The polyenic system of retinyl phosphate is required for its mannosyl donor activity but not for acceptor activity

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We investigated whether the polyenic and allylic phosphate systems of retinyl phosphate are essential for its mannosyl acceptor and donor activities in rat liver postnuclear membranes. Perhydromonoeneretinyll phosphate, a compound without growth-promoting activity in vitamin A-deficient animals, was prepared by catalytic hydrogenation of retinol and phosphorylation. Perhydromonoeneretinyll phosphate mannose synthesis from GDP-mannose showed continued accumulation for at least 60 min, while retinyl phosphate mannose synthesis showed a maximum at 20–30 min and then declined. Moreover, only retinyl phosphate stimulated transfer of mannose from GDP-mannose to endogenous proteins, which were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Thus, hydrogenation of side-chain double bonds in retinyl phosphate impaired only slightly its mannosyl acceptor activity, but caused loss of mannosyl donor activity.

Ret-*P* accepts mannose from GDP-mannose to form Ret-*P*-Man in a reaction catalysed by mammalian microsomal membranes (De Luca, 1977). Further, phosphorylation of the vitamin has been shown in intestinal cells (Frot-Coutaz *et al.*, 1976) and endogenous Ret-*P* was demonstrated in rat liver microsomes (De Luca *et al.*, 1982), using a recently developed incubation mixture containing BSA (Shidoji & De Luca, 1981).

We now ask whether the polyenic and allylic phosphate systems of Ret-*P* are necessary for its mannosyl acceptor and donor activities. pRet-*P* (structure shown below) was synthesized and utilized for this purpose.

were from Millipore, Boston, MA, U.S.A. Ret-*P* was synthesized by chemical phosphorylation (Bhat *et al.*, 1980) and pRet-*P* was made by catalytic hydrogenation of retinol (De Luca *et al.*, 1977) followed by chemical phosphorylation. GDP-[³H]mannose (sp. radioactivity 10 Ci/mmol) and GDP-[¹⁴C]mannose (sp. radioactivity 0.2 Ci/mmol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A.

Preparation of rat liver postnuclear membranes

Male rats (Osborne–Mendel strain), weighing 150–200 g, were starved overnight and killed by bleeding under light diethyl ether anaesthesia. The livers were homogenized with 2 vol. of medium A with a glass/Teflon homogenizer. After centrifugation at 2500 g for 20 min, the resultant supernatant was centrifuged at 105 000 g for 60 min. The pellet was resuspended in medium A and kept in liquid N₂ until use.

Materials and methods

Materials

Bovine serum albumin and GDP-mannose were from Sigma; Millipore filters (HA; pore size 0.45 μm)

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Abbreviations: Ret-*P*-Man, retinyl phosphate mannose; pRet-*P*-Man, perhydromonoeneretinyll phosphate mannose; Dol-*P*-Man, dolichyl phosphate mannose; Ret-*P*, retinyl phosphate; pRet-*P*, perhydromonoeneretinyll phosphate; Dol-*P*, dolichyl phosphate; BSA, bovine serum albumin; DTT, dithiothreitol; SDS, sodium dodecyl sulphate; medium A, 50 mM-Tris/HCl buffer, pH 7.6, containing 5 mM-MgCl₂, 25 mM-KCl and 0.25 M-sucrose.

Mannosylation of endogenous acceptors

The incubation mixture contained the following constituents at the indicated final concentrations: 30 mM-Tris/HCl buffer, pH 8.0; 4 mg of BSA/ml; 2.5 mM-MnCl₂; 8 mM-NaF; 4 mM-ATP; 5 mM-AMP; 0.4 μ Ci of GDP-[³H]mannose (GDP-mannose was added to final concentration of 24 μ M); and 1 mg of rat liver microsomal protein in 200 μ l final volume. Incubation was at 37°C in the presence or absence of either exogenous Ret-P or pRet-P (final concentration 0.15 mM). The reaction was stopped by the addition of 1 ml of ice-cold medium A and the resultant solution was immediately poured on to a Millipore filter (HA; 0.45 μ m) to trap microsomal membranes. An additional 1 ml of ice-cold medium A was added to wash out the unchanged GDP-[³H]mannose. The washed damp filter was placed into a counting vial that contained 5 ml of chloroform/methanol (2:1, v/v), and was kept at room temperature for 1 h. Then the filter paper was transferred to another counting vial and the chloroform/methanol [2:1, v/v, (5 ml)] extraction was repeated twice more. The combined extract was evaporated to dryness and the radioactivity was counted in 10 ml of Betafluor. After washing the filter twice with 5 ml of water, it was immersed in 5 ml of chloroform/methanol/water (10:10:3, by vol.) in a counting vial. This process was repeated twice. The radioactivity in the total chloroform/methanol/water (10:10:3, by vol.) fraction was counted in 10 ml of Betafluor after removing the solvent. The residual

radioactivity retained on the filter was extracted by Protosol (New England Nuclear, Boston, MA, U.S.A.) treatment; 50 μ l of Protosol was added to a filter and the vial was incubated at 50°C overnight in 10 ml of Betafluor.

Ret-P-Man and pRet-P-Man were analysed by t.l.c. The extract was applied to a silica-gel thin-layer plate (F-254; Merck) and developed in chloroform/methanol/water (45:35:6, by vol.). The silica gel (0.5 cm sections) was scraped into counting vials and the radioactivity was determined after addition of 0.25 ml of methanol and 10 ml of Betafluor (National Diagnostics, Sommerville, NJ, U.S.A.). Alternatively the filtration assay was employed as described by Shidoji & De Luca (1981).

SDS/polyacrylamide-gel electrophoresis of endogenous mannose acceptors

The procedure of Shimatake & Rosenberg (1981) was followed to build a gradient gel from 10 to 26% acrylamide, closely fashioned after the procedure of Laemmli (1970).

Other procedures

Protein was determined by the method of Lowry *et al.* (1951) with BSA as standard. Ret-P was measured spectrophotometrically at 325 nm in methanol ($\epsilon_{1\%}^{1\text{cm}} = 1440 \text{ M} \cdot \text{cm}^{-1}$). The concentration of Ret-P was determined by measurement of the phosphate content by the procedure of Chen *et al.* (1956).

Fig. 1. Comparison of Ret-P and pRet-P in mannosylation reactions of rat liver microsomes. GDP-[³H]mannose (0.4 μ Ci; 24 μ M) was incubated at 37°C with rat liver microsomes (1.5 mg of protein) in the absence ($\blacktriangle$) or presence of 1.15 mM-Ret-P ($\circ$) or -pRet-P ($\bullet$). After incubation, the membrane vesicles were collected by Millipore filter and extracted sequentially with 5 ml of chloroform/methanol (2:1, v/v) three times, 5 ml of water twice and 5 ml of chloroform/methanol/water (10:10:3) twice and the residues were digested with 50 μ l of Protosol at 50°C overnight. Each point represents an average of duplicate incubations differing by less than 15%. (a), (b) and (c) show results after chloroform/methanol extraction, chloroform/methanol/water extraction and Protosol digestion respectively.

Results and discussion

These experiments were specifically designed to study the requirement of the polyenic and allylic phosphate systems in Ret-*P* for its mannosyl acceptor and donor activity in postnuclear membrane of rat liver.

For this purpose retinol was hydrogenated catalytically to produce perhydromonoeneretol, which was then phosphorylated. This compound was inactive in promoting growth of vitamin A-deficient hamsters (Y. Shidoji, C. S. Silverman-Jones & L. M. De Luca, unpublished work).

Several striking differences between Ret-*P* and pRet-*P* were noted. Time-course studies showed an accumulation of pRet-*P*-Man (Fig. 1), whereas Ret-*P*-Man reached a maximum at about 30 min and then decreased, suggesting an intermediary function of this compound and/or hydrolysis.

Most (65–80%) of the radioactivity in the chloroform/methanol (2:1, v/v) extract co-migrated with standard Ret-*P*-Man, whereas pRet-*P*-Man showed a slightly more hydrophobic (higher R_F) behaviour on t.l.c. This transfer did not occur in incubations containing amphotycin, which inhibits Ret-*P*-Man synthesis (Shidoji & De Luca, 1981). Unlike Dol-*P*

Fig. 2. SDS/polyacrylamide-gel-electrophoretic analysis of (a) time-course study of Ret-*P* mediated transfer to endogenous acceptors and (b) endogenous acceptors of [¹⁴C]mannose from GDP-[¹⁴C]mannose in incubations containing Ret-*P* and pRet-*P*

(a) [¹⁴C]Mannose-labelled acceptors were prepared from incubations of rat liver microsomes containing 500 µg of microsomal protein and the usual incubation mixture. Incubations (1)–(5) contained 1 µCi each of GDP-[¹⁴C]-mannose (25 µM), whereas incubation (6) contained 1 µCi of [¹⁴C]mannose. Incubations (1), (2), (3), (4) and (6) contained Ret-*P* (0.15 mM). Incubations were run at 37°C for the following times: (1), for 5 min; (2), for 30 min; (3), for 60 min; (4), (5) and (6) for 3 h. They were terminated by adding 15 vol. of chloroform/methanol (2:1, v/v). The denatured pellets were washed twice with 1 ml of chloroform/methanol (2:1, v/v), twice with water and twice with chloroform/methanol/water (10:10:3, by vol.). The residues were solubilized in 45 µl of 10 M-urea, 15 µl of 0.8 M-dithiothreitol in 10% SDS and 80 µl of water and heated at 95°C for 10 min. The cooled solution was applied to gels for electrophoresis. Fluorographs were obtained as described by Shimatake & Rosenberg (1981), after exposure for 34 days. (b) Incubations contained the same components as in (a) and proceeded at 37°C for 60 min. Incubation (1) had no Ret-*P*; incubation (2) contained 10 µg of pRet-*P* and incubation (3) contained 10 µg of Ret-*P*. After incubation the reaction was stopped with 15 vol. of chloroform/methanol (2:1, v/v). The denatured residue was washed twice with 1 ml of chloroform/methanol (2:1, v/v), three times with 1 ml of water and three times with 1 ml of chloroform/methanol/water (10:10:3, by vol.). The resulting denatured pellet was suspended in 45 µl of 10 M-urea, 15 µl of 0.8 M-DTT in 10% SDS and 80 µl of water and heated at 95°C for 10 min. Equivalent amounts of protein (400 µg) were applied to the gel: 7600 c.p.m. was applied to (1); 5400 c.p.m. to (2); 31 870 c.p.m. to (3). Fluorographs were obtained as described in (a).

(results not shown) Ret-*P* was inactive in significantly stimulating the incorporation of mannose into the chloroform/methanol/water extract, which contains most of the lipid-oligosaccharide derivatives (Fig. 1). Ret-*P* but not pRet-*P* stimulated transfer to protein (Fig. 1).

SDS/polyacrylamide-gel-electrophoretic analyses of endogenous rat liver microsomal acceptors (Fig. 2*a*) show labelled prominent bands at mol.wts. about 17 000, 33 000, 45 000 and 65 000 from incubations containing Ret-*P*. The fluorograph shows a time-dependence with maximal labelling at 3 h. It also shows very little labelling in incubations without Ret-*P* (lane 5) or containing 1 μ Ci of [^{14} C]mannose instead of GDP-[^{14} C]mannose (lane 6). Fig. 2(*b*) shows a similar experiment with prominent labelling in the incubations containing Ret-*P*, but not pRet-*P*.

Labelled bands from Ret-*P*-containing incubations were not detected at mol.wt. higher than 14 000 after treatment with Pronase (Y. Shidoji, C. S. Silverman-Jones & L. M. De Luca, unpublished work).

The data demonstrate that saturation of the side-chain double bonds of Ret-*P* destroys its activity in mannosyl transfer to protein, in the presence of pRet-*P*-Man synthesis.

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