# PHOSPHORYLATION AND MANNOSYLATION OF DOLICHOL(C55) IN MICROSOMES FROM HEPATOCYTES PREINCUBATED WITH DOLICHOL

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## 1. Introduction

In liver and several other tissues dolichol phosphates are obligatory intermediates in defined steps of glycoprotein synthesis [1,2]. Several factors decide the nature and composition of the oligosaccharide chains synthesized. The structure of the polypeptide chain, its intramembraneous localization and the subcellular distribution, nature and number of the glycosyl transferases involved determine the sugar sequence. Dolichol is present in various amounts in different tissues and organs and a given tissue can contain several dolichols differing in their number of isoprene residues [3,4].

Dolichol phosphates appear to have a broad and uneven distribution in intracellular membranes [5]. In addition the number of isoprene residues influences the interaction of this lipid with specific glycosyl transferases [6]. These findings indicate that dolichol phosphates may play a regulatory role in glycoprotein synthesis.

In this study isolated hepatocytes were incubated with exogeneous dolichol in order to enrich the intracellular membranes with this lipid. It was found that this intermediate is transferred into hepatocytes, becomes phosphorylated, is enriched in the endoplasmic reticulum and becomes mannosylated to a large extent.

## 2. Materials and methods

Hepatocytes were prepared from nonstarved male rats using a collagenase perfusion procedure [7]. For fractionation the cells were submitted to a short sonication and the fractions were prepared by differential centrifugation. For preparation of liposomes 20 mg of egg lecithin (Lipid Products, South Nutfield, England) in chloroform-methanol was mixed with 1 μmol of labeled or unlabeled dolichol (C55). The solvent was evaporated and, after the addition of 4.5 ml 0.9% NaCl, sonication for 50 min at 1.5 A in the presence of $N_2$ and with cooling in an ice-water bath was performed.

The 25 ml incubation mixture contained $3 \times 10^8$ cells in Krebs-Henseleit buffer with or without 2 ml of the liposomal suspension. Incubation was carried out in a rotating flask in the presence of $O_2$:$CO_2$ (93.5:6.5) for 90 min in 37°C. At the end of this period the cells were pelleted by centrifugation at 50 g and washed four times by recentrifugation in 0.9% NaCl.

Dolichol and dolichol-P were extracted and isolated on DEAE-Sephadex (acetate) column by elution with chloroform:methanol:water (1:1:0.3) followed by 200 mM ammonium acetate in chloroform:methanol:water (1:1:0.3). GDP-mannosyl transferase activity was measured in the absence of detergent as described previously [8]. Dolichol phosphorylation with $^{32}P$ was achieved using the procedure described in [9]. In order to obtain dolichol [$^{32}P$]phosphate mannose, microsomes were incubated in the presence of unlabeled GDP-mannose and dolichol [$^{32}P$] phosphate and the mannosylated lipid was isolated by DEAE-Sephadex (acetate) chromatography eluting with increasing concentration of ammonium formate in chloroform:methanol:$H_2O$ (1:1:0.3). Chromatography of dolichol phosphate mannose was performed on Lipidex-1000 starting with water:methanol:*n*-butanol:chloroform (60:40:7:3). Elution of the

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Table 1
Transfer of liposomal [$^3$H]dolichol (C55) into isolated hepatocytes

| Fractions | Dolichol (total) | | | Dolichol-P (total) | | |
|---|---|---|---|---|---|---|
| | cpm | nmol | pmol | cpm | nmol | pmol |
| | total | total | mg/prot. | total | total | mg/prot. |
| 300 × *g* pellet | 107.101 | 17.6 | 49.1 | 18.327 | 3.0 | 8.3 |
| Mitochondria | 47.175 | 7.7 | 69.6 | 5.605 | 0.92 | 8.3 |
| 10 000 × *g* pellet | 40.680 | 6.7 | 118.0 | 4.142 | 0.68 | 11.9 |
| Microsomes | 39.155 | 6.99 | 155.8 | 12.417 | 2.22 | 49.5 |
| Supernatant | 31.416 | 5.2 | 25.4 | 2.808 | 0.46 | 2.2 |
| Yield | 88% | | | 86% | | |

The disrupted cells obtained by sonication were first centrifuged at 300 × *g* to remove unbroken cells and nuclei (300 × *g* pellet). The mitochondria were pelleted by centrifugation of the remaining supernatant at 3000 × *g* for 20 min. This pellet was then washed twice. The mitochondrial supernatant, together with the two washed supernatants, were centrifuged at 10 000 × *g* for 20 min. The resulting supernatant was centrifuged at 105 000 × *g* for 60 min to separate microsomes and supernatant. The supernatant was supplemented with the wash solution obtained after recentrifugation of the microsomes in 0.15 M Tris–HCl, pH 8.0

column was achieved with a linear gradient containing a decreasing concentration of water in the organic solvent. Radioactivity was measured with toluene-PPO scintillator. Protein was estimated by the Biuret procedure [10].

## 3. Results and discussion

Model experiments performed with liposomes prepared from pure egg lecithin have demonstrated that upon incubation hepatocytes take up the lipid material into the intracellular compartment. When the liposomes were prepared in the presence of dolichol (C55), uptake was equally efficient. Upon fractionation, unchanged dolichol could be found in all fractions prepared, including mitochondria, microsomes and the final particle-free supernatant (table 1).

The chromatographic analysis also demonstrated that during the incubation period 20–25% of the dolichol taken in is phosphorylated. The highest concentration of dolichol (C55) phosphate is in the microsomal fraction. The microsomal fraction prepared from cells preincubated with dolichol exhibits a large stimulation of mannose incorporation into the dolichol monophosphate upon incubation with GDP-[$^{14}$C]mannose (table 2). There is also some stimulation of mannose incorporation into the dolichol oligosaccharide extracted by chloroform:methanol:water (1:1:0.3), but no enhancement of incorporation into

Table 2
Mannosylation of dolichol (C55)-P in microsomes prepared from hepatocytes preincubated with [$^3$H]dolichol (C55)

| Preincubation | [$^3$H]Dolichol-P | [$^{14}$C]Mannose incorporated | | | Dolichol-P mannosylated | |
|---|---|---|---|---|---|---|
| | | L I | L II | Protein | Endogenous | Exogenous |
| | pmol/mg protein | cpm/mg protein | | | pmol/mg protein | |
| None | | 4.671 | 166 | 3.765 | 10.6 | |
| [$^3$H]Dolichol | 48.2 | 15.178 | 266 | 3.770 | 10.6 | 24.1 |

Microsomes were prepared as described in table 1 and washed with 0.15 M Tris–HCl, pH 8.0. L I represents the chloroform:methanol (2:1) extract and L II the chloroform:methanol:water (1:1:0.3) extract

the protein fraction is observed. It appears that endogeneous protein acceptor is present in only a limited amount. Incubation with GDP-mannose results in mannosylation of about half of the dolichol (C55) phospate present in the microsomes, which means that under the conditions employed the mannosyl acceptor capacity of this fraction was increased more than three-fold.

In order to exclude the possibility that incubation is stimulating the mannosylation of endogeneous and not exogeneous dolichol, chromatographic separation of the mannosylated polyprenol was performed. Among the various procedures examined, chromatography on Lipidex-1000 was found to be the most effective. The dolichol (C55) [$^{32}$P]phosphate standard is eluted in fractions 12–14 and the mannosylated standard in fractions 7–9 (fig.1, A and B). The mannosylated endogeneous dolichol obtained by incubation of microsomes with GDP-[$^{14}$C]mannose is eluted in fractions 13–15 on Lipidex-1000 (Fig.1, C). When microsomes from the preincubated cells were incubated with GDP-[$^{14}$C]mannose, a clear separation of two peaks was observed, a smaller peak corresponding to endogeneous and a larger peak corresponding to the exogeneous dolichol phosphate mannose (fig.1, D).

It appears from the experiments described here that exogeneous dolichol can under appropriate conditions be transferred to the cytoplasm of hepatocytes. Most probably, the quality of the cells isolated is an important factor in this process. The dolichol which enters the cell appears in several membranes and is also present in the cytoplasm. Its transport in the cytoplasm is probably facilitated by a carrier protein, as has been established for the transport of intracellular phospholipids and cholesterol. A part of this exogeneous dolichol is phosphorylated and at the end of the incubation displays a multimodal distribution resembling that of the endogeneous dolichol phosphate. The fact that the microsomal dolichol (C55) phosphate is mannosylated in the absence of detergent indicates that phosphorylated polyprenol is situated in the membrane in an appropriate manner. This approach for incorporating individual dolichol phosphates into microsomal membranes may be useful in future investigations to establish the specific roles of individual polyprenols in glycoprotein biosynthesis.

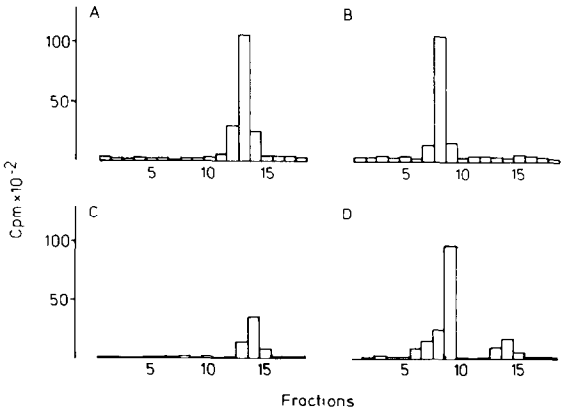


Fig.1. Chromatography of dolichol monophosphate sugars on Lipidex-1000. A, Dolichol (C55) [$^{32}$P]phosphate standard; B, dolichol (C55) [$^{32}$P]phosphate mannose standard; C, chloroform:methanol (2:1) extract of microsomes after incubation with GDP-[$^{14}$C]mannose; D, chloroform:methanol (2.1) extract of microsomes prepared from hepatocytes preincubated with dolichol (C55) and incubated with GDP-[$^{14}$C] mannose.

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