Mannosyl carrier functions of retinyl phosphate and dolichyl phosphate in rat liver endoplasmic reticulum

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Of the subcellular fractions of rat liver the endoplasmic reticulum was the most active in GDP-mannose:retinyl phosphate mannosyl-transfer activity. The synthesis of retinyl phosphate mannose reached a maximum at 20–30 min of incubation and declined at later times. Retinyl phosphate mannose and dolichyl phosphate mannose from endogenous retinyl phosphate and dolichyl phosphate could also be assayed in the endoplasmic reticulum. About 1.8 ng (5 pmol) of endogenous retinyl phosphate was mannosylated per mg of endoplasmic reticulum protein (15 min at 37°C, in the presence of 5 mM-MnCl₂), and about 0.15 ng (0.41 pmol) of endogenous retinyl phosphate was mannosylated with Golgi-apparatus membranes. About 20 ng (13.4 pmol) of endogenous dolichyl phosphate was mannosylated in endoplasmic reticulum and 4.5 ng (3 pmol) in Golgi apparatus under these conditions. Endoplasmic reticulum, but not Golgi-apparatus membranes, catalysed significant transfer of [¹⁴C]mannose to endogenous acceptor proteins in the presence of exogenous retinyl phosphate. Mannosylation of endogenous acceptors in the presence of exogenous dolichyl phosphate required the presence of Triton X-100 and could not be detected when dolichyl phosphate was solubilized in liposomes. Dolichyl phosphate mainly stimulated the incorporation of mannose into the lipid–oligosaccharide-containing fraction, whereas retinyl phosphate transferred mannose directly to protein.

The initial suggestion that vitamin A may be involved as a carrier of mannose in biological membranes was followed by findings that mammalian enzyme preparations synthesize glycophospholipids consisting of a retinol moiety linked by a phosphodiester bond to mannose. Reviews of this work have been published by De Luca (1977) and Wolf (1979). By using a recently developed assay system that utilizes bovine serum albumin (Shidoji & De Luca, 1981) the synthesis of Ret-*P*-Man from endogenous Ret-*P* was demonstrated in rat liver postnuclear membranes (De Luca *et al.*, 1982).

Abbreviations used: BSA, bovine serum albumin; Dol-*P*-Man, dolichyl phosphate mannose; DMSO, dimethyl sulphoxide; Dol-*P*, dolichyl phosphate; ER, endoplasmic reticulum; Ret-*P*-Man, retinyl phosphate mannose; Ret-*P*, retinyl phosphate; TMK, 0.05 M-Tris/0.005 M-MgCl₂/0.050 M-KCl (pH 7.6); medium A, TMK in 0.25 M-sucrose.

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Utilizing the same assay system we show in this report (1) that the ER of rat liver contains both the mannosyltransferase as well as the endogenous acceptor Ret-*P* for Ret-*P*-Man synthesis; (2) that Ret-*P* is active in stimulating mannosylation of endogenous protein acceptors of the ER, whereas Dol-*P* mainly stimulates the synthesis of lipid–oligosaccharides and (3) that Golgi-apparatus membranes, though containing the mannosyltransferase to synthesize Ret-*P*-Man from GDP-mannose and exogenous Ret-*P*, do not contain significant amounts of the endogenous acceptor Ret-*P*, thus suggesting a specific function for Ret-*P* in mannosylation in the ER.

Materials and methods

Materials

GDP-[¹⁴C]mannose (sp. radioactivity 166 Ci/mol) and GDP-[³H]mannose (sp. radioactivity 10.4 Ci/mmol) were obtained from Amersham—

Searle, Arlington Heights, IL, U.S.A., and Protosol was from New England Nuclear Corp., Boston, MA, U.S.A. Bovine serum albumin, dolichyl phosphate (grade III), GDP-mannose and synthetic dipalmitoylglycerophosphocholine were from Sigma Chemical Co., St. Louis, MO, U.S.A. Precoated plates of silica-gel G (F-254) were from E. Merck, A.G., Darmstadt, Germany. DEAE-Sephacel was obtained from Pharmacia, Piscataway, NJ, U.S.A. RP Royal X-Omat X-ray film was obtained from Eastman Kodak, Rochester, NY, U.S.A.

Methods

Isolation of membrane fractions. Livers were from male Holtzman rats (Holtzman Co., Madison, WI, U.S.A.) given food and water *ad libitum*. Golgi apparatus (Morré, 1973), ER (Morré *et al.*, 1974) and plasma membranes (Yunghans & Morré, 1973) were isolated by the methods referenced. Enzymic and morphometric analysis showed such cellular fractions to be contaminated to an extent of less than 10% with other cellular membranes (Morré *et al.*, 1974; Jelsema & Morré, 1978). For studies on endogenous Ret-*P* and MnCl₂ requirement, a 0.9% NaCl solution was used to resuspend the membranes. Proteins were determined by the method of Lowry *et al.* (1951) with BSA as standard.

Synthesis of Ret-*P* and Ret-*P*-Man. Ret-*P* was prepared by a modification (Bhat *et al.*, 1980) of a previously described procedure (Rosso *et al.*, 1975). DEAE-Sephacel (Sasak *et al.*, 1979) columns were used to purify Ret-*P*. Retinol content in subcellular organelles was measured as described previously for these fractions (Nyquist *et al.*, 1971).

Incubation mixtures. These were modelled on the recently described incubations for rat liver microsomes (Shidoji & De Luca, 1981). Standard incubations were prepared as follows. GDP-[¹⁴C]-mannose in the amounts specified and 10 µg of Ret-*P* in 99% methanol or solvent alone were transferred to test tubes. After removing the solvent under an N₂ stream, 4 mg of BSA/ml, 30 mM-Tris/HCl buffer (pH 8), 2.5 mM-MnCl₂ (or other concentrations as stated), 8 mM-NaF, 2 mM-ATP, 5 mM-AMP and ER, Golgi-apparatus or plasma-membrane protein, in the amounts specified, were added in a final volume of 200 µl. The mixture was incubated at 37°C. For incubations containing exogenous Ret-*P* the reaction was stopped by addition of 1 ml of ice-cold medium A and immediately poured on the MF-millipore (HA 0.45 µm) on the filter manifold (Millipore), allowing processing of several samples. The filter was washed with an additional 1 ml of medium A to remove unbound radioactive materials. The radioactivity retained on the filter was measured in 10 ml of Aquafuor (New England Nuclear). Counting efficiency for ¹⁴C was 70%. About 95% of this radioactivity represents Ret-*P*-Man at 2 min of incubation. For incubations without Ret-*P*, the

mixture was extracted with 1 ml of chloroform/methanol (2:1, v/v), yielding two phases; the lower phase, without further washing, was dried under N₂, immediately redissolved in 60 µl of 99% methanol with carrier Ret-*P* and applied to thin-layer plates of silica gel, which were then developed in chloroform/methanol/water (45:35:6, by vol). Duplicate plates were obtained for each sample. One was used for scraping and counting of radioactivity, the other for autoradiography, as described previously (De Luca *et al.*, 1982). The upper phase of this extract contains about 50% of total Ret-*P*-Man and less than 2% of total Dol-*P*-Man.

Thin-layer plates were dried and exposed to RP Royal X-Omat X-ray film for 1–2 weeks and the film was developed. Radioactive spots were located and the silica gel was then scraped into liquid-scintillation vials containing 0.3 ml of methanol. The vials were placed at room temperature for at least 2 h before the addition of 15 ml of toluene containing 0.5% diphenyloxazole and 0.01% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene. Radioactivity was measured by a Beckman LS-350 liquid-scintillation counter. Alternatively, 0.5 cm bands were scraped directly into liquid-scintillation vials and radioactivity was determined.

Incubation with Dol-*P* liposomes. Dol-*P* liposomes were prepared as described by Hanover *et al.* (1980). Dol-*P* [1 mg; Sigma; grade III; in 0.5 ml of chloroform/methanol (2:1, v/v)] was transferred to the tube and the solvent was evaporated under a stream of N₂. Synthetic dipalmitoylglycerophosphocholine (0.74 mg, in methanol; Calbiochem) was added to the tube and mixed well by vortex-mixer. After drying under an N₂ stream, the residue was dissolved in 80 µl of ethanol. The ethanolic solution was then dispersed in 1 ml of TMK buffer, and incubated at 37°C for the indicated times in the presence of 0.4 µCi of GDP-[³H]mannose adjusted to 24 µM with GDP-mannose. Incubation mixtures contained all the ingredients of the BSA incubation system as described for Ret-*P*-Man synthesis. The filtered microsomes were extracted with chloroform/methanol (2:1, v/v) (5 ml, five times), water (5 ml, three times) and chloroform/methanol/water (10:10:3, by vol.) (5 ml, four times). The residue was dried and treated with 40 µl of Protosol (New England Nuclear) at 50°C overnight before determination of radioactivity.

In general each experiment was performed at least twice with similar results.

Results

*Kinetics of Ret-*P*-Man synthesis from exogenous Ret-*P**

The endoplasmic reticulum was highly active in synthesizing Ret-*P*-Man from GDP-[¹⁴C]mannose with about 5.7% of the radioactivity transferred to

Fig. 1. Time dependence of Ret-P-Man formation. Conditions for incubation and filter assay were as described under 'Methods' for incubations containing BSA and Ret-P (10 μ g). GDP-[14 C]mannose (1.2 μ Ci) was used and the final concentration was adjusted to 29 μ M with GDP-mannose. Incubations contained 0.5 mg of ER protein (O) or 0.3 mg of Golgi-apparatus (GA) protein (●).

Ret-P in 20 min of incubation at 37°C per 0.5 mg of protein (Fig. 1). The Golgi apparatus contained approx. 14% of the ER activity and did not show loss of Ret-P-Man at later times. Plasma membranes contained less than 2% of the enzyme activity of ER (results not shown).

Studies on endogenous mannosyl lipid acceptors

ER membranes were active in synthesizing Ret-P-Man and Dol-P-Man from endogenous acceptor lipids (Fig. 2). Ret-P-Man synthesis displayed an absolute requirement for MnCl₂ (Fig. 2a), with optimal synthesis at 5 mM. Dol-P-Man synthesis, on the other hand, occurred at 85% of maximum value, in the absence of exogenous MnCl₂. Approx. 50% of the Ret-P-Man was recovered in the lower phase of the extract and the data in Fig. 2 are for the lower phase. About 50% of the Ret-P-Man was found in the upper phase. Fig. 2(b) shows the autoradiogram of an identical thin-layer chromatogram of the lower phase. Membranes from Golgi apparatus contained 8% of the Ret-P-Man- and 25% of the Dol-P-Man-synthesizing activity found in the ER at

Table 1. Mannosylation of endogenous protein and oligosaccharide lipid acceptors in the presence and absence of exogenous Ret-P or Dol-P

		Radioactivity (c.p.m./mg of ER protein)		
	Triton X-100 (%)	Ret-P-Man	Chloroform/methanol/water fraction	Unextracted residue
+ Ret-P	0	165 400	370	1350
	0.025	90 000	340	760
	0.050	71 500	380	730
	0.1	15 000	400	320
		Dol-P-Man		
+ Dol-P	0	43 000	620	230
	0.025	449 600	4400	730
	0.050	190 880	3170	310
	0.1	89 000	820	190
Control	0.1	3600	370	70

5 mM-MnCl₂. With regard to the crude microsomal fraction (De Luca *et al.*, 1982), Triton X-100 inhibited greatly the synthesis of Ret-P-Man and Dol-P-Man from endogenous acceptor lipids in the Golgi apparatus (results not shown) and the ER (Fig. 3).

Mannosyl-transfer activities of Ret-P and Dol-P to endogenous acceptors of ER and Golgi apparatus

Table 1 shows that Ret-P was active in stimulating transfer of mannose from GDP-mannose to endogenous non-lipid acceptors of ER, and that Triton X-100 inhibited this transfer. Golgi-apparatus membranes, which were poorly active in synthesizing Ret-P-Man and Dol-P-Man from endogenous and exogenous acceptor lipids, were also inactive in protein mannosylation (results not shown).

In contrast with Ret-P, Dol-P mainly stimulated transfer of mannose to the fraction (chloroform/methanol/water) containing the dolichyl pyrophosphate oligosaccharide compounds (Table 1), though transfer to protein was also stimulated. The detergent was required for the transfer activity and could not be replaced by liposomes, as shown in Fig. 4.

Discussion

The distribution of GDP-mannose:Ret-P mannosyltransferase activity studied in the BSA-based assay (Shidoji & De Luca, 1981) confirms that reported by Bergman *et al.* (1978) and Smith *et al.* (1979) and suggests that the main subcellular site of action of Ret-P is the ER. The specific activity of GDP-mannose:Ret-P mannosyltransferase is about 7-fold higher in ER than in Golgi apparatus in the

Fig. 2. MnCl_2 dependence of Ret-P-Man and Dol-P-Man synthesis from endogenous lipid acceptors of ER and Golgi apparatus

(a) Incubations with BSA were carried out as described under 'Methods' with $0.2 \mu\text{Ci}$ of $\text{GDP}-[^{14}\text{C}]\text{mannose}$ ($5 \mu\text{M}$) in the presence of either ER membranes (0.9 mg of protein) or Golgi apparatus (GA) (0.9 mg of protein). Incubations proceeded for 15 min at 37°C in the absence of Ret-P or Dol-P, were cooled to ice temperature and extracted with 5 vol. (1 ml) of chloroform/methanol ($2:1$, v/v), mixed and spun at low speed to yield a lower and an upper phase. The lower phase was dried under N_2 , redissolved in $60 \mu\text{l}$ of 99% methanol containing $600 \mu\text{g}$ of carrier Ret-P/ml, applied to thin-layers of silica gel under a stream of N_2 and chromatographed in chloroform/methanol/water ($45:35:6$, by vol.). The thin-layer was scraped in $250 \mu\text{l}$ of methanol and counted for radioactivity in Betafluor (New England Nuclear). ●, Dol-P-Man; O, Ret-P-Man. (b) An identical thin-layer plate was prepared as in (a) and autoradiography was performed by using a Kodak-made film XR-5 and exposure of 2 weeks after spraying with Enhance (New England Nuclear). Standard Ret-P-Man and Dol-P-Man were authenticated by normal-phase high-pressure liquid chromatography as reported by Kurokawa & De Luca (1982). Abbreviation: St., standards.

presence of excess retinyl phosphate ($136 \mu\text{M}$) (K_m $10 \mu\text{M}$) and GDP-mannose ($29 \mu\text{M}$) (K_m $13 \mu\text{M}$) (Shidoji & De Luca, 1981). Approx. 5.7% of the $[^{14}\text{C}]\text{mannose}$ added as $\text{GDP}-[^{14}\text{C}]\text{mannose}$ was transferred to exogenous Ret-P in 20 min in incu-

bations containing ER (0.5 mg of protein). However, at later times, Ret-P-Man decreased, suggesting a possible function for Ret-P-Man as an intermediate (or its hydrolysis) in the ER but not in the Golgi apparatus.

Fig. 3. Inhibitory effect of Triton X-100 on Ret-P-Man and Dol-P-Man synthesis from endogenous acceptor lipids in ER

(a) Incubations contained 5 mM-MnCl₂ and the other ingredients for the BSA incubations described under 'Methods' except that water or Triton X-100 replaced BSA. ER protein (0.9 mg) was incubated with 0.2 μCi of GDP-[^{14}C]mannose (5 μM) at 37°C for 15 min. The same procedure as described in the legend to Fig. 2 was used to analyse Ret-P-Man and Dol-P-Man. Values reported were obtained from the lower phase. ●, Dol-P-Man; ○, Ret-P-Man. (b) An identical plate was processed for autoradiography as described in the legend to Fig. 2(b). Abbreviation: St., standards.

apparatus. We found that 1.8 ng (5 pmol) of Ret-P/mg of ER protein was mannosylated in 15 min at 37°C. In contrast, in Golgi apparatus, only about 0.15 ng (0.41 pmol) of endogenous Ret-P was mannosylated. It should be emphasized that since about 50% of Ret-P-Man partitions in the upper phase, the amounts of Ret-P were calculated from the Ret-P found in the lower phase multiplied by a factor of 2. These and subsequent calculations for endogenous Dol-P refer only to the amounts of Ret-P and Dol-P actually mannosylated under the conditions of the assay and may represent a relatively small fraction of the total Ret-P and Dol-P in the membrane. When corrected for the total amount of ER and Golgi apparatus found in rat liver (Jelsema & Morr, 1978), 680 ng of Ret-P/10 g of liver would be accounted for in the ER whereas only 2 ng of Ret-P/10 g of rat liver would be contributed by the Golgi apparatus (Table 2). The latter was the range that might be expected from ER contamination of Golgi-apparatus fractions. Contrasting values for retinol are 153 μg in ER and 22 μg in Golgi apparatus per 10 g of liver (see also Krinsky & Ganguly, 1953) for retinol and retinol ester content of total microsomes. These values indicate that, although retinol may be concentrated in Golgi apparatus on a per mg of protein basis (Nyquist *et al.*, 1971), little is found phosphorylated within this organelle. The percentage of total retinol found in the ER in the phosphorylated form is low (less than 1%), indicating that phosphorylation of retinol may be rate-limiting for certain glycosylation processes, in a manner analogous to that suggested for Dol-P (Hemming, 1977).

The relative distribution of endogenous Dol-P followed closely that of Ret-P, except that more Dol-P was found in Golgi apparatus (22% of ER levels) than could be expected on the basis of ER contamination alone. ER was the main site of endogenous Dol-P (20 ng or 13.4 pmol/mg of ER protein), whereas about 4.5 ng or 3 pmol were found per mg of Golgi-apparatus protein. Since rat liver has been shown to contain 10–30 nmol (15–45 μg) of Dol-P per 10 g (Keller & Adair, 1981) therefore, based only on mannosylation of endogenous Dol-P, up to 50% of the total Dol-P in liver can be accounted for in the ER (Table 2). Measurements of 500 μg of dolichol per 10 g of liver (Dallner *et al.*, 1981) would indicate that only 3–9% of the total dolichol pool in liver is phosphorylated. However 89 ng of dolichol/mg of protein was found in microsomes (Rip *et al.*, 1981) (a major constituent is ER), which would suggest that at least 20% of the dolichol found in this subcellular fraction is phosphorylated. This would agree with the value of about 23% recently reported by Dallner *et al.* (1982).

These data also suggest that the function of Ret-P is distinct from that of Dol-P in that Ret-P is mostly

Furthermore, the BSA assay allowed the measurement of the amount of endogenous Ret-P and Dol-P available for mannosylation in the ER and Golgi

Fig. 4. Study of mannosyl transfer to Dol-P presented in liposomes

Dol-P-containing liposomes were prepared as described under 'Methods'. The final incubation medium contained 1 mM-phosphatidylcholine and 0.6 mM-Dol-P (●). Control liposomes contained only 1 mM-phosphatidylcholine (○). Extractions and other procedures are described under 'Methods'.

Table 2. Comparison between the levels of endogenous Ret-P and Dol-P and those of total retinol in subcellular fractions from rat liver

Cellular fraction	Ret-P*		Total retinol†		Dol-P*	
	(ng/mg of protein)	(ng/10 g of liver)	(ng/mg of protein)	(ng/10 g of liver)	(ng/mg of protein)	(ng/10 g of liver)
ER	1.8	680	400	153 200	20	7660
Golgi apparatus	0.15	2	1480	22 200	4.5	68

* Based on the amount of endogenous acceptor lipid mannosylated per 15 min in the BSA assay.

† From Nyquist *et al.* (1971).

active in a direct transfer to protein, whereas Dol-P mostly stimulated transfer to the fraction extracted in chloroform/methanol/water (10:10:3, by vol.) (Table 1). It should be emphasized that Dol-P specifically required the presence of detergent to show transfer activity. When Dol-P was provided as a liposomal preparation, Dol-P-Man synthesis was very high, but no significant transfer to lipid or protein could be detected, in agreement with data of Frot-Coutaz *et al.* (1979) who also failed to find transfer activity to endogenous acceptors for Dol-P liposomes, but found Ret-P liposomes to be active in mannosylation of endogenous protein. Thus the requirement for detergent in the Dol-P system may be due to the topology of the acceptors, in addition to its action merely as a solubilizing agent for Dol-P.

In conclusion, the ER of rat liver appears to host both Ret-P- and Dol-P-dependent mannosylation systems. Ret-P may well act at a postprocessing level on some of the same glycoproteins built through the Dol-P pathway, as also suggested by

recent findings (results not shown) that tunicamycin severely inhibits (60%) mannosylation of endogenous acceptors by both Ret-P and Dol-P. This scheme is also supported by findings that vitamin A deficiency leads to accumulation of endogenous Dol-P (De Luca *et al.*, 1982) and of dolichyl pyrophosphate *NN'*-diacetylchitobiose-(mannose)₃ (Rosso *et al.*, 1981) in rat liver microsomal membranes.

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References

- Bergman, A., Mankowski, T., Chojnacki, T., De Luca, L. M., Peterson, E. & Dallner, G. (1978) *Biochem. J.* **172**, 123-127
- Bhat, P. V., De Luca, L. M. & Wind, M. L. (1980) *Anal. Biochem.* **102**, 243-248

- Dallner, G., Chojnacki, T., Eggens, I. & Ekstrom, T. (1981) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40**, 1884
- Dallner, G., Chojnacki, T., Edlund, C. & Eggens, I. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 666
- De Luca, L. M. (1977) *Vitam. Horm. (N.Y.)* **35**, 1–57
- De Luca, L. M., Brugh, M., Silverman-Jones, C. S. & Shidoji, Y. (1982) *Biochem. J.* **208**, 159–170
- Frot-Coutaz, J., Letoublon, R. & Got, R. (1979) *FEBS Lett.* **107**, 375–378
- Hanover, J. A., Lennarz, W. J. & Young, Y. D. (1980) *J. Biol. Chem.* **255**, 6713–6716
- Hemming, F. W. (1977) *Biochem. Soc. Trans.* **5**, 1223–1231
- Jelsema, C. & Morré, D. J. (1978) *J. Biol. Chem.* **253**, 7960–7971
- Keller, R. K. & Adair, W. L. (1981) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40**, 26 (abstr.)
- Krinsky, N. I. & Ganguly, J. (1953) *J. Biol. Chem.* **202**, 227–232
- Kurokawa, T. & De Luca, L. M. (1982) *Anal. Biochem.* **119**, 428–432
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Morré, D. J. (1973) in *Molecular Techniques and Approaches in Developmental Biology* (Chrispeels, M. J., ed.), pp. 1–27, Wiley, New York
- Morré, D. J., Yunghans, W. N., Vigil, E. L. & Kennan, T. W. (1974) in *Methodological Developments in Biochemistry* (Reid, E., ed.), vol. 4, pp. 195–236, Longmans, London
- Nyquist, S. E., Crane, F. L. & Morré, D. J. (1971) *Science* **173**, 939–941
- Rip, J. W., Rupar, C. A., Chandhary, N. & Carroll, K. K. (1981) *J. Biol. Chem.* **256**, 1929–1934
- Rosso, G. C., De Luca, L., Warren, C. D. & Wolf, G. (1975) *J. Lipid Res.* **16**, 235–243
- Rosso, G. C., Bendrick, C. J. & Wolf, G. (1981) *J. Biol. Chem.* **256**, 8341–8347
- Sasak, W., Silverman-Jones, C. S. & De Luca, L. M. (1979) *Anal. Biochem.* **97**, 298–301
- Shidoji, Y. & De Luca, L. M. (1981) *Biochem. J.* **200**, 529–538
- Smith, M. J., Schreiber, J. B. & Wolf, G. (1979) *Biochem. J.* **180**, 449–453
- Wolf, G. (1979) *Nutr. Rev.* **35**, 97–99
- Yunghans, W. N. & Morré, D. J. (1973) *Prep. Biochem.* **3**, 301–312