Subcellular Localization of the Enzyme that Forms Mannosyl Retinyl Phosphate from Guanosine Diphosphate [^{14}C]Mannose and Retinyl Phosphate

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The subcellular distribution of the enzyme catalysing the conversion of retinyl phosphate and GDP-[^{14}C]mannose into [^{14}C]mannosyl retinyl phosphate was determined by using subcellular fractions of rat liver. Purity of fractions, as determined by marker enzymes, was 80% or better. The amount of mannosyl retinyl phosphate formed (pmol/min per mg of protein) for each fraction was: rough endoplasmic reticulum, 0.48 ± 0.09 (mean $\pm$ s.d.); smooth membranes (consisting of 60% smooth endoplasmic reticulum and 40% Golgi apparatus), 0.18 ± 0.03 ; Golgi apparatus, 0.13 ± 0.03 ; and plasma membrane 0.02.

Mannosyl retinyl phosphate can be synthesized from GDP-[^{14}C]mannose and retinyl phosphate in a reaction catalysed by a crude rat liver microsomal membrane fraction (Rosso *et al.*, 1975). Evidence has been obtained indicating that mannose is transferred from mannosyl retinyl phosphate to acceptor(s) located in the same fraction (Rosso *et al.*, 1977). The suggestion has been made that mannosyl retinyl phosphate might be formed and might function in the surface membrane of liver cells (Arnold *et al.*, 1976). To investigate this possibility and perhaps to obtain clues to the specificity and function of mannosyl retinyl phosphate in mannose transfer, the microsomal membrane fraction was further fractionated to localize the enzyme involved in mannosyl retinyl phosphate synthesis. While this work was in progress, a report appeared (Bergman *et al.*, 1978) on the subcellular distribution of the synthesis of polyprenyl-linked sugars, including retinol, which reached conclusions somewhat different from those of the present paper.

Materials and Methods

Preparation of liver membrane fractions

Three male Sprague–Dawley albino rats (200–280 g) were starved for 18 h, then decapitated and exsanguinated. Livers were excised, blotted, and weighed (each liver weighed about 9 g). They were immersed in cold 0.25 M-sucrose, cut into large pieces, and homogenized in 5 vol. of 0.25 M-sucrose/10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid] buffer (pH 7.6) in a Potter–Elvehjem homogenizer as described by Fleischer & Kervina (1974), except that only two, instead of three, up-and-down strokes were used during homogenization to preserve the structure of the Golgi apparatus.

Fractionation was carried out exactly as described by Fleischer & Kervina (1974). Rough-endoplasmic-reticulum and smooth-membrane fractions were resuspended after isolation in Tris buffer and repelleted to wash away adsorbed protein and free ribosomes (Beaufay *et al.*, 1974). Purity of the fractions was checked by published methods as indicated in the legend to Fig. 1. In addition, the Golgi apparatus marker enzyme, galactosyltransferase (Morré, 1969; Mookerjee & Yung, 1975; Bauer *et al.*, 1976), was shown to transfer galactose to desialylated degalactosylated fetuin linearly with time up to 40 min, and with protein concentrations up to 0.8 mg/ml of Golgi apparatus. Protein was assayed by the method of Lowry *et al.* (1951).

Enzyme assay

Despite the losses of mannosyl retinyl phosphate in the aqueous (upper) phase of a Folch-extraction procedure (Silverman-Jones *et al.*, 1976), we developed a method based on this procedure to provide a rapid analytical assay of mannosyl retinyl phosphate formation. The reproducibility of results obtained with this method was checked by a double-label procedure. For the standard assay, retinyl phosphate (20 μg ; final concentration, 0.26 mM), synthesized and purified as described by Rosso *et al.* (1975), was placed in an incubation tube with GDP-[^{14}C]mannose (0.5 μCi ; sp. radioactivity 200 mCi/mmol; final concentration, 12.5 μM). Solvents were removed under N_2 . This and subsequent procedures were carried out in dim incandescent light. The substrates were then taken up in 20 μl of aqueous 5% Triton X-100, and the following substances were added to give the final concentrations indicated: Tris/HCl, pH 7.5, 30 mM; MnCl_2 , 10 mM; ATP, 2.7 mM; EDTA, 2.5 mM; and enzyme, to a final volume of 0.2 ml. The

following amounts of protein from each fraction were added per incubation: homogenate, 3.5–4.0 mg; rough endoplasmic reticulum, 1.1 mg; smooth membranes, 0.7–0.9 mg; Golgi apparatus, 0.1–0.2 mg; plasma membrane, 0.3 mg. After enzyme addition, the incubations were flushed with N_2 gas, closed, and incubated for 20 min at 30°C. The reaction was terminated by the addition of 3 ml of chloroform/methanol (2:1, v/v), followed by 0.6 ml of 0.9% NaCl. The mixture was shaken on a vortex mixer, the lower (organic) phase was removed (first extract) and washed twice with 0.4 ml of 0.9% NaCl/methanol (2:1, v/v). These aqueous washings were added to the aqueous residue from the first extraction, and the washings and residue were re-extracted with 3 ml of chloroform/methanol (2:1, v/v). The organic (lower) phase was again removed (second extract), and the aqueous residue was extracted as described above. The organic (lower) phase from this extraction was combined with the first and second extracts. The solvents from the combined extracts were removed in a rotary evaporator under N_2 ; the residue was taken up in 0.1 ml of methanol and chromatographed on silica gel G thin-layer plates in chloroform/methanol/water (60:35:6, by vol.) with retinyl phosphate as a marker for mannosyl retinyl phosphate (Silverman-Jones *et al.*, 1976), which was localized by u.v. light. The spot corresponding to mannosyl retinyl phosphate was then scraped from the plate, and radioactivity was counted. Mannosyl retinyl phosphate was clearly separated from mannose and dolichyl mannosyl phosphate (Fig. 2). Freeze-drying of the aqueous solutions followed by t.l.c. showed that about

40% of the mannosyl retinyl phosphate was lost in the aqueous phase by this procedure. To determine accurately the extent of the loss and to test the reproducibility of the method, we conducted four assays with rough endoplasmic reticulum and homogenate as described above, except that GDP-[3H]mannose was used (sp. radioactivity 12.6 Ci/mmol, diluted to 400 mCi/mmol with non-radioactive GDP-mannose; 1 μ Ci was used per incubation). At the end of the incubation, [^{14}C]mannosyl retinyl phosphate (4000–6000 d.p.m.), prepared and purified by the procedure of Silverman-Jones *et al.* (1976), was added to the incubation mixture, and the loss of mannosyl retinyl phosphate in the aqueous phase under the conditions of the assay described above was found to be $34.9 \pm 4.3\%$ (mean $\pm$ s.d.). This value was then used to correct for losses of mannosyl retinyl phosphate in the standard assay.

To isolate GDP-[^{14}C]mannose and to assay for any that might remain in the medium after incubation, we applied a small portion of the incubation mixture to a cellulose thin-layer plate, which was then chromatographed for 5 h in ethyl acetate/n-butanol/water/glacial acetic acid (6:8:8:5, by vol.), dried, and chromatographed again in the same solvent (Richards & Hemming, 1972). The nucleotide sugar was located by a guide spot of the pure substance, scraped from the plate, and counted for radioactivity.

Materials

GDP-[U- ^{14}C]mannose, synthesized by the method of Braell (1976), was kindly supplied by Dr. P. W.

Fig. 1. Purity of subcellular fractions

(a) Glucose 6-phosphatase as endoplasmic-reticulum marker, assayed as described by Aronson & Touster (1974). (b) Succinate-2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium reductase (succinate-INT reductase) as mitochondrial marker (Morré, 1971). (c) 5'-Nucleotidase as plasma-membrane marker (Aronson & Touster, 1974), with the modification of using 0.2 ml of 2.48% ascorbate instead of 1-amino-2-naphthol-4-sulphonic acid for phosphorus assay. (d) Galactosyl transferase as Golgi-apparatus marker (Mookerjee & Yung, 1975; Bauer *et al.*, 1976). (e) RNA assays (Munro & Fleck, 1966) as a marker for rough endoplasmic reticulum. Marker-enzyme assays show results of determinations on single preparations, or means for two to three preparations, where vertical lines give the range, except for the RNA assay, which shows the mean $\pm$ s.d. for six different preparations. Abbreviations used: H, homogenate; PM, plasma membrane; RER, rough endoplasmic reticulum; SM, smooth membrane; GA, Golgi apparatus.

Robbins, Department of Biology, Massachusetts Institute of Technology. GDP-[^3H]mannose was purchased from New England Nuclear Corp., Boston, MA, U.S.A. UDP-[$^6\text{-}^3\text{H}$]galactose (sp. radioactivity 16.3 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, IL, U.S.A. UDP-galactose, ATP (disodium salt), thymidine 5'-monophosphate *p*-nitrophenyl ester (grade 1), *p*-iodonitrotetrazolium violet [2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyltetrazolium chloride], (grade 1), Hepes, and sucrose (grade 1) were all purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results

Fig. 1 shows the results of purity tests of the membrane fractions. Endoplasmic reticulum (both rough endoplasmic reticulum and smooth membranes) was characterized by its high glucose 6-phosphatase activity (Fig. 1a); it was only insignificantly contaminated by mitochondria (Fig. 1b) or plasma membrane (Fig. 1c). Rough endoplasmic reticulum had only 8% Golgi apparatus contamination; smooth membranes were clearly a mixture of 60% smooth endoplasmic reticulum and 40% Golgi apparatus (Fig. 1d). The difficulty in removing Golgi apparatus from smooth endoplasmic reticulum has been a common problem found by previous researchers (e.g., Fleischer & Kervina, 1974). Golgi apparatus was characterized by a high amount of galactosyltransferase activity (Fig. 1d); it was only insignificantly contaminated by endoplasmic reticulum (Fig. 1a) or plasma membrane (Fig. 1c). Plasma membrane was characterized by 5'-nucleotidase; it contained only small amounts of endoplasmic reticulum (Fig. 1a), mitochondria (Fig. 1b), or Golgi apparatus (Fig. 1d). To distinguish between rough endoplasmic reticulum and smooth membranes, we assayed the RNA content, which showed high concentrations in rough endoplasmic reticulum (Fig. 1e). In conclusion, all fractions (except smooth membranes) were about 80% pure, or better.

A rapid analytical assay for mannosyl retinyl phosphate formation was developed and its reproducibility checked as described in the Materials and Methods section. As shown in Fig. 2, mannosyl retinyl phosphate was readily separable from mannose and dolichyl mannosyl phosphate.

To assay for mannosyl retinyl phosphate formation in the various cell fractions, we found it necessary to demonstrate that this reaction is protein dependent; Fig. 3 shows that it is, at least for rough endoplasmic reticulum and smooth membranes. These data were replotted to reveal the effect of the ratio of the concentrations of Triton X-100 to protein on the reaction, since Nilsson *et al.* (1978) showed that this detergent had a profound effect on the transfer of mannose from GDP-mannose to lipid acceptors.

Fig. 4 demonstrates that, although smooth membranes were not much affected at high concentration ratios, the detergent appeared to inhibit the reaction catalysed by rough endoplasmic reticulum.

Fig. 5 shows the localization of mannosyl retinyl phosphate formation. The data are derived from five preparations, each made from a separate rat liver. Smooth membranes had about one-half the activity of rough endoplasmic reticulum and about the same activity as Golgi apparatus; cytosol and plasma membrane had very little activity. The determinations were made for rough endoplasmic reticulum and smooth membranes at the same relatively low ratio of Triton X-100 to membrane protein (w/w ratio of Triton/protein for each was

Fig. 2. Thin-layer chromatogram of combined Folch extracts of incubation of GDP-[^{14}C]mannose and retinyl phosphate with rough endoplasmic reticulum (a) or smooth membranes (b), showing residual [^{14}C]mannose (M), dolichyl mannosyl phosphate (DMP), and mannosyl retinyl phosphate (MRP), the last named coinciding with the retinyl phosphate (RP) guide spot

Chloroform/methanol/water (60:35:6, by vol.) on silica gel G was used as solvent.

Fig. 3. Protein dependence of mannosyl retinyl phosphate formation

The assay was performed as described in the Materials and Methods section for mannosyl retinyl phosphate formed with increasing amounts of protein of rough endoplasmic reticulum (●) or smooth membranes (○) incubated.

Fig. 4. Dependence of mannosyl retinyl phosphate formation on detergent/protein ratio

Results shown in Fig. 3 are replotted to show dependence of enzyme specific radioactivity (c.p.m./mg of protein per min) on the Triton X-100/protein (w/w) ratio of the rough-endoplasmic-reticulum (●) and smooth (○) membrane preparations.

Fig. 5. Subcellular localization of mannosyl retinyl phosphate formation

Assays for mannosyl retinyl phosphate formation were as described in the Materials and Methods section. Abbreviations used: PM, plasma membrane; RER, rough endoplasmic reticulum; SM, smooth membrane; GA, Golgi apparatus. The vertical bars represent standard deviations.

approx. 1), which gave the highest activities for both rough endoplasmic reticulum and smooth membranes, as shown in Fig. 4. Because of the shortage of available Golgi apparatus and plasma membrane materials, the effect of detergent concentration ratio on these fractions could not be investigated. The results in Fig. 5 for Golgi apparatus and plasma membrane were obtained with preparations containing a high ratio of Triton X-100/protein (6.7 and 3.3 respectively). Based on the results reported by Nilsson *et al.* (1978) for Golgi apparatus, one might expect a greater transfer activity for lower ratios of Triton/protein, at least in this fraction.

The low activity in Golgi apparatus was not caused by the high amount of phosphodiesterase activity peculiar to this fraction, which might have

destroyed the substrate, GDP-mannose. We determined that over 30% of the GDP-[^{14}C]mannose still remained at the end of the incubation period, no doubt protected by the presence of the ATP.

Discussion

Bergman *et al.* (1978) found that, in the presence of added retinyl phosphate, the highest enzymic activity for mannosyl retinyl phosphate formation resided in the smooth membrane fraction. It was 50% higher than the activity in rough endoplasmic reticulum. Under similar conditions, in our hands, the rough endoplasmic reticulum was by far the most active fraction. In the present studies, the activity in smooth membranes was about equal to that in Golgi apparatus, a fraction found to have very low activity by Bergman *et al.* (1978). The only substantial differences between the conditions used by Bergman *et al.* (1978) and those used by us that could account for the differences in the results obtained are: (1) their use of microsomal phospholipid in the incubation mixture; and (2) their use of dimethyl sulphoxide as solvent where we used the detergent Triton X-100. We conducted tests with whole-microsomal-membrane fractions and found that the omission of phospholipid had no effect on mannosyl retinyl phosphate formation.

With respect to the use of Triton X-100, we determined the dependence of the reaction on both protein and detergent concentrations and then carried out the reaction at the detergent/protein concentration ratio giving the highest activity, at least for rough endoplasmic reticulum and smooth membranes. Bergman *et al.* (1978) used dimethyl sulphoxide in their incubations, a solvent for which the effect on the transfer of mannose from GDP-mannose to lipid is unknown. Their solvent/protein ratios varied for the different fractions.

We were unable to test the effect of detergent on Golgi apparatus activity; it is likely, however, that at lower Triton/protein ratios than we used, the activity of this fraction would have been higher and approached that observed in smooth membranes (Nilsson *et al.*, 1978). In that case, the difference between our data and those of Bergman *et al.* (1978), who found very low Golgi apparatus activity, would have been further accentuated.

Other reasons for believing our results to be more reliable than those of Bergman *et al.* (1978) are: (1) we quantitatively standardized our isolation procedure for mannosyl retinyl phosphate by using doubly labelled mannosyl retinyl phosphate to test reproducibility; (2) we evaluated our results statistically (for both reproducibility of the assay and distribution of enzyme activity) and reported standard deviations, whereas Bergman *et al.* (1978) reported median values only; (3) we tested the purity of each

fraction and performed the reaction for mannosyl retinyl phosphate formation on the same fractions so tested; and (4) we checked the efficacy of our ATP concentration (2.7 mM) in preventing GDP-mannose breakdown. Bergman *et al.* (1978) used a smaller concentration of ATP (1.8 mM) and made no mention of determining the preservation of the substrate throughout the course of their reaction.

Arnold *et al.* (1976) reported that plasma membrane of chick embryo liver was active in mannosyl retinyl phosphate formation. In the present work this fraction had negligible mannosyl retinyl phosphate-forming activity. It is possible that, at a lower detergent concentration than we used, this fraction may have shown some activity. However, it seems likely that the difference in data is caused by a species and tissue difference.

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