Retinyl phosphate mannose synthesis in rat liver membranes

Phospholipase sensitivity and phospholipid requirement

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A remarkable and immediate decrease in GDP-mannose : retinyl phosphate mannosyltransferase activity was found on pre-incubation of rat liver postnuclear membranes with phospholipase A₂ or phospholipase C. Under the same conditions of pre-incubation (1 min at 37°C) trypsin did not affect the enzyme activity, whereas pre-incubation for 30 min with trypsin and Pronase abolished enzyme activity. The lipid extract of untreated rat liver membranes partially restored enzyme activity after phospholipase treatment. Sphingomyelin was as active as the endogenous lipids. Other phospholipids were less active in the following order: phosphatidylcholine > phosphatidylethanolamine > phosphatidylinositol = phosphatidylserine. Dolichyl phosphate mannose synthesis was inhibited less (33%) by phospholipase C than was Ret-*P*-Man synthesis (98.5%) under identical conditions of incubation, which included 0.025% Triton. However, retinyl phosphate mannose synthesis by purified endoplasmic reticulum was found to be resistant to phospholipase C. Mixing experiments failed to demonstrate an inhibitory effect of the phospholipase-treated postnuclear membrane fraction on the synthetic activity of the endoplasmic reticulum, thus excluding the release of an inhibitory factor from the postnuclear membranes.

Recently, the BSA-based incubation system of PNM from rat liver has permitted a characterization of the conditions for optimal synthesis of Ret-*P*-Man from exogenous (Shidoji & De Luca, 1981) and endogenous (De Luca *et al.*, 1982) Ret-*P*.

In the present paper we find an inhibitory effect of phospholipase C on Ret-*P*-Man synthesis in postnuclear membranes, but fail to observe the same effect on Dol-*P*-Man synthesis or on the synthesis of Ret-*P*-Man in purified endoplasmic reticulum.

Materials and methods

Materials

L- α -Phosphatidylcholine (type III-E) and sphingomyelin from chicken egg yolk, L- α -phos-

phatidylethanolamine (L- α -cephalin; type V) from *Escherichia coli*, L- α -phosphatidylinositol (grade III) from soya bean, phosphatidylserine from bovine brain, BSA, PL-ase A₂ (bee venom), PL-ase C (type 1, *Clostridium welchii* or *Bacillus cereus*), trypsin, Pronase, ribonuclease, mannose and GDP-mannose were all from Sigma. Millipore filters (HA; pore size 0.45 μ m) were from Millipore, Boston, MA, U.S.A. Amphomycin was from Bristol Myers. Ret-*P* was synthesized by chemical phosphorylation (Bhat *et al.*, 1980). GDP-[³H]-mannose (sp. radioactivity 10 Ci/mmol) and GDP-[¹⁴C]mannose (sp. radioactivity 0.2 Ci/mmol) or of specific radioactivity as stated were obtained from New England Nuclear Corporation, Boston, MA, U.S.A.

Preparation of rat liver postnuclear membranes

Osborne-Mendel-strain male rats (weighing 150–200 g), starved overnight, were 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 or as stated and kept in

Abbreviations used: PL-ase, phospholipase; Ret-*P*-Man, retinyl phosphate mannose; Dol-*P*-Man, dolichyl phosphate mannose; Ret-*P*, retinyl phosphate; Dol-*P*, dolichyl phosphate; BSA, bovine serum albumin; medium A, 50 mM-Tris/HCl, pH 7.6, containing 5 mM-MgCl₂, 25 mM-KCl and 0.25 M-sucrose; ER, endoplasmic reticulum; PNM, postnuclear membrane fraction.

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liquid N₂ until use. ER membranes were prepared as described previously (Morré *et al.*, 1974).

Phospholipase treatment

Rat liver PNM (1mg of protein) were incubated at 37°C with 100m-units, or as stated, of either PL-ase A₂ or PL-ase C, or with 5% glycerol as a control for different time intervals from 1 to 20min. Ret-*P*-dependent mannosyltransferase activity was then measured by a filter assay as previously described (Shidoji & De Luca, 1981). The standard incubation was as follows: 0.4μCi of GDP-[³H]mannose (or 0.1μCi of GDP-[¹⁴C]mannose, final concn. 24μM) was incubated with postnuclear or ER membrane protein in the presence or in the absence of 10μg of Ret-*P* (0.15mM) in a final volume of 200μl. Ret-*P* mannosyltransferase activity was measured at 37°C for 20min and was expressed as c.p.m. on the filter/mg of protein per min, after subtraction of the radioactivity on the filter from the incubation without exogenous Ret-*P*. Other details are given in the Table legends. In comparative studies between Ret-*P*-Man and Dol-*P*-Man synthesis, 0.025% Triton X-100 was used to solubilize Dol-*P* (50μg/0.2ml). All other constituents remained the same.

Ret-*P*-Man was also analysed by t.l.c. After 20min incubation, 15vol. of chloroform/methanol (2:1, v/v) was added to yield a monophasic extract. 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 (0.5cm sections) was scraped into counting

vials and the radioactivity was determined after addition of 0.25ml of methanol and 10ml of Beta-fluor (National Diagnostics, Somerville, NJ, U.S.A.).

Measurement of stability of GDP-mannose and recovery of membrane proteins in the presence and in the absence of PL-ase C

Rat liver PNM was pre-incubated for 1 min with PL-ase C (sp. activity 1000 units/2mg per ml). The ratio of PL-ase C to rat liver membranes was 100 units per mg of protein. After 1 min of pre-incubation, the equivalent of 667μg of membrane protein was incubated for 2 min at 37°C with the usual components and 0.4μCi of GDP-[³H]mannose (final concentration 24μM). It was then cooled to ice temperature and 1ml of medium A was added. The mixture was filtered through HA filters of pore size 0.45μm and washed to yield 2.0ml of filtrate. A portion (40μl) was used for chromatography on Whatman no. 1 paper in ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.) for 42h. GDP-[¹⁴C]mannose and [¹⁴C]mannose were used as standards. Alternatively, the reaction was stopped with 5vol. of chloroform/methanol (2:1, v/v) and the well-mixed solution was centrifuged to separate two phases. The lower phase was then washed with 0.6ml of methanol/water (1:1, v/v) and spun. The combined lower phases were used for determination of radioactivity. The latter extraction procedure was used in incubations containing 0.025% Triton X-

Fig. 1. Dose-response (a) and time course (b) of phospholipase inactivation of rat liver postnuclear membrane GDP-mannose: Ret-*P* mannosyltransferase

(a) Rat liver PNM (1mg of protein) was pre-incubated at 37°C for 1 min with the indicated amounts of PL-ase A₂ (○) or PL-ase C (▲). (b) Rat liver PNM (1mg of protein) was pre-incubated at 37°C with PL-ase A₂ (100m-units; ○), PL-ase C (100m-units; ▲), or with glycerol (10μl; ●) as a control, for the indicated times. After the pre-incubation, the initial velocity of Ret-*P*-Man synthetase was determined in a 2min incubation by the filter assay as described in the Materials and methods section.

100 when comparisons between Ret-*P*-Man and Dol-*P*-Man synthesis were made.

For the protein measurement in the filtrate, essentially the same procedure was followed except that no BSA was included and the 2 ml filtrate was dried and redissolved in 500 μ l of distilled water. The results are shown in Table 2.

Other procedures

Protein was determined by the method of Lowry *et al.* (1951), with BSA as standard. Ret-*P* concentration was measured spectrophotometrically at 325 nm in methanol ($A_{1\text{cm}}^{1\%} = 1440$).

Results

Phospholipase treatments

The hepatic GDP-mannose:Ret-*P* mannosyltransferase is known to be a microsomal membrane-bound enzyme (Bergman *et al.*, 1978; Smith *et al.*, 1979). As shown in Fig. 1, either PL-ase A₂ or PL-ase C decreased the Ret-*P* mannosyltransferase activity in a dose-dependent manner up to 10 m-units/mg of protein (Fig. 1a), and 100 m-units/mg caused about an 80% inhibition within 1 min (Fig. 1b). If cleavage of membrane phospholipids by PL-ase(s) causes a release of the Ret-*P* mannosyltransferase and/or Ret-*P*-Man itself, the filter assay for Ret-*P*-Man synthetase might not be adequate to measure enzyme activity after PL-ase treatment. Therefore after GDP-[³H]mannose was incubated with rat liver PNM in the presence or in the absence of PL-ase A₂, the radioactive compounds in the total chloroform/methanol extract were analysed by t.l.c. Synthesis of a mannose-labelled compound migrating at a rate similar to that of Ret-*P*-Man was selectively diminished by PL-ase A₂ treatment (results not shown). When Ret-*P*-[³H]Man (2370 c.p.m.) purified by DEAE-Sephacel (Sasak *et al.*, 1979) was incubated at 37°C with 100 m-units of PL-ase A₂ for 10 min, almost all the radioactivity (2290 c.p.m.) was recovered in the 10 mM-ammonium acetate fraction (which contains Ret-*P*-Man) from the DEAE-Sephacel column, suggesting that the mannophospholipid itself is unaffected by PL-ase A₂. The same result was obtained with phospholipase C (results not shown).

Inasmuch as these PL-ases inhibit the Ret-*P* mannosyltransferase activity immediately and without destroying Ret-*P*-Man, these lipolytic enzymes were added at 10 min incubation time and the radioactivity retained on the filter was measured (Fig. 2). There was no further increase in ³H radioactivity on the filter, and the radioactivity retained on the membrane was kept relatively constant in the presence of phospholipase C as well as amphomycin, an inhibitor of Ret-*P* mannosyltransferase (Shidoji & De Luca, 1981). PL-ase A₂, however, significantly decreased the radioactivity

Fig. 2. Effects of phospholipase A₂ and phospholipase C on the membrane-bound Ret-*P*-Man

GDP-[³H]mannose (0.4 μ Ci) was incubated at 37°C with rat liver PNM (1.5 mg of protein) in the presence of 0.13 mM-Ret-*P*. At 10 min incubation time, PL-ase A₂ (100 m-units; ○), PL-ase C (100 m-units; ▲), amphomycin (200 μ g; △), or glycerol (10 μ l; ●) was added to the incubation mixture. At the indicated times, the radioactivity associated with the membranes was measured by the Millipore filter trapping method described in the Materials and methods section. Each point represents the mean $\pm$ s.d. from triplicate incubations.

retained on the filter. At 20 min after PL-ase A₂ treatment, most of the mannanolipid in the filtrate behaved as Ret-*P*-Man on a t.l.c. plate (results not shown), suggesting that PL-ase A₂ released intact Ret-*P*-Man from the membranes. PL-ase C did not cause release of Ret-*P*-Man from the membrane.

A comparative study (Table 1) shows that trypsin was inactive in causing a decrease in GDP-mannose:Ret-*P* mannosyltransferase in pre-incubations lasting 1 and 2 min, whereas PL-ase C caused a decrease of from 33 to 72%. However, both trypsin and Pronase abolished enzyme activity when incubated with microsomes for 30 min, under conditions that did not cause a significant inhibition by ribonuclease. α -Mannosidase inhibited by 28% at 30 min. Moreover, the action of phospholipase C was totally inhibited by 0.05% sodium dodecyl sulphate, a known inhibitor (MacFarlane & Knight, 1941) of PL-ase activity, suggesting that the observed inhibition is due to the lipolytic effect of the PL-ase (Expt. VI, Table 1).

Table 1. *Effect of phospholipase C and trypsin pre-incubation on Ret-P-Man synthesis by rat liver postnuclear membranes*

Rat liver PNM was pre-incubated with PL-ase C from *Bacillus cereus* (Sigma) (1000 units/2 mg of protein per ml) using 100 m-units (0.2 μ g) of phospholipase C per mg of membrane protein in 0.9% NaCl. The pH was adjusted to 7 with 0.03 M-Tris/HCl buffer to yield a concentration of 660 μ g of membrane protein per 20 μ l. Pre-incubation at 37°C lasted for 1 min in Expts. I and II and 2 min in Expts. III and IV. At the end of pre-incubation, the mixture was equilibrated to ice temperature and 20 μ l (660 μ g) was used as the source of enzyme for Ret-P-Man synthesis in the presence of 24 μ M-GDP-[3 H]mannose (0.4 μ Ci). The reaction proceeded for 2 min at 37°C and was stopped with 1 ml of ice-cold medium A, filtered, and the filter washed with 1 ml of medium A. The filters were assayed for radioactivity in 15 ml of Hydrofluor (New England Nuclear). Rat liver PNM was pre-incubated with other enzymes, by the same procedure. Trypsin from bovine pancreas (Sigma) (9235 BAEE units/mg of protein) was used in the ratio of 1855 m-units (0.2 μ g) per mg of membrane protein in Expts. I (1 min at 37°C) and III (2 min at 37°C). For Expt. V 100 μ g of trypsin, 100 μ g of Pronase, 10 m-units of α -mannosidase or 500 μ g of ribonuclease were utilized in a 30 min pre-incubation with 1.3 mg of membrane protein. For Expt. VI, rat liver PNM was pre-incubated with PL-ase C from *B. cereus* (Sigma) (1667 units/2.5 mg of protein per ml) using 107 m-units (0.16 μ g) of phospholipase C per mg of membrane protein in 0.9% NaCl. The pH was adjusted to 7 with 0.03 M-Tris/HCl buffer to yield a concentration of 666 μ g of membrane protein per 20 μ l. Pre-incubation at 37°C lasted for 1 min. At the end of pre-incubation, the mixture was equilibrated to ice temperature and 20 μ l (666 μ g) was used as the source of enzyme for Ret-P-Man synthesis in the presence of 24 μ M-GDP-[14 C]mannose (0.1 μ Ci). The reaction proceeded for 2 min at 37°C and was stopped with 1 ml of ice-cold medium A, filtered, and the filter washed with 1 ml of medium A. The filters were assayed for radioactivity in 10 ml of Betafluor (National Diagnostics). GDP-[14 C]mannose (0.1 μ Ci; final concn. 0.24 μ M) was incubated with 0.67 mg of protein in the presence or in the absence of 10 μ g of Ret-P (0.15 mM) in a final volume of 200 μ l or in the presence or in the absence of 71.42 m-units of PL-ase C (107 m-units/mg of rat liver postnuclear membrane vesicles) and/or 0.1 mM-CaCl₂. Values in parentheses are means. Abbreviation used: SDS, sodium dodecyl sulphate.

	Exogenous Ret-P-dependent Ret-P-Man synthesis		
	(c.p.m.)	(3 H c.p.m./ 2 min per 0.6 mg of protein)	(% of control)
Expt. I			
- Ret-P	2290 3470 4230 } (3300)	0	0
+ Ret-P	14360 17020 12860 } (14740)	11410	100
+ Ret-P + PL-ase C	7310 6250 6150 } (6570)	3240	28
+ Ret-P + trypsin	14760 15440 13510 } (14570)	11240	99
Expt. II			
- Ret-P	2530 3130 2640 } (2760)	0	0
+ Ret-P	14370 13120 11360 } (12950)	10180	100
+ Ret-P + PL-ase C	10120 9300 9230 } (9550)	6780	67
Expt. III			
- Ret-P	3550 3120 3640 } (3430)	0	0
+ Ret-P	13400 13200 16060 } (14220)	10780	100
+ Ret-P + PL-ase C	8780 7130 8290 } (8067)	4630	43
+ Ret-P + trypsin	16150 14690 16850 } (15890)	12460	115

	Exogenous Ret- <i>P</i> -dependent Ret- <i>P</i> -Man synthesis		
	(c.p.m.)	³ H c.p.m./ 2 min per 0.6 mg of protein)	(% of control)
Expt. IV			
– Ret- <i>P</i>	3830 } (3050)	0	0
+ Ret- <i>P</i>	2270 } (18660)	15610	100
+ Ret- <i>P</i> + PL-ase C	19550 } 17770 } (7360)	4310	27.6
Expt. V			
– Ret- <i>P</i>	4700 } (4550)	0	0
+ Ret- <i>P</i>	4400 } (20100)	15550	100
+ Ret- <i>P</i> + Pronase	22700 } 17500 } (3440)	0	0
+ Ret- <i>P</i> + trypsin	3480 } (3940)	0	0
+ Ret- <i>P</i> + α-mannosidase	3400 } 3700 } (15750)	11200	72
+ Ret- <i>P</i> + ribonuclease	4180 } 15970 } (19000)	14450	93
Expt. VI			
– Ret- <i>P</i>	15530 } (3900)	0	0
+ Ret- <i>P</i>	17200 } (22600)	18600	100
+ Ret- <i>P</i> + CaCl ₂	20800 } (25900)	21900	117
+ Ret- <i>P</i> + CaCl ₂ + PL-ase C	18600 } (10700)	6700	36
+ Ret- <i>P</i> + CaCl ₂ + PL-ase C	26500 } (25800)	21900	117
+ SDS (0.05%)	27200 } (11300)	7300	33
+ Ret- <i>P</i> + PL-ase C	24600 } 11250 } 10200 }		

Table 2. *Stability of GDP-mannose and recovery of postnuclear membrane proteins in the presence of phospholipase C* PL-ase C (specific activity 500 units/mg of protein per 0.5 ml) was incubated with rat liver PNM in the ratio of 100 units/mg of membrane protein. After pre-incubation, 667 μg of protein was incubated as described in the legend to Table 1 with 0.4 μCi of GDP-[³H]mannose (24 μM final concentration). Incubation proceeded for 2 min at 37°C. Samples were processed as specified in the Materials and methods section. Loss of membrane protein in the filtrate was measured as described in the Materials and methods section.

	³ H radioactivity as Ret- <i>P</i> -Man (c.p.m./0.6 mg per 2 min)	³ H radioactivity in GDP- [³ H]mannose (c.p.m.)	Ratio of protein in filtrate/total protein
– PL-ase C	12000	165000	161:667
+ PL-ase C (1 min)	6300	195000	174:667

The decrease in apparent enzyme activity of the crude PNM caused by PL-ase C was not due to either substrate depletion or the non-specific solubilization of membrane protein as shown in Table 2.

Phospholipid reconstitution experiments

Lipid extract obtained from an equivalent amount of PNM as used in the assay was tested for Ret-*P*-Man synthesis in membranes that had been pre-incubated with PL-ase C as described in the

legend to Table 3. The lipid extract was able to partially restore the activity from 22.8 to 46.2% in the absence of sodium cholate (0.15%) and from 11.9 to 46.8% of control in the presence of sodium cholate, as shown in Expt. I of Table 3. Of the various phospholipids tested, sphingomyelin was nearly as active as the lipid extract (Table 3, Expt. II). Fig. 3 shows a dose-response study for the effect of sphingomyelin. At 300 μg of sphingomyelin for 0.48 mg of microsomal protein the Ret-*P*-Man-syn-

Table 3. *Effect of phospholipase C treatment and phospholipid addition on Ret-P-Man synthesis by rat liver postnuclear membranes*

In Expt. I PNM (10mg of protein in 1 ml) was added to 5 ml of chloroform/methanol (2:1, v/v). After centrifugation (3600 rev./min; 5 min), the lower organic phase was transferred to another tube, washed with 500 μ l of distilled water, dried under vacuum and solubilized in 1 ml of chloroform/methanol (2:1, v/v). For PL-ase C treatment of PNM a solution was prepared containing 250 μ l of microsomes (69 mg/ml), 1.5 ml of medium A, 250 μ l of a solution of BSA (40 mg/ml) and 20 μ l of a solution of 0.1 M-CaCl₂. Of this mixture (1 ml) was incubated at 37°C for 30 min after addition of 100 μ l of a solution containing PL-ase C (1 mg/ml). Control incubations contained 100 μ l of 50% glycerol. Ice-cold medium A (5 ml) was added to terminate the action of the phospholipase C and the sample was centrifuged at 105000g for 60 min to obtain the membrane pellet. The pellet was then suspended in 5 ml of medium A and centrifuged at 105000g for 60 min. The washed pellet was resuspended in 1 ml of medium A. Portions (250 μ l) were used for PL-ase C-treated and control membranes. Lipid extract from the equivalent amounts of membrane preparation was added to the PNM after evaporation to dryness. Each mixture was then sonicated for 30 s at 4°C at short intervals for 10 consecutive times (total time 5 min). The described suspensions were then used as enzyme source for Ret-P-Man biosynthesis. The solution (60 μ l or 4.1 mg of protein) was incubated with or without Ret-P (10 μ g) and 0.4 μ Ci of GDP-[³H]mannose (sp. radioactivity 10.4 Ci/mmol) at 37°C for 5 min in the BSA-containing incubation system. In Expt. II a solution containing 0.3 ml of PNM (69 mg/ml), 0.5 ml of BSA (40 mg/ml), 25 μ l of CaCl₂ (100 mM) and 1.77 ml of medium A was used for PL-ase C or glycerol control treatments. To the above solution (1.2 ml) was added 100 μ l of a solution of PL-ase C (1 mg/ml) or 100 μ l of 50% glycerol. The mixture was incubated for 30 min at 37°C, diluted with 5 ml of ice-cold medium A, centrifuged at 105000g for 60 min to yield a pellet that was resuspended in 5 ml of ice-cold medium A and re-centrifuged at 105000g for 60 min. The resulting pellet was suspended in 1.2 ml of medium A (8 mg of membrane protein per ml). The equivalent of 1.6 mg of protein (200 μ l) of control and of treated enzyme suspensions was then reconstituted with 50 μ l (500 μ g) of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol or sphingomyelin. The resulting mixtures were sonicated for 30 s for 10 consecutive times at 4°C. Ret-P-Man synthesis was measured in incubations containing 0.4 μ Ci of GDP-[³H]mannose (10.4 Ci/mmol) and 0.4 mg of protein (150 μ g of phospholipid) at 37°C for 5 min in duplicate.

Expt.	Treatments	Exogenous Ret-P-dependent Ret-P-Man synthesis	
		(³ H c.p.m./5 min per 0.6 mg of protein)	(% of control)
Expt. I			
	+ Ret-P	11400	100
	+ Ret-P + sodium cholate (0.15%)	7710	67.7
	+ Ret-P + lipid extract	10700	94
	+ Ret-P + sodium cholate (0.15%) + lipid extract	10770	94.5
	+ Ret-P + PL-ase C	2600	22.8
	+ Ret-P + PL-ase C + sodium cholate (0.15%)	1350	11.9
	+ Ret-P + PL-ase C + lipid extract	5270	46.2
	+ Ret-P + PL-ase C + sodium cholate (0.15%) + lipid extract	5330	46.8
		(³ H c.p.m./5 min per 0.48 mg of protein)	
Expt. II			
	+ Ret-P	18100	100
	+ Ret-P + PL-ase C	3000	16
	+ Ret-P + PL-ase C + phosphatidylserine	2730	15
	+ Ret-P + PL-ase C + phosphatidylinositol	3060	16
	+ Ret-P + PL-ase C + phosphatidylethanolamine	4180	23
	+ Ret-P + PL-ase C + phosphatidylcholine	5130	28
	+ Ret-P + PL-ase C + sphingomyelin	7600	42

thesizing activity was restored from 11% to 47% of control (Fig. 3a). Fig. 3(b) shows the fluorography of the t.l.c. of the lower phase extract of the various incubations.

The effect of PL-ase treatment appeared specific for Ret-P-Man synthesis inasmuch as Dol-P-Man synthesis under the same conditions of incubation including 0.025% Triton was relatively resistant

Fig. 3. Sphingomyelin dependence of Ret-P-Man synthesis

(a) PL-ase C-treated PNM was prepared by pre-incubation at 37°C for 1 min and incubated at 0.48 mg of protein per incubation with increasing concentrations of sphingomyelin (0, 30, 90, 150 and 300 μg). The sphingomyelin solution was dried and then resuspended in the presence of the enzyme by sonication (10 times for 30 s each at 4°C). Control membranes were treated in the same way. Incubations were carried out as usual at 37°C for 5 min and contained $0.4 \mu\text{Ci}$ ($10.4 \text{ Ci}/\text{mmol}$) of $\text{GDP}-[{}^3\text{H}]\text{mannose}$ and 0.48 mg of membrane protein in the presence or in the absence of $10 \mu\text{g}$ of Ret-P. The filter assay was used to measure Ret-P-Man synthesis. (b) An identical experiment was carried out using $\text{GDP}-[{}^{14}\text{C}]\text{mannose}$ ($0.2 \mu\text{Ci}$; sp. radioactivity $269 \text{ Ci}/\text{mol}$) at a final GDP-mannose concentration of $24 \mu\text{M}$. In this experiment the incubation was terminated by the addition of 5 vol. (1 ml) of chloro-

(Table 4). However, Ret-P-Man synthesis in purified ER was not affected by the PL-ase (Table 4). Moreover, mixing experiments failed to demonstrate the release of a factor from the PNM responsible for inhibition of Ret-P-Man synthesis. In fact Ret-P-Man synthesis on the ER was not affected by the presence of the PL-ase-treated PNM (Table 4). Thus, if a factor is generated by the PL-ase, it must remain in the membrane, where it affects Ret-P-Man synthesis.

Discussion

Most mammalian glycosyltransferases, involved in glycolipid and glycoprotein synthesis, are membrane-bound enzymes and some of them are known to be sensitive to PL-ase treatments. PL-ase A, for example, inhibits UDP-glucuronyltransferase activity (Graham & Wood, 1969). In the present paper, we show that rat liver GDP-mannose:Ret-P mannosyltransferase activity present in the crude PNM was rapidly inhibited by either PL-ase A₂ or PL-ase C treatment under conditions that did not cause inhibition by trypsin (Fig. 1, Table 1). The inhibitory activity of the PL-ase C preparation was abolished by 0.05% sodium dodecyl sulphate, a known inhibitor of PL-ase C activity (MacFarlane & Knight, 1941).

Interestingly, Dol-P-Man synthesis appeared relatively resistant to the action of PL-ase C under conditions (Table 4) that showed more than 90% inhibition of Ret-P-Man synthesis. This finding may be of potential usefulness in the study of the topology and phospholipid requirement of the synthesis and utilization of the two lipid intermediates.

In contrast with PNM, the synthesis of Ret-P-Man on purified ER was not inhibited by PL-ase C treatment, suggesting that the crude membrane fraction may have released a soluble factor ultimately responsible for the inhibitory effect on Ret-P-Man synthesis on the ER. However, this view is

form/methanol (2:1, v/v) to yield a lower organic phase upon centrifugation at 3600 rev./min for 5 min. A portion (20 μl) of the lower phase was used for radioactivity determination (Fig. 3a). The remaining solution was evaporated to dryness, solubilized in 100 μl of chloroform/methanol (2:1, v/v) and applied to thin layers of silica gel, which were developed in chloroform/methanol/water (43:35:6, by vol.) and used for fluorographic analysis. The positions of standard Ret-P-Man and Dol-P-Man are indicated by arrows. The 'unknown' refers to a minor radioactive compound of undetermined structure.

Table 4. Comparison of the effect of phospholipase C on Ret-P-Man and Dol-P-Man synthesis in postnuclear membranes and lack of effect in endoplasmic reticulum

Values in parentheses are means. Expt. I was conducted on PNM to test the activity of PL-ase C against Dol-P-Man and Ret-P-Man synthesis. The membranes were pre-incubated as described for 1 min at 37°C with PL-ase C (71 m-units per incubation). The mixture was then cooled to about 4°C and incubated as usual for 2 min at 37°C. The only difference in this experiment was the addition of 0.025% Triton X-100 to all incubations to solubilize Dol-P used at 50 µg/200 µl. Also the biphasic extraction as outlined in the Materials and methods section was used for measurement of Dol-P-Man and Ret-P-Man. GDP-[¹⁴C]mannose (0.1 µCi, final concentration 1.86 µM) was used in this experiment with 0.67 mg of PNM protein. In Expt. II, ER was pre-incubated with PL-ase C from *B. cereus* (Sigma) (1667 units/2.5 mg of protein per ml) using 101 m-units (0.16 µg) of PL-ase C per mg of membrane protein in 0.9% NaCl. The pH was adjusted to 7 with 0.03 M-Tris/HCl buffer to yield a concentration of 696 µg of membrane protein per 80 µl. Pre-incubation at 37°C lasted for 1 min. At the end of pre-incubation, the mixture was equilibrated to ice temperature and 80 µl (696 µg) was used as the source of enzyme for Ret-P-Man synthesis in the presence of 24 µM (0.1 µCi) GDP-[¹⁴C]mannose. The reaction proceeded for 2 min at 37°C and was stopped with 1 ml of ice-cold medium A, filtered, and the filter washed with 1 ml of medium A. The filters were assayed for radioactivity in 7 ml of Betafluor from National Diagnostics. For the incubation mixture GDP-[¹⁴C]mannose (0.1 µCi; final concn. 24 µM) was incubated with 0.67 mg of protein (ER) in the presence or in the absence of 10 µg (0.133 mM) of Ret-P in a final volume of 200 µl, and in the presence or in the absence of 71.10 m-units of PL-ase C (103 m-units/mg of ER). For Expt. III procedures were essentially the same as for Expt. II, except that 0.33 mg of ER protein and 0.67 mg of PNM protein was used. The biphasic extraction procedure was used to measure Ret-P-Man and Dol-P-Man as outlined in the Materials and methods section.

Exogenous Ret-P- or Dol-P-dependent mannlipid			
Expt. I		(c.p.m./2min per 0.67 mg of protein)	(% of control)
- Ret-P	4600 } (4150) 3700 }		
+ Ret-P	27350 } (28670) 30000 }	24500	100
+ Ret-P + PL-ase C	4660 } (4600) 4500 }	420	1.5
- Dol-P	3500 } (3680) 3800 }		
+ Dol-P	27400 } 21200 } (24500) 35000 } 23800 }	20800	100
+ Dol-P + PL-ase C	23200 } 25900 } (17600) 11750 } 18100 } 17100 } 15200 }	13900	67
Exogenous Ret-P-dependent Ret-P-Man synthesis			
Expt. II (ER)		(¹⁴ C c.p.m./2min)	(% of control)
- Ret-P	3160 } (3100) 3100 }	0	0
+ Ret-P	29800 } (27100) 24400 }	23900	100
+ Ret-P + PL-ase C	34900 } (34500) 34100 }	31400	131
Exogenous Ret-P- or Dol-P-dependent mannlipid			
Expt. III		(c.p.m./2min per 0.67 µg of protein)	(% of control)
- Ret-P + PNM	8100 } (6100) 4070 }		
+ Ret-P + PNM	25700 } (27300) 28800 }	21230	100
+ Ret-P + PL-ase C + PNM	9200 } (7080) 5000 }	980	4

		Exogenous Ret- <i>P</i> - or Dol- <i>P</i> -dependent mannlipid	
		(c.p.m./2 min per 0.67 µg of protein)	(% of control)
+ Ret- <i>P</i> + ER	15400 } (18270)	12170	100
	21100 }		
+ Ret- <i>P</i> + PL-ase C + ER	22900 } (18100)	12000	98
	13000 }		
+ Ret- <i>P</i> + PL-ase C + ER + PNM	14800 } (11800)	5700	47
	8800 }		

incompatible with the results of the mixing experiments shown in Table 4, where Ret-*P*-Man synthesis on the purified ER was unaffected by the presence of the crude membrane fraction treated with PL-ase C.

These results exclude the possibility that the PL-ase acts directly on either Ret-*P* or Ret-*P*-Man and demonstrate that the inhibitory effect is mediated by a domain in the crude membrane (and not a released factor) that has been lost during the preparation of the ER. Upon binding of the PL-ase, phospholipid cleavage may, in principle, cause the inhibitory effect because of the production of lyso-phosphatidylcholine, fatty acid and diacylglycerol, which may remain bound to the membranes, and may by themselves have an inhibitory effect on Ret-*P*-Man synthesis.

The ability of lipidic extracts from rat liver microsomes and of sphingomyelin to restore, at least partially, PL-ase-sensitive Ret-*P*-Man synthesis strongly supports the concept that the phospholipid environment of the membrane is required for this activity; however, the mechanism whereby the inhibition of Ret-*P*-Man synthesis occurs as the result of PL-ase C treatment remains unknown.

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