COMPARATIVE EFFECTS OF EXOGENOUS RETINYLPHOSPHATE AND DOLICHYLPHOSPHATE ON THE IN VITRO MANNOSYLATION OF GLYCOPROTEINS

Jacques FROT-COUTAZ, Robert LETOUBLON and René GOT

Laboratoire de Biologie et Technologie des Membranes, Université Claude Bernard de Lyon, 43, Bd du 11 Novembre 1918, 69621 Villeurbanne, France

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1. Introduction

Retinol [1,2] and dolichol [3] have been considered as the polyprenol intermediates in the biosynthesis of glycoproteins. When microsomes are incubated with GDP-mannose, the amount of dolichol phosphomannose formed is quite important while very little retinol phosphomannose-like material appears [4]; true retinol phosphomannose is synthesized when exogenous retinol phosphate is added [4]. In an 'in vitro' situation one cannot eliminate the possibility that synthesis of retinol phosphate mannose is due to the enzyme responsible for the mannosylation of dolichol phosphate which mistakes exogenously added retinol phosphate for dolichol phosphate.

Here we have: (i) developed conditions that achieve a controlled transfer of either dolichol phosphate or retinol phosphate from liposomes to microsomal vesicles [5]; (ii) demonstrated that vitamin A takes place in some mannosylation reactions which form a pathway different from the one involving dolichols as lipid intermediates [3].

2. Materials and methods

2.1. *Reagents*

Dolichol phosphate, phosphatidic acid (from egg yolk), phosphatidylethanolamine (from *Escherichia coli*), phosphatidyl choline (from egg yolk) were purchased from Sigma Chemical Co.

Phosphatidyl [*N-methyl*-^{14}C]choline, 50–60 mCi/mmol (1.85–2.2 GBq/mmol) and guanosine diphospho-[U-^{14}C]mannose (166 mCi/mmol) and [*carbinol*-^{14}C] vitamin A were from the Radiochemical Centre, Amersham.

Retinol phosphate was made chemically according to [6]; ATP, disodium salt was obtained from Boehringer; Sintix from Isotec.

2.2. *Preparation of microsomes*

Male Wistar rats (200–300 g) were provided a standard diet and drinking water ad libitum; they were killed by cervical dislocation and the livers successively drained of blood, excised, minced and homogenized in lots of 10 g liver in 40 ml chilled homogenization buffer consisting of 50 mM Tris–HCl (pH 7.8), 0.25 M sucrose. Homogenization was for 50 s at 2000 rev./min using a Teflon–glass homogenizer; microsomes were then prepared as in [7].

Microsomal proteins were determined by the Hartree method [8] after delipidation [9].

2.3. *Preparation of phospholipid vesicles*

Suspension of phospholipid vesicles in 50 mM Tris–HCl buffer (pH 7.8) were prepared by sonicating for 4 min (at a 35 W setting microprobe, Branson Sonifier, Model B-12) 18–30 mM phospholipids.

Centrifugation for 20 min at 150 000 × *g* served to remove the largest vesicles; the resulting supernatant is referred to as liposomes.

2.4. *Fusion of microsomes with phospholipid vesicles*

Fusion of microsomes with phospholipid vesicles

was obtained by incubation of 0.6 ml liposomes with 6 mg (as proteins) of microsomes resuspended in 0.6 ml buffer used for the phospholipid vesicle preparation. The resulting mixture is referred to as particulate enzyme preparation.

2.5. *Assay for incorporation of [^{14}C]mannose into mannosylphosphorylpolyisoprenols and endogenous glycoproteins: conditions and extraction procedures*

The final concentrations of the components in the standard incubation mixture (0.2 ml) were as follows: 50 mM Tris–HCl (pH 7.8), 2.5 mM $MgCl_2$, 3 mM ATP (disodium salt), GDP-[^{14}C]mannose, 0.125 μCi (spec. act. 166 mCi/mmol); 180 μl particulate enzyme preparation (900 μg protein) were added to initiate the reaction, which was at 22°C.

The reaction was terminated by adding 3 ml chloroform/methanol mixture (2/1, v/v) to the incubation mixture; dolichol monophosphate [^{14}C]mannose and retinol phosphate [^{14}C]mannose were determined by thin-layer chromatography as in [4]. This procedure [4] results in the formation of an insoluble material whose radioactivity considered as [^{14}C]-mannose incorporation into glycoproteins was measured as follows: the precipitate was layered on glass microfibre paper (GF/C, Whatman) and washed free of GDP-[^{14}C]mannose and [^{14}C]mannolipids successively by 10 ml 5% phosphotungstic acid in 2 N HCl, 50 ml water, 10 ml chloroform/methanol mixture (2/1, v/v), 10 ml chloroform/methanol/water mixture (1/1/0.3, by vol.), 10 ml ethanol; the filter was transferred to a scintillation vial and the radioactivity measured in presence of 10 ml PPO/POPOP/toluene scintillation mixture (4 g/0.1 g/1 litre).

All the operations in sections 2.3–2.5 were at 22°C and under dim light and nitrogen stream for the experiments in sections 2.3 and 2.4.

3. Results and discussion

3.1. *Kinetics of fusion between phospholipid vesicles and microsomes*

Sonicated vesicles of various lipid composition have been successfully used as acceptors for the assays of lipid glycosyltransferases, UDP-galactose:ceramide galactosyltransferase and UDP-glucose:ceramide glucosyltransferase [10] from rat brain.

Since our system was different, a preliminary experiment was carried out to study the interaction between microsomes and liposomes of known and defined composition similar to that of natural membranes [11]; phosphatidyl [*N-methyl*-^{14}C]choline or [^{14}C]retinol phosphate were used as markers to follow the fusion of liposomes with microsomes.

Figure 1 shows that it takes ~ 30–60 min for fusion between liposomes and microsomes to reach a plateau and that ~ 40% of the initial radioactivity ([^{14}C]retinolphosphate) is transferred into microsomes; as expected, we found that the rate and extent of fusion is the same for phosphatidyl [*N-methyl*-^{14}C] choline as for retinol phosphate (results not shown).

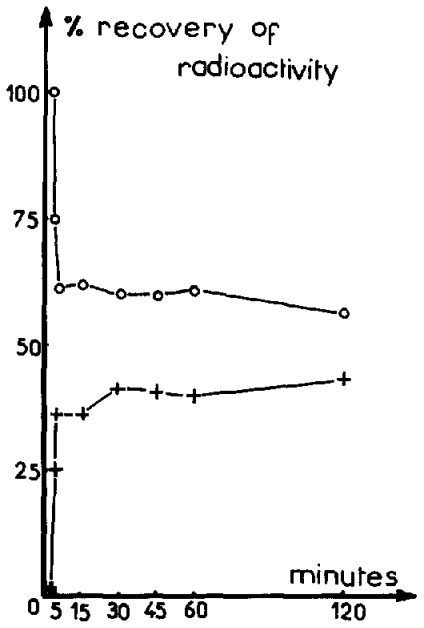

Fig.1. Typical kinetics of fusion between phospholipid vesicles and microsomes. Liposomes were prepared with the following phospholipids: phosphatidylcholine (5 mg); phosphatidylethanolamine (2.5 mg); phosphatidic acid (0.5 mg); retinol phosphate (0.100 mg); [^{14}C]retinol phosphate (spec. act. 10 mCi/mmol), 0.25 μCi. Sonication was in 0.6 ml buffer solution. At different times of incubation aliquots (0.150 ml) of the particulate enzyme preparation were centrifuged for 2 min at 150 000 × *g* using an air-driven ultracentrifuge (Beckman) and the radioactivity present in both the supernatants (o——o) and the pellets (+——+) measured in presence of 20 ml Scintix (the pellets were first dissolved in 0.1 ml formic acid).

3.2. *Mannophospholipid biosynthesis*

It is obvious from the previous experiment that part of the phospholipid vesicles is transferred into microsomes but the question is raised as to know whether substrates like dolichol phosphate or retinol phosphate are available to the microsomal enzymes responsible for their mannosylation.

Figure 2 shows that incubation of particulate enzyme preparations obtained from retinol phosphate or dolichol phosphate-containing liposomes with GDP-[^{14}C]mannose results in a highly significant increase in the synthesis of retinol phosphate [^{14}C]mannose and dolichol phosphate [^{14}C]mannose as compared to the controls.

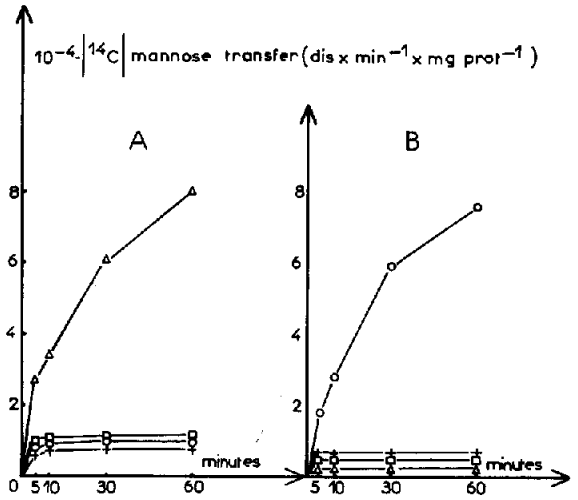

Fig.2. Time course of incorporation of [^{14}C]mannose into dolichol phosphate [^{14}C]mannose and into retinol phosphate [^{14}C]mannose. [^{14}C]Mannose transfers from GDP-[^{14}C]mannose to dolichol phosphate (A) or to retinol phosphate (B) were studied in presence of 4 different particulate enzyme preparations (a–d) obtained under the following conditions. (1) Liposome composition: (a) (+) phosphatidylcholine (5 mg), phosphatidylethanolamine (2.5 mg), phosphatidic acid (0.750 mg). Sonication was in 0.6 ml buffer solution; (b) (○) same as (a) but 0.5 mg retinol phosphate and no phosphatidic acid; (c) (△) same as (a) but 0.150 mg phosphatidic acid and 0.600 mg dolicholphosphate; (d) (□) liposomes replaced by the buffer solution used for sonication of the lipid mixture. (2) Fusion was for 60 min. (3) [^{14}C]mannose transfer: Incubations of the 4 different particulate enzyme preparations (a–d) with GDP-[^{14}C]-mannose and the determination of dolichol phosphate [^{14}C]mannose (A) and retinol phosphate [^{14}C]mannose (B) were as in section 2.

In order to keep constant the gross negative charge of the different particulate enzyme preparations, the composition in acidic phospholipids (phosphatidic acid + retinol phosphate or dolichol phosphate) was about the same in the corresponding phospholipid vesicles.

3.3. *In vitro mannosylation of endogenous glycoproteins*

The increased synthesis of dolichol phosphate mannose and retinol phosphate mannose due to exogenously added dolichol phosphate and retinol phosphate does not result in both cases in a proportional enhancement of [^{14}C]mannose transfer into glycoproteins: while a 20-fold increase in dolichol phosphate mannose biosynthesis has no effect, a striking enhancement is obtained from retinol phosphate mannose (fig.3).

On the basis of the above results it is possible:

1. To confirm the function of retinol phosphate mannose as donor of mannosyl residues to endogenous acceptor [2].

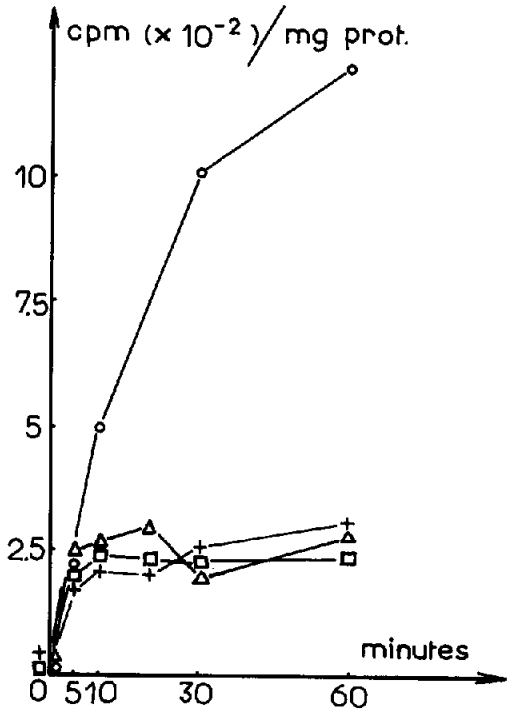

Fig.3. Time dependance of uptake of [^{14}C]mannose into glycoproteins; for conditions and symbols, see fig.2.

2. To conclude that there are two distinct pathways for glycoprotein mannosylation: one involving dolichol, the other one retinol.

In vitro, the dolichol pathway seems to have a poor capacity for incorporating [^{14}C]mannose into endogenous glycoproteins with a rather important amount of dolicholphosphate while the vitamin A pathway is characterized by a high amount of endogenous acceptors available for mannosylation with very little retinol phosphate. In favor of two separate pathways is the report [13] that in rat liver the esterification of dolichol is not depressed in the presence of a several-fold excess of retinol.

A double pathway for glycoproteins mannosylation has already been considered as possible [14] but its significance remains to be elucidated.

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