MANNOSYLTRANSFER FROM GDP-MANNOSE TO OLIGOSACCHARIDE-LIPIDS

W. T. Forsee, J. A. Griffin, and J. S. Schutzbach

Department of Microbiology
and Institute of Dental Research
University of Alabama in Birmingham
Birmingham, Alabama 35294

Received February 24,1977

SUMMARY: Rabbit liver microsomes catalyzed mannosyltransfer from GDP-mannose to oligosaccharide-lipids isolated from porcine liver. The transfer occurred in the presence of 10 mM EDTA, a condition under which the formation of dolichol-P-mannose and other chloroform soluble mannosyl-lipids was almost completely inhibited, indicating that the mannosyl-oligosaccharide linkage was formed by a direct transfer of mannose from the nucleotide sugar. Virtually all of the mannose incorporated into the oligosaccharides was released by α-mannosidase, demonstrating the formation of an α-mannosyl-linkage in the oligosaccharide-lipid product. An enzyme catalyzing the divalent cation independent transfer of mannose from GDP-mannose to exogenous oligosaccharide-lipids was solubilized from rabbit liver microsomes and purified over 10 fold.

A number of investigations in recent years have shown that dolichol-P-mannose is a glycosyl-donor in the formation of α-mannosyl-linkages in mammalian glycoproteins (1-5). Adamany and Spiro have demonstrated the direct transfer of mannose from dolichol-P-mannose to α-methylmannoside and aryl-mannosides with the resultant formation of α-1,2-mannosyl-mannose linkages using an enzyme preparation from calf thyroids (6,7). We have recently shown that rabbit liver microsomes catalyzed the formation of α-1,2-, α-1,3-, and α-1,6-mannosyl-mannose when incubated with GDP-[^{14}C]mannose and free mannose[1] under the appropriate reaction conditions (8). Our results demonstrated that formation of α-1,3-mannosyl-mannose occurred in the presence of 10 mM EDTA, a condition in which synthesis of mannosyl-P-lipid was almost completely inhibited, and implied that formation of the α-linked disaccharide occurred by mannosyltransfer directly from nucleotide sugar to the exogenous acceptor. Free mannose, however, was required at relatively high concentrations and the physiological significance of the reaction was uncertain. We would now like to present evidence demonstrating that the formation of some

1 All sugars are of the D-configuration.

ISSN 0006-291X

α-mannosyl-linkages found in mammalian glycoproteins occurs by mannosyltransfer directly from GDP-mannose to an oligosaccharide-lipid.

MATERIALS AND METHODS: Oligosaccharide-lipids were isolated from porcine liver according to the procedures of Forsee and Elbein (9). One unit of oligosaccharide-lipid acceptor was defined as that amount of oligosaccharide-lipid which had the capacity to accept 40,000 cpm when saturated with [^{14}C]mannose under optimal assay conditions. Dolichol-P was purchased from Sigma Chemical Co. All other chemicals and enzymes were also purchased from commercial sources. Paper chromatography was carried out on Whatman No. 1 paper in the following solvent systems: (1) 1-butanol-pyridine-water, 40:30:40; (2) 1-propanol-ethyl acetate-water, 7:1:2; and (3) ethyl acetate-pyridine-water, 8:2:1. Carbohydrates were detected with silver nitrate or with p-anisidine phthalate (10) and protein was determined by the procedure of Lowry et al.(11) using bovine serum albumin as the standard.

ENZYME ASSAYS: Mannosyltransfer from GDP-[^{14}C]mannose to oligosaccharide-lipid was measured in reaction mixtures containing GDP-[^{14}C]mannose (0.05 μCi, 246 mCi/mmol, New England Nuclear), EDTA (1 μmol), oligosaccharide-lipid (1 unit), and enzyme in a total volume of 0.125 ml of 0.1 M Tris-acetate buffer, pH 7.0, containing 5% methanol and 0.8% Triton X-100 (V/V). The amount of enzyme added was 0.5 mg of crude microsomes or 0.05-0.1 mg of soluble enzyme. After 30 min at 25°, the reaction was terminated by the addition of 0.05 ml of water, 0.25 ml of methanol, and 0.25 ml of chloroform in succession. The mixture was centrifuged at 8,000 x g for 3 min and the lower chloroform phase was removed and saved. The upper phase and interface were extracted with a second 0.25 ml of chloroform and following centrifugation the upper and lower phases were removed. The combined chloroform layers were washed with 0.25 ml of CH_3OH-H_2O (1:1), dried, and counted. The pellet at the interface of the aqueous and organic phases was washed twice with 0.5 ml of 50% methanol and was extracted with $CHCl_3$-CH_3OH-H_2O (10:10:3). The 10:10:3 extract contained the bulk of the [^{14}C]mannosyl-oligosaccharide-lipid and the residual pellet contained material tentatively identified as glycoprotein.

Mannosyltransfer from GDP-[^{14}C]mannose to dolichol-P was assayed according to the procedures previously described (8) except that transfer to exogenous dolichol-P was assayed in the presence of 0.8% Triton X-100.

Preparation of Rabbit Liver Microsomes: Livers were excised from 1.2-1.5 kg fasted New Zealand White rabbits and a crude microsomal fraction was prepared therefrom as previously described (8).

RESULTS

Rabbit liver microsomes were incubated with GDP-[^{14}C]mannose and oligosaccharide-lipid in reaction mixtures containing 10 mM EDTA. After 30 min at 25°, the reaction was terminated by the addition of methanol and chloroform. The upper aqueous phase contained unreacted GDP-[^{14}C]mannose while the lower phase did not contain significant radioactivity. Essentially all of the newly formed product was retained in the pellet found at the interface of the two phases. After washing the pellet with 50% methanol, 95% of

Table I

Stimulation of Mannosyltransfer by Oligosaccharide-lipids

Reaction mixtures were scaled up 10 fold over standard reactions and the products were fractionated as described for the standard assay except the extraction volumes were correspondingly increased, and Reaction I did not contain added oligosaccharide-lipid acceptor.

	Addition to Reaction Mixture	Radioactivity (cpm) Incorporated into		
		Dolichol-P-mannose	Oligosaccharide-lipids	Glycoprotein
I	None	150	16,900	1,100
II	Oligosaccharide-lipids	760	189,000	10,200

the radioactivity was extracted with $CHCl_3$-CH_3OH-H_2O (10:10:3). The results of the fractionation are presented in Table I. The addition of exogenous oligosaccharide-lipid as an acceptor stimulated the formation of the 10:10:3 product over 10 fold and also stimulated the incorporation of [^{14}C]mannose into the insoluble pellet over 10 fold. A number of investigations have demonstrated that similar fractionation procedures isolated dolichol-P-mannose and related compounds in the chloroform layer, oligosaccharide-lipids in the 10:10:3 extract, while glycoproteins remained in the residual pellet (2,4,5).

Solubilization and Purification of Mannosyltransferase: The crude microsomal preparation from rabbit liver (5 ml) was diluted 1:4 in 0.1 M Tris-acetate, pH 7.0, and was solubilized by the addition of an equal volume of 2.5% Triton X-100 in the same buffer. The mixture was gently agitated on a Vortex mixer for 1 min, and was immediately diluted 1:3 into water containing 30% glycerol and mercaptoethanol (0.1 ml/liter). After centrifugation at 100,000

Table II

Purification of Mannosyltransferase

FRACTION	VOLUME (ml)	TOTAL ACTIVITY[1]	SPECIFIC ACTIVITY[2]	TOTAL RECOVERY (%)
Crude Microsomes	4	2.2×10^3	11	100
Triton X-100 supernatant	120	6.0×10^3	25	272
DEAE-Cellulose	7	1.5×10^3	180	68

1. One unit of enzyme is defined as the amount of enzyme catalyzing the transfer of 1000 cpm of [^{14}C]mannose to oligosaccharide-lipid under the conditions of the standard assay in 30 min.

2. Specific activity is defined as units/mg protein.

x g for 60 min, the enzyme activity was found in the supernatant (Table II). The solubilized enzyme was applied to a column of DEAE-cellulose (1.5 x 18 cm) that had been equilibrated with 0.001 M Tris-acetate, pH 7.0, containing 0.1% Triton X-100 and 20% glycerol. The column was eluted with a NaCl gradient (400 ml) from 0 to 0.5 M in the equilibrating buffer. The bulk of the enzyme was eluted at a salt concentration of approximately 0.05 M NaCl, and recovery of enzyme activity from the column ranged from 20-50% with an overall purification of up to 16 fold (Table II).

Mannosyltransfer from GDP-mannose to oligosaccharide-lipid by the partially purified enzyme was linear with time for up to 50 min and was proportional to enzyme concentration. The partially purified enzyme did not catalyze mannosyltransfer to any endogenous oligosaccharide-lipids, or to either endogenous or exogenous dolichol-P.

Characterization of the Product: [^{14}C]Mannosyl-oligosaccharide-lipid was prepared with solubilized enzyme in reaction mixtures scaled up 10 fold over standard incubations and the product was extracted with $CHCl_3$-CH_3OH-

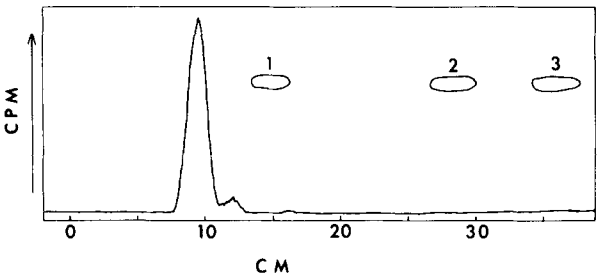

Fig. 1. Paper chromatography of [^{14}C]mannosyl-oligosaccharide. Carbon-14 labeled mannosyl-oligosaccharide-lipid was prepared in reaction mixtures scaled up 10 fold over standard assay reactions and the product was isolated and hydrolyzed in mild acid as described in the text. The product was applied to paper and the chromatogram was developed descending in solvent 1 for 24 hrs. The figure shows a radioactive strip scan of the chromatogram. Full scale deflection equals 30,000 cpm. The standards are (1) stachyose, (2) maltose, and (3) mannose.

H_2O (10:10:3). The radioactive oligosaccharide-lipid was dried under nitrogen, dissolved in 1 ml of 50% 1-propanol containing 0.02 N HCl and hydrolyzed at 80° for 15 min. The water soluble radioactive products released by mild acid hydrolysis were then separated by chromatography in solvent 1 (Fig. 1). The major product had an R_{Man} of 0.25 and migrated more slowly than the stachyose standard.

The [^{14}C]labeled product was isolated after chromatography in solvent 1 and a sample was treated with α-mannosidase (Fig. 2). All of the radioactivity was liberated as free [^{14}C]mannose as judged by chromatography in solvents 2 and 3, establishing that the anomeric configuration of the newly formed mannosyl-linkage was alpha.

DISCUSSION

The results presented in this communication show that rabbit liver microsomes synthesize the formation of a α-mannosyl-linkage in an oligosaccharide-lipid by mannosyltransfer from GDP-mannose. Mannosyl-lipids with the solubility properties of dolichol-P-mannose were not synthesized and therefore could not have been involved as intermediates in the formation of the newly synthesized α-mannosyl-linkages. Also, the reaction occurred at a

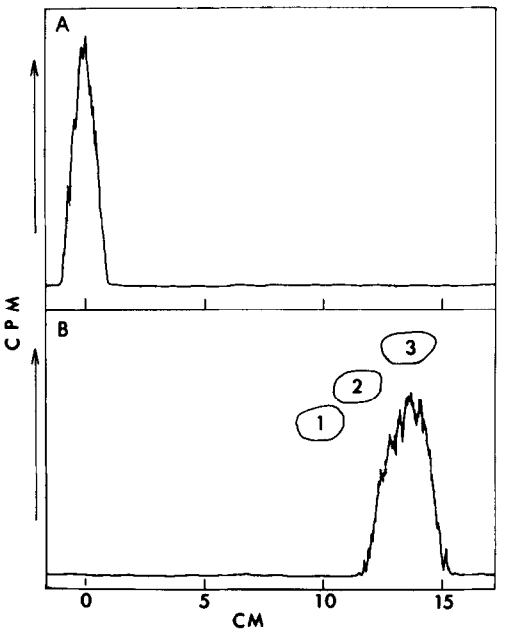

Fig. 2. Hydrolysis of [^{14}C]mannosyl-oligosaccharides by α-mannosidase. The carbon-14 labeled product was synthesized by mannosyltransfer from GDP-[^{14}C]mannose to oligosaccharide-lipid using Triton X-100 solubilized enzyme. The labeled oligosaccharide was isolated after mild acid hydrolysis and was purified by paper chromatography as in Fig. 1. Samples of the radioactive oligosaccharide were incubated with either α-mannosidase (B) or with enzyme inactivated by heating at 100° for 3 min (A). Reaction mixtures contained sample (1200 cpm), and α-mannosidase (1 μg; Boehringer Mannheim) in 0.2 ml of 0.5 M ammonium acetate, pH 5.0, and were incubated for 4 hrs at 25°. Reaction products were separated by chromatography in solvent 2. The figure shows radioactive strip scans of the chromatograms. Full scale deflection equals 1000 cpm. The standards are (1) galactose, (2) glucose, and (3) mannose.

maximal rate in the presence of 10 mM EDTA, whereas, enzymes catalyzing the formation of dolichol-P-mannose require divalent cations for activity.

The major oligosaccharide-lipid product obtained in these studies was distinctly different from a previously characterized trisaccharide-lipid which was also formed by a direct transfer of mannose from GDP-mannose to a lipid-linked acceptor, di-N-acetylchitobiosyl-P-P-lipid (12-14). The trisaccharide-lipid was extracted into the chloroform layer during fractionation (12,14) and was shown to contain a β-linked mannosyl-unit. The [^{14}C]mannosyl-oligosaccharide-lipid described in this paper was only extracted with $CHCl_3$-CH_3OH-

H_2O (10:10:3) and contained an α-mannosyl-linkage. Additionally, the free oligosaccharide released after mild acid hydrolysis migrated more slowly than the tetrasaccharide, stachyose, upon paper chromatography. Free trisaccharide has been shown to migrate faster than stachyose in the same solvent system (9).

This is the first report of the solubilization of a mammalian mannosyltransferase which catalyzes the incorporation of mannose into an exogenous acceptor.

ACKNOWLEDGMENTS: This investigation was supported by NIH research grants CA 16777 from the National Cancer Institute and DE 2670 from the National Institute of Dental Research. The expert technical assistance of Janet Dodson and Martha Gurley is greatfully acknowledged.

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