THE BIOSYNTHESIS OF MANNOSE-CONTAINING GLYCOPROTEINS: A POSSIBLE LIPID INTERMEDIATE*

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SUMMARY

Particulate fractions from liver, oviduct, and myeloma tumor incorporated mannose-^{14}C from GDP-mannose-^{14}C into glycoprotein and lipid. Smooth membranes from rabbit liver were purified 8-fold with respect to incorporation of mannose-^{14}C into endogenous lipid acceptor; the mannolipid appeared similar in many respects to the mannosyl-1-phosphoryl-polyisoprenol compound found in bacteria. Incorporation into lipid was 7 times greater than into protein when incubated in the presence of 6×10^{-3}M $MnCl_2$, 2.5×10^{-3}M EDTA, and 0.15% Zonyl A. The role of lipid as a possible intermediate in the biosynthesis of secreted, soluble glycoproteins is discussed; a minimum of two mannosyl transferases are required.

The biosynthesis of the carbohydrate units of membrane glycoproteins (1, 2), submaxillary mucins (3, 4, 5, 6), and collagen (7, 8) appears to proceed by sequential attachment of each sugar unit to the growing carbohydrate chain (9, 10). In HeLa cells, the glycosyl transferases involved in this process form a multienzyme group (11) strongly bound to the smooth endoplasmic reticulum, possibly the Golgi apparatus (1, 12, 13). The question arises about the general application of this mechanism; are other glycoproteins such as those secreted into the blood plasma or egg white assembled in a similar manner? Attention was focused in the present study to the incorporation of mannose, a monosaccharide component found in soluble, secreted glycoproteins, but rarely in mucins.

EXPERIMENTAL

Preparation of fractions. In a typical experiment, rabbit liver, wet wt. 77 g, was minced with scissors and homogenized in 175 ml of 0.25M sucrose. The homogenate was centrifuged at 20,000 x g for 10 minutes; the resulting supernatant

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fluid was adjusted to 0.015M in CsCl. Aliquots of 15 ml were layered onto 23 ml of 1.3M sucrose in 0.015M CsCl, and centrifuged at 131,000 x g for 2 hours (14, 15). The rough endoplasmic reticulum (rough ER) was obtained as a pellet; the smooth membrane fraction (smooth endoplasmic reticulum), which was formed at the boundary between the two solutions, was collected, diluted with 5 volumes of 0.05M Tris buffer, pH 7, and centrifuged at 131,000 x g for 2 hours; the smooth membrane fraction was obtained as a pellet. The pellets were kept frozen and were suspended in Tris buffer as needed. A 6:1 ratio of RNA in the rough ER and smooth membranes was found.

Assay for bound mannose-^{14}C. After incubation with appropriate components, the membranes were precipitated and washed 3 times with 3 ml of 5% CCl_3COOH. The resulting pellet was extracted twice with $CHCl_3$:CH_3OH (2:1); the residue and lipid extract were evaporated down to dryness at 80°. The protein and lipid residues were dissolved in 0.2 ml N NaOH and 0.1 ml aliquots applied to filter paper, dried, and counted with a Beckman liquid scintillation counter.

Preparation of the mannolipid-^{14}C. The lipid extracts from two separate incubation mixtures, one with GDP-mannose-^{14}C and the other with unlabeled substrate, were each washed with 0.2 volumes of 0.9% NaCl. After the two phases had cleared, the bottom layers were concentrated to about 50 ml under reduced pressure. At this point, the two extracts were combined.

The lipid (2.4 x 10^5 cpm) was applied to a silicic acid column, 5.5 x 36.5 cm, and eluted with 900 ml of $CHCl_3$, followed by a $CHCl_3$:CH_3OH gradient. The fraction (1.4 x 10^5 cpm) recovered between 1500 to 1700 ml was saponified in 80 ml by incubation at 37° for 10 minutes after addition of 7.5 ml N NaOH. The solution was neutralized with CH_3COOH and extracted according to Scher et al. (16); most of the radioactivity appeared in the organic phase.

The lipid material, 8.5 x 10^4 cpm, was applied to a 2 x 23 cm column of DEAE-cellulose, prepared according to Dankert et al. (17). The column was eluted with 750 ml of 99% CH_3OH followed by a linear gradient of

ammonium acetate in CH_3OH (17). Most of the recovered radioactivity, 2.72 x 10^4 cpm, appeared in the elution volume of 168 to 203 ml.

Further purification was carried out by paper chromatography. The sample was applied on silicic acid impregnated paper and chromatographed in $CHCl_3$:CH_3OH:H_2O (12:6:1) for 2 hours. The radioactivity, which was found in one band (R_F=0.225), was eluted with CH_3OH, and evaporated to dryness.

Analytical methods. Protein was determined by the Lowry procedure; RNA was estimated spectrophotometrically following hydrolysis by ribonuclease at 37° for 45 minutes at pH 7.8 (12). Sugars were identified following chromatography on Whatman 3MM paper for 22 hours using the solvent system n-butanol:pyridine: 0.1N HCl (5:3:2). The sugars were detected with $AgNO_3$.

RESULTS AND DISCUSSION

A number of tissues were investigated for the ability to incorporate mannose from GDP-mannose-^{14}C into lipid and protein fractions as shown in Table I. Those tissues such as liver and oviduct which manufacture and secrete

TABLE I

THE INCORPORATION OF MANNOSE-^{14}C INTO LIPID AND PROTEIN BY VARIOUS SPECIES

Source	cpm/g tissue x 10^4 protein	lipid	lipid/protein*
Hen oviduct[1]	1.75	2.9	1.65-3.3
Rabbit liver[2]	1.1	2.72	1.45-3.5
Rat liver[2]	0.54	1.78	3.3
Guinea pig liver[2]	1.22	4.5	3.7
Myeloma cells[3]	2.12	15.5	7.3
HeLa cells	0	0	-

*Calculated after 1 hour incubation at 37° under the conditions given in Table III. In all cases, except for HeLa cells where some fucose-^{14}C was incorporated, the transferred sugar was identified as mannose by paper chromatography. There was a wide variation in the ratio depending on the state of the tissue.

[1]Triton X-100 extract of the 5,000 x g pellet.

[2]Determined on the 5,000 x g pellets.

[3]Membrane fraction prepared by Dr. Hagopian; cells kindly supplied by Dr. K. Horibata, Salk Institute, from a subline of P3K (18); values are expressed as cpm/10^9 packed cells.

plasma and egg white glycoproteins, respectively, contain the enzymes for transfer of mannose. By contrast, mannose was not incorporated significantly into HeLa cells, which do not secrete glycoproteins (except for collagen) and where virtually all of the glycoprotein is incorporated into membranes. In the myeloma tumor, which secretes an IgG immunoglobulin (18), a very high activity for mannose incorporation was found; the lipid:protein ratio even exceeded that of liver and varied from 6-10:1. These results reveal that mannose is transferred from GDP-mannose to both lipid and protein components by cells actively engaged in secretion of glycoproteins containing mannose.

In order to locate the cellular site of mannose incorporation, rabbit liver was used; the distribution of transferase activities in different fractions is shown in Table II. It is apparent thatthe enzymes are most concentrated in the smooth membranes; the incorporation into lipid and protein in this fraction is 9-fold and 3-fold, respectively, over the crude homogenate. By contrast, the rough ER contain little of the total activity. Although 29% of the total cellular activity was associated with the smooth membranes, it should be noted that over 43% of the activity is found in the 20,000 x g pellet. It is likely that the activity found in the pellet is due to adsorbed smooth membrane since the specific activity is not increased; the possibility of other sites of incorporation is not excluded, however.

TABLE II

DISTRIBUTION OF TRANSFERASE ACTIVITY IN THE RABBIT LIVER

Fraction	% Yield		Purification*	
	protein	lipid	protein	lipid
Crude homogenate	100	100	1	1
20,000 x g pellet	18	43	0.5	1
Smooth membranes	9	29	3	9
Rough endoplasmic reticulum	2	8	1	3

*Expressed as cpm/mg protein under conditions shown in Table III.

The requirements for mannose incorporation into protein and lipid into smooth membranes are presented in Table III. Maximal activity was obtained at 6 x 10^{-3}M Mn^{++}, 2.5 x 10^{-3}M EDTA, and 0.15% Zonyl A. The enzymes and acceptor molecules appear to be strongly bound to these membranes; neither sonication nor detergents was successful in solubilizing the system.

TABLE III

ASSAY FOR INCORPORATION OF MANNOSE INTO PROTEIN AND LIPID

	cpm protein	lipid
Complete*	226	1047
- EDTA	34	116
- Mn^{++}	44	122
- Zonyl A	69	466
- Mn^{++}, + Mg^{++}	72	346
+ boiled enzyme	9	0

*The complete system contained 50 µl smooth membrane fraction, 10 µl 0.1M $MnCl_2$, 10 µl 0.025M EDTA, 10 µl GDP-mannose-^{14}C (45 µC/µmole), 10 µl 1.5% Zonyl A, and 10 µl of 0.3M Tris buffer, pH 7.

The time course of incorporation into protein and lipid is shown in Fig. 1. It is interesting to note the rapid rate of labeling into lipid compared to the protein. After 1 hour, further incorporation into these fractions did not occur.

The results of this work are of special interest in that they suggest a more complicated mechanism for the biosynthesis of secreted glycoproteins (containing mannose) than with membrane glycoproteins where a "one linkage-one enzyme" mechanism apparently prevails. It is highly probable that the mannolipid reported here represents an intermediate in the biosynthesis of mannose-containing glycoproteins; mannose is not found as a component of glycolipids (19). In addition, the mannolipid exhibits solubility and chromatographic properties which differ markedly from those of glycolipids and the known phospholipids. Its properties fall close to those of the

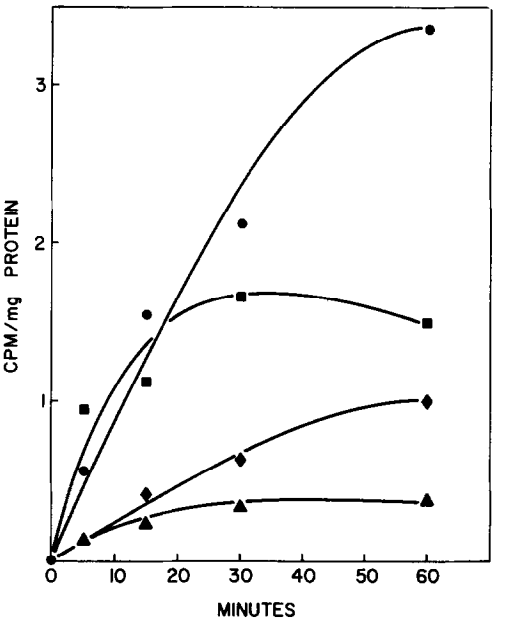

Fig. 1. Time course of incorporation of mannose-^{14}C into protein and lipid of smooth and rough membranes. ●—●, smooth (lipid); ◆—◆ smooth (protein); ■—■, rough (lipid); ▲—▲ rough (protein).

mannosyl-1-phosphoryl-polyisoprenol compound isolated by Scher et al. and shown to be an intermediate in the synthesis of mannans in bacteria. Mild acid hydrolysis (0.01N HCl, 5 min, 100°) of the purified or unfractionated mannolipid released mannose-^{14}C as the only radioactive product; similar results were obtained with hydrolysis in 2 N HCl for 6 hours at 100°. Analysis of the purified material revealed a hexose:PO_4 ratio which varied from 1:1 to 2:1. No hexosamine was detected in the mannolipid. Also, its resistance to alkaline hydrolysis and chromatographic behavior all indicate the similarity of these two compounds. Another indication comes from preliminary chase experiments whereby after 10 minutes labeling, followed by addition of unlabeled GDP-mannose (25-fold in excess), further incubation results in a fall in the radioactivity in the lipid accompanied by a gradual increase in the amount of radioactivity in the protein.

Thus, it appears that a minimum of two mannosyl transferases are

involved in the attachment of mannose to glycoproteins; one enzyme which synthesizes the mannolipid, and another which transfers mannose from the mannolipid to glycoprotein. Further investigation of this problem and characterization of the mannolipid are currently under way.

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