demonstrated that the oligosaccharide moiety of N-linked glycoproteins involves transfer *en bloc* of a preformed oligosaccharide chain (GlcNAc₂Man₉Glc₃) linked to a polyisoprenoid lipid carrier [dolichyl pyrophosphate (DolPP)] to a nascent peptide chain. This peptide-bound oligosaccharide is then processed by removal, and then further addition, of sugars to form either the high-mannose or the complex-type glycoprotein [1,2]. Dolichyl phosphate (DolP) is also the lipid carrier of the mannose donor, dolichyl phosphate mannose (DolPMan), for the addition of the last four mannose moieties to the growing oligosaccharide chain [3].

Recently, an important regulatory site of the lipid-mediated glycosylation of proteins has been found to be at the synthesis of lipid-linked oligosaccharides (OLL) [4–9]. The discovery of a retinyl mannanolipid (retinyl phosphate mannose, RetPMan) by De Luca *et al.* [10], and the study *in vivo* by Rosso *et al.* [11] showing that vitamin A deficiency caused accumulation of DolPP-GlcNAc₂Man₅, led to the formulation of a hypothesis that vitamin A might regulate the synthesis of OLLs. In the present work an ‘*in vitro*’ model was used in order to avoid the possibility of other physiological influences caused by vitamin A deficiency, which occurs in the ‘*in*

vivo’ model. We isolated a rough endoplasmic reticulum (RER) membrane fraction from the livers of vitamin A-deficient and pair-fed control rats to study the effect of vitamin A deficiency on the following lipid-linked glycosylation reactions in glycoprotein synthesis: the synthesis of DolPMan, DolPGlc, RetPMan and OLL, and the transfer of oligosaccharide chains to the protein acceptors.

MATERIALS AND METHODS

DolP (grade III), UDP-GlcNAc (sodium salt), UDP-glucose (from yeast), GDP-mannose (type III), AMP (type III), ATP (from equine muscle), BSA (fatty-acid- and globin-free) and β -mercaptoethanol (type I) were obtained from Sigma (St. Louis, MO, U.S.A.), UDP-[³H]glucose (10.4 Ci/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.), and GDP-[¹⁴C]mannose from Dr. P. W. Robbins (Center for Cancer Research, this Institute). Chemically synthesized RetP was a gift from Dr. L. De Luca (National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.) made as described by Bhat *et al.* [12]. Endo- β -N-acetylglucosaminidase H from *Streptomyces plicatus* (specific activity 40 units/mg) was obtained from Genzyme (Boston, MA, U.S.A.), and Triton X-100 (enzyme

Abbreviations: DolPMan, dolichyl phosphate mannose; DolPGlc, dolichyl phosphate glucose; DolP, dolichyl phosphate; RetPMan, retinyl phosphate mannose; RetP, retinyl phosphate; OLL, oligosaccharide-lipid; DolPP, dolichyl pyrophosphoryl; BSA, bovine serum albumin; RER, rough endoplasmic reticulum; endo-H, endo- β -N-acetylglucosaminidase H; PMSF, phenylmethanesulphonyl fluoride.

* Present address: Dana Farber Cancer Institute, Boston, MA 02115, U.S.A.

† To whom correspondence and reprint requests should be sent.

grade) was from Schwarz-Mann (Orangeburg, NY, U.S.A.).

Albino rats (Holtzman strain) were from Charles River Laboratories (Wilmington, MA, U.S.A.). Silica-Gel 60_{F-254} t.l.c. plates (0.25 mm thick) from Merck (Darmstadt, Germany) were obtained from Brinkman (Westbury, NY, U.S.A.).

Preparation of vitamin A-deficient and pair-fed control rats

Vitamin A deficiency was induced in 20-day-old male weanling Holtzman rats (30–40 g) by the method of Kiorpes *et al.* [13]. The onset of vitamin A deficiency was taken to be the time that the deficient rats had lost 10–15% of body weight and showed other symptoms of vitamin A deficiency (eye lesions, oily fur and impairment of walking pattern). Only animals without any signs of infection were sampled for experimentation.

Preparation of liver microsomal membranes

The rats were fasted overnight and killed by decapitation. The livers were homogenized in 50 mM-Tris/HCl buffer, pH 7.4 (or pH 6.9 in later experiments as indicated), with 0.25 M-sucrose (4 ml of buffer/g liver). The homogenate was centrifuged at 1000 *g* for 10 min, the supernatant fraction at 10000 *g* for 15 min and the resulting supernatant fraction at 48000 *g* for 60 min. The 48000 *g* pellet representing the microsomal membrane fraction was resuspended in 50 mM-Tris/HCl buffer, pH 7.4 (pH 6.9 in later experiments). The membrane fraction was used as an enzyme source and was prepared fresh for every experiment. The pellet obtained at 48000 *g* had higher (30%) specific enzyme activity than the 100000 *g* pellet (results not shown). Microsomal protein concentration was determined by the method of Lowry *et al.* [14], with BSA as standard.

Incubation conditions

The standard incubation mixture contained 175 μ M exogenous DolP in Triton X-100 (final concn. 0.05%), 7.5 μ M-GDP-[¹⁴C]mannose (5 μ Ci) for mannose labelling or UDP-[³H]glucose (5 μ Ci) for glucose labelling, 30 μ M-UDP-glucose, 30 μ M-UDP-GlcNAc, 4 mM-ATP, 5 mM-AMP, 8 mM-NaF, 5 mM-MgCl₂, 5 mM-MnCl₂ and liver microsomal membranes (3–4 mg of protein) in 50 mM-Tris/HCl buffer, pH 7.4. The final volume was 0.3 ml. The reaction was started by the addition of microsomal membranes and incubation was at 37 °C for 12.5 min or indicated times. In the later experiments, as indicated in the Results section, a pH of 6.9 was used for all preparations and incubations.

Extraction of OLLs

The incubation described above was stopped by addition of a 20-fold volume of cold chloroform/methanol (2:1, v/v) with gentle mixing to denature the microsomal proteins and to remove the phospholipids. The suspension was left at 4 °C for 30 min without further mixing, then centrifuged in a table-top centrifuge for 10 min (600 *g*), and the supernatant fraction was collected. This was followed by two extractions with 2 ml of cold chloroform/methanol (2:1, v/v) as in the previous step. The three extracts were combined and saved for t.l.c. analysis of DolPMan and DolPGlc synthesis. Next, three additions of 2 ml of cold water were applied to

wash out the residual salts and labelled substrate at 30 min intervals at 4 °C with occasional gentle mixing; these washings were discarded. The pellet was then partially dried with a stream of N₂. The OLL was extracted three times with 3 ml of chloroform/methanol/water (10:10:3, by vol.) at room temperature at 30 min intervals with vigorous vortex-mixing, and the pellet was dispersed by sonication at the first extraction. The combined supernatants were dried with a stream of nitrogen. The residue was subjected to mild acid hydrolysis for 45 min at 100 °C in 2 ml of 0.02 M-HCl [15] (to produce oligosaccharide from OLL) and freeze-dried ready for h.p.l.c. fractionation.

H.p.l.c. fractionation of oligosaccharides

H.p.l.c. fractionation was carried out on Beckman model 344 h.p.l.c. system with Bio-Sil Amino-5S 4 mm $\times$ 250 mm column (Bio-Rad Laboratories) with an amino-guard column. The starting solvent condition was acetonitrile/water (7:3, v/v); a linear gradient to final proportions of 2:3 over 60 min was then used. The flow rate was 1 ml/min, and 0.4 ml fractions were collected; radioactivity was determined by liquid-scintillation counting. The principal oligosaccharide peaks were identified by comparison of the retention time of, and co-migration with, known standards. Standards (GlcNAc₂-Man₅, GlcNAc₂-Man₉ and GlcNAc₂-Man₉-Glc₃) were obtained as a gift from Dr. C. S. Hubbard (Center for Cancer Research, this Institute). Total OLL synthesis is reported as a summation of h.p.l.c. peaks from GlcNAc₂-Man₅ to GlcNAc₂-Man₉-Glc₃. Results are reported as means for duplicate experiments, differences being less than 10% of the mean.

T.l.c. analysis of DolPMan, DolPGlc and RetPMan synthesis

DolPMan, DolPGlc and RetPMan were analysed by t.l.c. [16]. The radiolabelled bands corresponding to DolPMan, DolPGlc and RetPMan were scraped off and counted for radioactivity with Liquiscint. The results were reported as means for duplicate experiments, differences being less than 10% of the mean; repeated experiments showed similar results.

Endo- β -N-acetylglucosaminidase H (endo-H) digestion

After extractions, the protein pellet was partially dried and extracted with buffer (1% SDS/0.15 M-NaCl/0.02% NaN₃/25 μ M-PMSF/10 mM-Tris/HCl, pH 7.1) for 1 h at 70–90 °C with occasional sonication. The suspension was centrifuged in a microcentrifuge at room temperature for 15 min. The residual pellet was counted for radioactivity; it accounted for only 14–20% of the radioactivity left unextracted. The glycoprotein in the supernatant was re-precipitated with 20 vol. of cold acetone (–20 °C overnight) in order to remove the excess salt, SDS and radiolabelled non-protein compounds. Then the glycoprotein pellet was dried and redissolved in endo-H incubation buffer with minimal SDS (0.1 M-citrate buffer, pH 5.6, with 0.2 mM-PMSF and 0.02% NaN₃) [17,18]. The suspension was sonicated at 70–90 °C in a water bath and SDS was added dropwise until the suspension cleared (the final SDS concentration was less than 0.05%). Then this clear solution was heated at 90–95 °C for 3 min with 0.1 M- β -mercaptoethanol [19]. After the mixture had been cooled to room temperature, endo-H was added at a final

concentration of 150 munits/ml and it was incubated overnight at 37 °C in a shaking waterbath.

After endo-H digestion, the mixture was precipitated with 20 vol. of chloroform/methanol/water (10:10:3, by vol.) and the protein pellet was removed and counted for radioactivity after being dissolved with Protosol (New England Nuclear). The radioactivity left in the protein accounted for only 5–9% of the total. The chloroform/methanol/water mixture was dried in a stream of N₂ and the residue was dissolved in 0.1 ml of water. Excess SDS (which could affect h.p.l.c. analysis) from this final step was removed by microcentrifugation at 4 °C [18]. The remaining solution was subjected to h.p.l.c. analysis.

Preparation of [*glucose*-³H]OLL substrate

The [*glucose*-³H]OLL was prepared by incubation of vitamin A-deficient microsomes (microsomal fractions) with UDP-[³H]glucose (30 μCi) and the standard incubation mixture. The incubation was scaled up 3-fold. After incubation, the OLL was extracted and hydrolysed as described above. The resulting oligosaccharide was then analysed by h.p.l.c. to check for purity, DolPP-GlcNAc₂Man₉[³H]Glc₃ accounting for 87% of the total [³H-glucose]OLLs.

Assay for transfer-labelled oligosaccharide to protein acceptors

The reaction mixture contained [*glucose*-³H]OLL (3880 c.p.m.), 5 mM-MgCl₂, 5 mM-MnCl₂, 2 mM-dithiothreitol and different concentrations of Triton X-100 (as shown) in 50 mM-Tris/HCl, pH 6.9 (adapted from [17]). The reaction was initiated by the addition of 800 μg of microsomal protein to the incubation mixture, the final volume of which was 150 μl and which was incubated at 30 °C for 10 min. After incubation, the microsomal protein was sonicated and washed intensively with chloroform/methanol (2:1, v/v), water, and chloroform/methanol/water (10:10:3, by vol). Then the pellets were dissolved with Protosol and the radioactivity was determined after addition of Econofluor and dark-adaptation for 10 h.

RESULTS

Effect of exogenous DolP on OLL formation

When an isolated membrane fraction (mainly RER) from rat liver was incubated with the sugar-nucleotide mix (UDP-*N*-acetylglucosamine, UDP-glucose and GDP-[¹⁴C]mannose, plus Mn²⁺ and Mg²⁺), only trace amounts of labelled oligosaccharides could be obtained; however, in the presence of exogenous DolP, OLL synthesis by the RER fraction was greatly enhanced and showed concentration-dependence (Fig. 1). A concentration of 170 μM (0.1 mg/0.4 ml) DolP, at the lowest saturation point, was used routinely. In the presence of 4 mM-ATP, total OLL synthesis was enhanced by 30–35%. Fractionation of the oligosaccharide mixture revealed that this enhancement was not due to the increase of any particular OLL. It is not likely that ATP is involved in the reactions of OLL synthesis, but it probably enhances OLL synthesis by its protective role on GDP-mannose.

OLL synthesis in vitamin A-deficient and pair-fed control membranes from rat livers

When incubations were performed at pH 7.4, the time course of incorporation of labelled mannose into OLL

Fig. 1. Requirement of exogenous DolP for the synthesis of OLLs

DolP was dissolved in Triton X-100 (final concn. 0.05%). OLLs were obtained from incubation of membranes from livers of normal rats with GDP-[¹⁴C]mannose at pH 7.4, and were hydrolysed to release free oligosaccharides, which were fractionated by h.p.l.c. After h.p.l.c. fractionation, total OLL was calculated by subtracting the mannose peak from the total radioactivity obtained. Each value represents the mean for duplicate experiments, with differences less than 10% of the mean.

was as shown in Fig. 2. Deficient membranes incorporated 1.6–2.0 times as much labelled mannose as compared with control membranes. H.p.l.c. fractionation of the oligosaccharide mixture after acid hydrolysis of the OLLs revealed that DolPPGlcNAc₂Man₅ and DolPPGlcNAc₂Man₆ were the principal OLL formed by both deficient and control membrane preparations (Fig. 3).

Since only small amounts of glucosylated OLLs were formed under these incubation conditions, in contrast with the findings reported from cell cultures and tissue slices, and also because the amount of radioactivity incorporated into the endogenous glycoprotein was very small, it was essential to optimize DolPPGlcNAc₂Man₅-Glc₃ synthesis in order to study further the cause of the observed differences in OLL synthesis in deficient and control membranes. A recent report by Murphy & Spiro [18] showed that a relatively high pH, 7.4, and the presence of saline inhibited the activities of the glucosyltransferase(s) in thyroid slices by almost 70%.

Therefore a new series of experiments was carried out with incubations at pH 6.9 (instead of 7.4), the pH value at which activity of the glucosyltransferase(s) is optimal,

Fig. 2. Time course for total OLL synthesis by liver membrane preparations from vitamin A-deficient and pair-fed control rats

OLL were obtained from incubation of membranes with GDP-[^{14}C]mannose at pH 7.4, as described for Fig. 1.

Fig. 3. H.p.l.c. chromatogram of mannose-labelled oligosaccharides obtained from control membranes at pH 7.4

OLLs were obtained after incubation of membranes for 30 min with GDP-[^{14}C]mannose at pH 7.4, were hydrolysed to release free oligosaccharides and fractionated by h.p.l.c. Peaks I, II and III were standardized by co-chromatography with standards obtained from Dr. C. Hubbard (Center for Cancer Research, this Institute). Deficient membranes showed similar profiles. The large peak on the left is mannose from residual labelled substrate. Peaks: I, $\text{GlcNAc}_2\text{Man}_5$; II, $\text{GlcNAc}_2\text{Man}_9$, III, $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$.

and eliminating saline from all membrane preparations and incubation media. The h.p.l.c. profile was found to be altered. As shown in Figs. 4(a) and 4(b), $\text{DolPPGlcNAc}_2\text{Man}_5$, $\text{DolPPGlcNAc}_2\text{Man}_9$ and $\text{DolPPGlcNAc}_2\text{Man}_9\text{Glc}_3$ were the three principal peaks; the

Fig. 4. H.p.l.c. chromatogram of mannose-labelled oligosaccharides obtained from incubation of membranes at pH 6.9

OLLs were obtained from incubations of membranes with GDP-[^{14}C]mannose at pH 6.9, were hydrolysed to release free oligosaccharides and were fractionated by h.p.l.c. (Duplicate and repeat experiments showed similar results.) The peak at the left of the chromatogram is mannose from residual labelled substrate. Peaks I, II and III are as in Fig. 3. (a) Membranes from control rats; (b) membranes from vitamin A-deficient rats.

accumulation of $\text{DolPPGlcNAc}_2\text{Man}_9\text{Glc}_3$ was at the expense of $\text{DolPPGlcNAc}_2\text{Man}_9$. Under these incubation conditions (which were used from this point on), the result still holds that deficient membranes incorporated 2.0–2.5 times as much labelled mannose into total OLL as control membranes at both time points (5.0 and 12.5 min; Table 1). Fractionation of the oligosaccharide mixtures (Table 1) showed that this higher level of OLL synthesis in deficient membranes was contributed to by all OLL intermediates studied ($\text{DolPPGlcNAc}_2\text{Man}_5$ to $\text{DolPPGlcNAc}_2\text{Man}_9\text{Glc}_3$), not one at the expense of the other intermediates (Figs. 4a and 4b). Next we investigated the effect of deficiency by specifically labelling the glucose moiety, by incubating membranes with labelled UDP-[^3H]glucose. As shown in Figs. 5(a) and 5(b), we observed two labelled oligosaccharide peaks, a principal peak plus a minor peak with a shorter retention time (the first peak at the left of the chromatogram is free glucose, a contaminant from the residual labelled precursors). The principal peak was identified as $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$ by co-migration with a standard, and the minor peak was probably Glc_1 - or Glc_2 -oligosaccharide or both (since it was adjacent to $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$, with a lower M_r , and glucose was the only

Table 1. Synthesis of individual oligosaccharide lipid intermediates by liver membranes from vitamin A-deficient and pair-fed control rats at pH 6.9

OLLs obtained from incubation of control and deficient membranes with GDP-[¹⁴C]mannose at pH 6.9, for 5.0 or 12.5 min, were hydrolysed to release free oligosaccharides, and these were fractionated by h.p.l.c. Radioactivity of the individual oligosaccharide peaks of the h.p.l.c. chromatogram were computed by a program designed in our laboratory.

Membranes	Incubation time (min)	Radioactivity (c.p.m./per mg of microsomal protein)				
		GlcNAc ₂ Man ₅	GlcNAc ₂ Man ₆ – GlcNAc ₂ Man ₈	GlcNAc ₂ Man ₉	GlcNAc ₂ Man ₉ Glc ₁ – GlcNAc ₂ Man ₉ Glc ₃	GlcNAc ₂ Man ₉ – GlcNAc ₂ Man ₉ Glc ₃
Control	5.0	956	876	408	1368	3604
	12.5	1356	997	640	1564	4552
Deficient	5.0	2584	1396	880	2376	7232
	12.5	4616	2472	1868	2200	11 152
Deficient/control	5.0	2.7	1.6	2.2	1.7	2.0
	12.5	3.4	2.5	2.9	1.4	2.5

Fig. 5. H.p.l.c. chromatogram of glucose-labelled oligosaccharides obtained from incubation of membranes at pH 6.9

Procedures were as in Fig. 4, except that UDP-[³H]glucose was incubated instead of GDP-[¹⁴C]mannose. The peak at the left of the chromatogram is glucose from residual labelled substrate. Peak I is GlcNAc₂Man₉Glc₃. (a) Membranes from control rats; (b) from deficient rats.

label). When the radioactivity of the oligosaccharide peaks was determined, deficient membranes incorporated 1.6 times as much labelled glucose into OLL as did control membranes (results not shown).

In addition, because of the accumulation of DolPPGlcNAc₂Man₅ in vitamin A deficiency *in vivo* [11], a question has been raised as to whether deficient mem-

branes could glucosylate DolPPGlcNAc₂Man₅, as occurs in the E-1-negative mutant mouse lymphoma cell line [20]. Figs. 5(a) and 5(b) show that no such incomplete intermediate was formed. Only the normally glucosylated DolPPGlcNAc₂Man₉Glc₃ component in both control- and deficient-membrane preparations appeared.

DolPMan, DolPGlc and RetPMan

RetPMan has been hypothesized to be a mannose carrier assisting DolPMan synthesis for the elongation of the OLL intermediate DolPPGlcNAc₂Man₅ to DolPPGlcNAc₂Man₉Glc₃, its final product [11]. We attempted to explore such a mechanism in our isolated microsomal membrane system, and at the same time determined the effect of RetPMan on the whole series of glycosylation reactions in glycoprotein synthesis, i.e. formation of DolPMan, DolPGlc, OLL and protein glycosylation.

Three incubation conditions were studied: (1) exogenous RetP (1.7 × 10⁻⁴ M [21]) was preincubated with microsomal membranes and the substrate mixture for 5 min before exogenous DolP was added; (2) exogenous RetP was incubated together with DolP, microsomal membranes and the complete incubation mixture; and (3) the complete standard incubation mixture was incubated with microsomal membranes without exogenous RetP, as a control.

Table 2 summarizes the results obtained with GDP-[¹⁴C]mannose and UDP-[³H]glucose as radiolabels. Regardless of the incubation conditions or whether exogenous RetP was incubated at the same time or before the exogenous DolP was added, we did not detect any significant effect of RetP on any of the glycosyl intermediates being analysed (DolPMan, DolPGlc and total OLL). When oligosaccharide intermediates (from GlcNAc₂Man₅ to GlcNAc₂Man₉Glc₃) were examined individually after h.p.l.c., RetPMan did not show any effect on any particular oligosaccharide peak. Again, we observed that deficient membranes incorporated 2–4 times as much labelled mannose or glucose into glycosyl intermediates (DolPMan, DolPGlc) and total OLL. The increment was contributed to by the overall increase in all OLL intermediates. Furthermore, RetPMan synthesis from exogenous RetP did not differ significantly between control and deficient membranes. In addition (Table 3), when the resulting protein pellets from these experiments

Table 2. RetPMan synthesis from exogenous RetP and its effect on glycosylation reactions

RetP (final concn. 1.7×10^{-4} M) solution was prepared fresh and solubilized in BSA (4 mg/ml) [16]. The carbohydrate moiety was labelled with either GDP-[14 C]mannose or UDP-[3 H]glucose. RetPMan, DolPMan and DolPGlc were analysed by t.l.c. from chloroform/methanol (2:1, v/v) extractions. Labelled and/or fluorescent bands corresponding to RetPMan, DolPMan and DolPGlc were scraped off and counted for radioactivity with Liquiscint. OLLs were obtained from chloroform/methanol/water (10:10:3, by vol.) extractions, hydrolysed to release free oligosaccharides and analysed by h.p.l.c. The resulting protein pellets were dissolved with Protosol. The results are reported, as the means for duplicate experiments, in c.p.m./mg of microsomal protein, with differences being not more than 10% of the mean.

Membranes	Incubation conditions	Radioactivity (c.p.m./mg)				
		DolPMan*	DolPGlc†	RetPMan*	OLL*	OLL†
					(GlcNAc ₂ Man ₅ -GlcNAc ₂ Man ₅ Glc ₃)	(GlcNAc ₂ Man ₅ Glc ₂ and GlcNAc ₂ Man ₅ Glc ₃)
Control	Pre-incubation with RetP	153 000	25 960	45 700	5 324	1 588
	Incubation along with RetP	199 000	20 020	41 150	9 020	1 434
	No exogenous RetP	242 000	25 410	1 403	10 068	1 502
Deficient	Pre-incubation with RetP	321 000	51 810	62 250	17 212	7 097
	Incubation along with RetP	388 000	51 590	47 060	36 264	5 888
	No exogenous RetP	438 000	77 550	435	33 724	5 803
Deficient/control	Pre-incubation with RetP	2.1	2.0	1.36	3.2	4.5
	Incubation along with RetP	2.0	2.6	1.14	4.0	4.1
	No exogenous RetP	1.8	3.1	0.31	3.3	3.9

* Labelled with GDP-[14 C]mannose.

† Labelled with UDP-[3 H]glucose.

Table 3. Effect of exogenous RetP on the incorporation of [14 C]mannose* or [3 H]glucose† into microsomal glycoproteins‡

Incubation conditions	Membranes... Label...	Radioactivity (c.p.m./mg of microsomal protein)					
		Control		Deficient		Ratio: deficient/control	
		[14 C]Man	[3 H]Glc	[14 C]Man	[3 H]Glc	[14 C]Man	[3 H]Glc
Pre-incubation with RetP		38 420	8 152	15 600	4 636	0.41	0.57
Incubation along with RetP		40 290	5 508	18 570	3 652	0.46	0.66
No exogenous RetP		43 180	7 446	25 260	4 434	0.59	0.60

* Labelled with GDP-[14 C]mannose.

† Labelled with UDP-[3 H]glucose.

‡ Microsomal glycoprotein pellets were from experiments in Table 2; experimental conditions were as in Table 2.

were dissolved in Protosol and the radioactivity was determined, deficient membranes incorporated into microsomal glycoproteins only 40–66% of the labelled mannose or glucose incorporated as did the controls, and exogenous RetP could not reverse this defect. Retinol at 1.7×10^{-6} M suspended in BSA (4 mg/ml) also did not show any effect on any of the glycosylation reactions (results not shown).

The results suggested that the possible site of defect could be at the transfer of oligosaccharide to polypeptides. For the purpose of completeness and consistency, the whole series of events in the glycosylation process (DolPMan, DolPGlc, OLL formation and

glycosylation of the protein acceptor) were analysed under the same experimental conditions. Table 4 summarizes the results. When GDP-[14 C]mannose was used as radiolabelled tag, deficient membranes synthesized 4.0 times as much DolPMan as did controls, 2.3 times as much OLL as did controls, but incorporation of labelled mannose into microsomal glycoprotein was only 0.56 times that of controls.

When UDP-[3 H]glucose was used similarly, deficient membranes synthesized 2.6 times as much DolPGlc as did controls, 2.7 times as much OLL as did controls, but only 0.7 times as much glucose-labelled glycoprotein as did controls.

Table 4. Effect of vitamin A deficiency on glycosylation reactions with GDP-[¹⁴C]mannose and UDP-[³H]glucose

DolPMan and DolPGlc were analysed by t.l.c. from chloroform/methanol (2:1, v/v) extractions, and the labelled band corresponding to t.l.c. mobility was scraped off and counted for radioactivity with Liquiscint. OLLs were obtained by chloroform/methanol/water (10:10:3, by vol.) extraction, hydrolysed to release free oligosaccharides and analysed by h.p.l.c. The resulting protein pellets were dissolved in SDS, the oligosaccharide moiety was separated from its protein core by endo-H digestion and then analysed by h.p.l.c. The radiolabelled h.p.l.c. peak represents the mannose- and glucose-labelled oligosaccharide moiety isolated from microsomal glycoproteins. The results are reported as the means for duplicate experiments, with differences being not more than 10 % of the mean.

Membranes	Radioactivity (c.p.m./mg of microsomal protein)					
	DolPMan*	OLL* (GlcNAc ₂ Man ₅ - GlcNAc ₂ Man ₉ Glc ₃)	Mannose- labelled glycoprotein*	DolPGlc†	OLL† (GlcNAc ₂ Man ₉ Glc ₂ and GlcNAc ₂ Man ₉ Glc ₃)	Glucose- labelled glycoprotein†
Control	39040	10181	15829	8780	1705	2836
Deficient	157440	23440	8807	26480	5880	1516
Deficient/control	4.0	2.3	0.56	3.0	3.4	0.53

* Labelled with GDP-[¹⁴C]mannose.
† Labelled with UDP-[³H]glucose.

Characterization of the oligosaccharide moiety after endo-H digestion of microsomal glycoproteins

The possibility had to be considered that the oligosaccharides of the deficient-membrane glycoproteins were qualitatively different from that of the controls, as suggested by Rosso *et al.* [11], since, in their '*in vivo*' study, those investigators found that vitamin A-deficient rat liver accumulated more of the shorter-chain OLL intermediate (DolPPGlcNAc₂Man₅) and had less of its final glucosylated form (DolPPGlcNAc₂Man₉Glc₃). Therefore, characterization of the oligosaccharide moiety was undertaken.

We observed only one single peak isolated from both pair-fed control and deficient microsomes (Figs. 6a and 6b), using GDP-[¹⁴C]mannose as the radiolabelled tag. When oligosaccharides from control and deficient microsomal membranes were combined, they co-migrated as one single peak (Fig. 6c). Similar experiments were done with UDP-[³H]glucose. Again, control and deficient oligosaccharides contained only a single species, and they co-migrated as one single peak (results not shown). When oligosaccharide species from GDP-[¹⁴C]mannose and UDP-[³H]glucose experiment were combined, they also co-migrated as one single peak (results not shown).

At this point we had established that the oligosaccharide from the glycoprotein produced by the microsomes was endo-H-sensitive, contained mannose and glucose and was identical for deficient and control animals.

For further characterization, glucose-labelled GlcNAc₂Man₉Glc₃ (Fig. 7a) was treated with endo-H in order to obtain GlcNAcMan₉Glc₃. H.p.l.c. fractionation then gave a new peak of lower *M_r*, in addition to, and at the expense of, the residual GlcNAc₂Man₉Glc₃ (Fig. 7b). This new peak did not co-migrate with the following oligosaccharides (obtained as standards from Dr. W. Sasak and Dr. C. Hubbard): GlcNAcMan₉Glc, GlcNAcMan₈Glc, and the series GlcNAcMan₅-GlcNAcMan₉ (results not shown).

Thus the peak with an *M_r* lower than that of the GlcNAc₂Man₉Glc₃ peak obtained after endo-H digestion of GlcNAc₂Man₉Glc₃ was concluded to be GlcNAcMan₉Glc₃. When this substance was combined with the oligosaccharide species isolated from the endo-H-digested microsomal glycoproteins of control and deficient membranes labelled by incubation with GDP-[¹⁴C]mannose or UDP-[³H]glucose, the single oligosaccharide species from all the experiments co-migrated with the GlcNAcMan₉Glc₃ (Fig. 7c). This new peak accounted for the summation from both sources.

Thus these experiments demonstrated that the oligosaccharide moiety isolated from control- and deficient-microsomal-membrane glycoproteins was the same species and was identified as GlcNAcMan₉Glc₃. Deficient membranes incorporated approx. 50% of labelled mannose or glucose into this glycoprotein compared with controls, despite a 2–4-fold accumulation of all dolichyl-linked intermediates.

Transfer of labelled oligosaccharides from exogenous OLLs to endogenous protein acceptors

The above results suggested that the defect in glycoprotein synthesis caused by vitamin A deficiency was at the protein-glycosylation step. To show the effect of deficiency on a direct transfer of the oligosaccharide moiety, we incubated exogenous lipid-linked GlcNAc₂-Man₉[³H]Glc₃ with endogenous microsomal protein acceptors at various Triton X-100 concentrations (Fig. 8). In the absence of Triton X-100, no significant amount of labelled oligosaccharide was transferred to the endogenous protein acceptors in both control and deficient microsomes. However, at higher Triton X-100 concentrations, control membranes transferred 4–6 times the amount of labelled oligosaccharide (15–35.6% of the labelled substrate) into endogenous protein acceptors as compared with deficient microsomes.

These data therefore further substantiate the conclusion that the defect in vitamin A deficiency is at the transfer of oligosaccharide from its lipid carrier to the protein acceptors.

Fig. 6. H.p.l.c. chromatogram of oligosaccharide obtained from endo-H treatment of the microsomal glycoprotein from incubation with GDP-[^{14}C]mannose

After extraction with chloroform/methanol (2:1, v/v), water and chloroform/methanol/water (10:10:3, by vol.), the residual protein pellet was solubilized with SDS. Then the extracted microsomal glycoproteins were treated with endo-H and the labelled oligosaccharide moiety was analysed by h.p.l.c. Peak I is $\text{GlcNAcMan}_9\text{Glc}_3$. (a) Glycoprotein from control rats; (b) from deficient rats. (c) is a combined co-migration h.p.l.c. chromatogram of oligosaccharides obtained after endo-H treatment of both control (a) and deficient (b) microsomal glycoprotein from incubation with GDP-[^{14}C]mannose.

DISCUSSION

An important regulatory site of lipid-mediated glycosylation of proteins is in the synthesis of lipid-linked oligosaccharides [4–9]. The vast literature concerning studies *in vivo* also strongly suggests that vitamin A is involved in the glycosylation process [11,21]. Owing to

Fig. 7. H.p.l.c. chromatograms of (a) [^3H]glucose-labelled $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$ from incubation with UDP-[^3H]glucose and (b) endo-H-treated [^3H]glucose-labelled $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$, and (c) co-migration h.p.l.c. chromatograms of endo-H-treated [^3H]glucose-labelled $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$ of (b) and [^{14}C]mannose-labelled oligosaccharides from control microsomal glycoproteins after endo-H treatment (from the experiment of Fig. 6a)

(a) The peak at the left of the chromatogram is glucose from residual labelled substrate. Peak I is $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$. Procedures were as in Fig. 5. (b) The new peak between the residual glucose and $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$ (peak I) was characterized as $\text{GlcNAcMan}_5\text{Glc}_3$ (peak II). (c) Other combined co-migration chromatograms such as (b) with deficient microsomes showed similar h.p.l.c. profiles (results not shown). Peaks I, II are as in (b). —, ^3H radioactivity; ----, ^{14}C radioactivity.

the shortcomings of the '*in vivo*' model caused by physiological and metabolic interferences, the results obtained from these studies were only suggestive, not conclusive.

Fig. 8 Transfer of labelled oligosaccharide from exogenous [*glucose-³H*]OLL to endogenous protein acceptors at various Triton X-100 concentrations

[*glucose-³H*]OLL was used as a substrate for the transfer reaction. After incubation the microsomal membranes were washed exhaustively with chloroform/methanol (2:1, v/v), water and chloroform/methanol/water (10:10:3, by vol.). The protein pellets were dissolved in Protosol and the radioactivity was determined after addition of Econofluor and dark-adaptation for 10 h. Numbers in parentheses represent percentages of total ³H-labelled oligosaccharides transferred to endogenous protein.

Our '*in vitro*' model enabled us to study the effect of vitamin A deficiency and re-addition of retinoids on glycosylation processes more directly. We were able to show that RetPMan does not function as a cofactor in OLL elongation, as had been suggested [11]: deficient membranes made more DolPPGlcNAc₂Man₅; larger intermediates from DolPPGlcNAc₂Man₆ to DolPPGlcNAc₂Man₉Glc₃ also accumulated. DolPMan synthesis was not halted, but increased 2–3 times over the control. Exogenous RetP, as a source of RetPMan, did not reverse these effects. Therefore RetPMan was shown not to function biochemically as a mannose carrier.

These results accord with current thinking on the role of RetPMan. Recently, De Luca and co-workers [22] reported that the previously identified endogenous RetPMan-like compound differed from that obtained from chemically prepared RetP and was identified as phosphatidylmannose. They also concluded from that study that a single enzyme, mannosyltransferase, catalysed the transfer of mannose from GDP-mannose to DolP and RetP, as well as to phosphatidic acid. These results are consistent with a recent report by Stoll *et al.* [23] that a single enzyme in rat liver and hamster ovary cells catalysed the synthesis of DolPMan and RetPMan. Our studies demonstrated that vitamin A deficiency increased the incorporation of labelled mannose or glucose into lipid-linked glycosylation intermediates (DolPMan, DolPGlc and OLL), but decreased incorporation into the protein acceptors. Furthermore, the data revealed the unique finding that the oligosaccharide moiety isolated from microsomal glycoproteins by endo-H digestion was not being processed in the microsomal vesicles we used. This result eliminated the possibility that the observed differences in deficiency were contributed to by the processing reactions (i.e. removal of

glucose). Therefore, these studies enable us to conclude that the decrease in glycosylation of the vitamin A-deficient microsomal glycoproteins is the result of a defect in the transfer of the oligosaccharide moiety from its lipid carrier to the protein acceptors, resulting in accumulation of lipid-linked sugar intermediates, but decreased protein glycosylation. A more direct experiment, using DolPPGlcNAc₂Man₉[³H]Glc₃ as an exogenous substrate to study the protein glycosylation step directly, confirmed the hypothesis that the defect in glycoprotein synthesis in vitamin A deficiency is at the step where transfer of oligosaccharide to protein acceptors occurs. However, we could not determine whether the difference was in the activity or level of the glycosyltransferase or in the structure or level of the acceptor protein, nor was it possible to determine whether the vitamin deficiency affected an extra-nuclear event or influenced expression of the glycosyltransferase gene. Recently, Kim & Wolf [24] determined that vitamin A deficiency affected the rate of transcription of mRNA for fibronectin, another glycoprotein, by rat liver and hepatocytes.

Another possibility is the influence of the viscosity of the lipid constituting the membrane vesicles on the formation of OLL and glycoprotein. Spiro & Spiro [9] have recently shown that increased mobility of the vesicle lipid (or its disruption by detergent) results in decreased OLL and glycoprotein synthesis compared with DolPGlc synthesis by thyroid endoplasmic reticulum. Recent experiments (M. Hamm, V. T. Chan & G. Wolf, unpublished work) showed a radically altered lipid composition and increased viscosity of the vitamin A-deficient vesicles we used in our experiments. Such changes in mobility may account for different rates of DolPMan, DolPGlc, OLL and glycoprotein synthesis in vitamin A-deficient rat liver, since the pathway requires trans-membrane movement of substrates.

In conclusion, this is the first time that the effect of vitamin A deficiency and re-addition of retinoids on different glycosylation steps has been studied, starting from precursor DolPMan and DolPGlc synthesis to the final transfer of the oligosaccharide moiety to the protein acceptors, in an '*in vitro*' liver microsomal model. The results from this project allow us to pinpoint the defect in glycoprotein synthesis caused by vitamin A deficiency at the protein-glycosylation step, not at the lipid-mediated glycosylation steps.

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