Recovery of dolichyl diphosphate oligosaccharide in methanolic aqueous phase prepared from rat liver microsomal fractions

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The synthesis of dolichyl diphosphate oligosaccharide was studied by incubating rat liver microsomes (microsomal fractions) with GDP-[¹⁴C]mannose, UDP-glucose, UDP-*N*-acetylglucosamine and [³H]dolichol phosphate. The labelled products obtained by the first step of extraction of the microsomes in methanolic aqueous phase (MAP fraction in chloroform/methanol/water; 3:2:1, by vol.) and in CMW fraction (chloroform/methanol/water; 10:10:3, by vol.) obtained by extraction of the interphase after the first step of extraction were analysed on a DEAE-cellulose column. With the progress of incubation, the radioactivity in unchanged GDP-mannose decreased, whereas the labelled dol-*P-P*-oligo in the MAP fraction increased about 5–6-fold. The lipid oligosaccharide in this fraction accounted for about 50–60% of the GDP-mannose used, whereas the recovery of the labelled lipid oligosaccharide in the CMW fraction was about 10%. The lipid oligosaccharide from both reactions after mild acid hydrolysis were analysed by gel filtration on Bio-Gel P-4. The oligosaccharide from the MAP fraction gave a peak of higher *M_r*, distinctly separate from the lower-*M_r* peak obtained from the CMW fraction. Microsomes incubated with labelled lipid oligosaccharide from the MAP fraction showed incorporation of the label into endogenous protein.

INTRODUCTION

The synthesis of the dolichol-linked oligosaccharides soluble in CMW fraction (chloroform/methanol/water; 1:1:0.3, by vol.) has been accomplished by particle-bound enzymes from liver (Parodi *et al.*, 1972; Oliver & Hemming, 1975a), hen oviduct (Waechter *et al.*, 1973) and mouse myeloma (Hsu *et al.*, 1974) and a role for these compounds in the glycosylation of proteins has been proposed. The carbohydrate composition of some of these compounds was shown to consist of two *N*-acetylglucosamine, nine mannose and three glucose residues (Staneloni *et al.*, 1980). We report here that a significant portion of lipid oligosaccharide synthesized in rat liver microsomes is extractable in methanolic aqueous phase (MAP fraction in chloroform/methanol/water, 3:2:1, by vol.) and this lipid contains an oligosaccharide of larger size than that normally extracted in CMW fraction. This lipid oligosaccharide present in MAP fraction is usually discarded in conventional extraction procedures (Behrens *et al.*, 1971, 1973; Spiro *et al.*, 1976).

MATERIALS AND METHODS

Reagents and chemicals

GDP-[U-¹⁴C]mannose (sp. radioactivity 269 mCi/mmol) and (*R,S*)-[5-³H]mevalonolactone (sp. activity 30 Ci/mmol) were purchased from New England Nuclear Corp., Lachin, Quebec, Canada. Dol-*P*, UDP-glucose and UDP-*N*-acetylglucosamine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DEAE-cellulose (microgranular) was from Whatman Ltd. (Maidstone, Kent, U.K.). All other reagents were of analytical grade.

Preparation of [³H]Dol-*P*

Hepatocytes from normal rats were isolated as described by Sarkar & Mookerjea (1984) and were divided into four incubation flasks in Waymouth's medium (40 ml). A 1 mCi portion of (*R,S*)-[5-³H]mevalonolactone (sp. activity 30 Ci/mmol; New England Nuclear) in ethanol was evaporated to dryness, dissolved in 1 ml of Waymouth's medium and was equally distributed into four flasks. The flasks were incubated under a continuous stream of carbogen (O₂/CO₂, 19:1) at 37 °C for 4 h. After incubation, the cells were transferred into centrifuge tubes and washed twice with ice cold Hanks balanced salts solution, pH 7.4, and extracted with 20 ml of chloroform/methanol (2:1, v/v) and 20 ml of water. After centrifugation the chloroform phase was removed and the pellets were re-extracted. The chloroform phases from these extracts were pooled together. T.l.c. was performed in silica-gel F-254 (E. Merck, Darmstadt, Germany) after evaporation of the solvent and redissolving the lipid in 0.5 ml of chloroform. Chromatography was performed in a solvent system consisting of chloroform/methanol/28% (w/v) NH₃ (13:7:1, by vol.). The spot corresponding to standard Dol-*P* was scraped off and extracted with chloroform/methanol (2:1, v/v) and further purified by h.p.l.c. in a C₁₈ reverse-phase column, with methanol/isopropyl alcohol (1:1, v/v) containing 10 mM-phosphoric acid as solvent.

Assay for transfer of [¹⁴C]mannose from GDP-[¹⁴C]mannose to lipid oligosaccharide

The reaction mixture contained 60 mM-Tris/HCl, pH 7.4, 2.5 mM-MnCl₂, GDP-[¹⁴C]mannose (0.14 nmol),

Abbreviations used: Dol-*P-P*-oligo, dolichyl diphosphate oligosaccharide; MAP-fraction, methanolic-aqueous-phase fraction; CMW fraction, chloroform/methanol/water fraction; Dol-*P* (-Man), dolichyl phosphate (mannose).

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0.23 mM unlabelled GDP-mannose, 2 mM-UDP-*N*-acetylglucosamine, 1.2 mM-UDP-glucose, Dol-*P* (7.5 nmol), 0.15% Triton X-100 and 1.5–2 mg of microsomal protein in a total volume of 135 μ l. Dol-*P* in chloroform was evaporated under N_2 to dryness, then solubilized in the required amount of Triton X-100 by sonication followed by other reagents, and finally the microsomes were added to start the reaction. Incubations were done at 37 °C for the indicated times.

Extraction of lipid oligosaccharide

At the end of incubation, the reaction was stopped with chloroform/methanol/water (3:2:1, by vol.), mixed and centrifuged at 2500 rev./min (1240 *g*) for 5 min. The lower chloroform layer was removed with a Pasteur pipette and the insoluble interphase and the upper phase was extracted two more times by adding 2 ml of chloroform/methanol/water (3:2:1, by vol.). After removal of the lower layer containing the residual chloroform, the mixture of upper phase and interphase was centrifuged. The upper phase thus obtained after centrifugation, termed 'methanolic aqueous phase' (MAP), was removed and stored at -20 °C for further purification of the lipid oligosaccharide. To obtain the lipid oligosaccharide from the interphase, the pellet was extracted twice with chloroform/methanol/water (10:10:3, by vol.; 'CMW'), the mixture used by Behrens *et al.* (1971). This extraction procedure is the conventional method for the extraction of lipid oligosaccharide from the interphase (Behrens *et al.*, 1971, 1973).

Purification of lipid oligosaccharide by DEAE-cellulose chromatography

DEAE-cellulose (DE-52, microgranular) was converted into the acetate form by overnight soaking in 1 M-acetic acid. Excess acid was removed by filtering the resin, which was then washed first with water followed by methanol (2 litres). The resin was packed to a height of 7 cm in a 0.8 cm-diameter column and washed with methanol (50 ml) and then with chloroform/methanol/water (10:10:3, by vol.; 50 ml). The sample was applied in chloroform/methanol/water (10:10:3, by vol.) and eluted with a sequential discontinuous gradient of 0, 10, 25, 100 mM-ammonium acetate in the same solvent. A flow rate of 30 ml/h was maintained and the samples were taken for liquid-scintillation counting of radioactivity. Appropriate fractions were pooled and the solvent evaporated on a rotary evaporator under reduced pressure at 37 °C. Ammonium acetate was removed by repeated addition of water during freeze-drying.

Release of oligosaccharide from lipid oligosaccharide and its separation by Bio-Gel P-4 chromatography

Oligosaccharides were released from the purified lipidoligosaccharides in MAP and CMW fractions by hydrolysis in 0.02 M-HCl in 20% (v/v) methanol for 20 min at 100 °C (Hubbard & Robbins, 1980). The samples were then evaporated to dryness and dissolved in 0.1 M-pyridine acetate buffer, pH 5.0, and applied to a Bio-Gel P-4 (85 cm $\times$ 1.7 cm) column equilibrated with the same buffer. The column was eluted with the same pyridine/acetate buffer by using a flow rate of 10 ml/h and calibrated with *p*-nitrophenylmannose and *p*-nitrophenyl maltoheptaoside. Fractions from the column were counted for radioactivity.

Fig. 1. DEAE-cellulose chromatography of MAP and CMW extracts

MAP and CMW fractions were obtained as described in the Materials and methods section and applied to a DEAE-cellulose column (7 cm $\times$ 0.8 cm). The column was equilibrated with chloroform/methanol/water (10:10:3). The elution of MAP (●) and CMW extract (□) was compared with that of Dol-*P*-Man (○) and GDP-[¹⁴C]-mannose (△). A discontinuous gradient (----) of ammonium acetate in the above solvent was used to elute the radioactive components.

Assay for transfer of oligosaccharide from lipid oligosaccharide to proteins

[¹⁴C]Mannose-labelled lipid oligosaccharide from the MAP fraction was purified by DEAE-cellulose chromatography as described above. The incubation mixture for the transfer experiment contained about 80000 c.p.m. of lipid oligosaccharide, 0.1 M-Tris/HCl, pH 7.0, 10 mM-MnCl₂, 2 mM-2-mercaptoethanol, 1% Triton X-100 and microsomes (2.5 mg of protein) in a total volume of 150 μ l. The reaction mixture was incubated at 25 °C for 0–60 min. The reaction was stopped at various times by adding 2 ml of chloroform/methanol/water (10:10:3, by vol.). The contents were mixed and centrifuged at 2000 *g* at room temperature for 10 min. The pellets were washed two more times with the same solvent, with water, and finally dissolved in tissue solubilizer and counted for radioactivity.

RESULTS

DEAE-cellulose chromatography of MAP and CMW fractions

DEAE-cellulose chromatography of the lipid oligosaccharides soluble in MAP or CMW fractions showed a single peak of lipid oligosaccharide component eluted with 100 mM-ammonium acetate (tube nos. 4–8, Fig. 1). Under the same conditions of chromatography a standard Dol-*P*-Man emerged from the column at a concentration of 10 mM-ammonium acetate (Godelaine *et al.*, 1979). The standard labelled GDP-mannose was eluted at 100 mM-ammonium acetate concentration, but was well separated (tube nos. 8–12) from the peaks obtained for MAP and CMW. A significant (about 25%)

Fig. 2. Time course of lipid oligosaccharide synthesis in the MAP fraction

Synthesis of lipid oligosaccharide in the MAP fraction at 30 s, and at 5 min was analysed by DEAE-cellulose chromatography. The chromatography was performed as described in Fig. 1. Profile at 30 s (○) and at 5 min (●). The GDP-mannose peak disappeared at 5 min.

amount of radioactivity which did not bind to the column was not identified (tube nos. 0–1).

Time-dependent synthesis of lipid oligosaccharide in the MAP fraction

DEAE-cellulose chromatography of the labelled MAP fraction (68 000 c.p.m.) obtained after 30 s of incubation showed one small peak (tube nos. 4–8) and a second large peak (tube nos. 8–12) (Fig. 2).

The small (tube nos. 4–8) and the large (tubes nos. 8–12) peaks correspond to the lipid oligosaccharides and GDP-mannose peaks obtained for Fig. 1. Furthermore incubation for 5 min yielded only one large lipid-oligosaccharide peak (tube nos. 4–8), and there was no free GDP-mannose peak. Also there was one unidentified peak (tube nos. 0–1), which did not bind to the column.

Incorporation of [^3H]Dol-*P* and [^{14}C]mannose into lipid oligosaccharide

In order to test the presence of dolichol in the lipid oligosaccharide obtained from MAP fraction, the microsomes were assayed for the synthesis of lipid oligosaccharide with (i) [^3H]Dol-*P* and GDP-[^{14}C]mannose, (ii) with [^3H]Dol-*P* and unlabelled GDP-mannose, or (iii) with unlabelled Dol-*P* and GDP-[^{14}C]mannose for 10 min. The MAP fractions obtained from the assays were chromatographed on DEAE-cellulose. This elution profile of lipid oligosaccharide from the MAP fraction was identical with that shown in Fig. 1. Both [^3H]Dol-*P* and [^{14}C]oligosaccharide were recovered from the column with 100 mM-ammonium acetate in tube nos. 4–8 (Fig. 3). These results therefore support the conclusion that the lipid oligosaccharide in the MAP fraction is linked with Dol-*P*.

Isolation of labelled oligosaccharide from purified lipid oligosaccharides in MAP and CMW extracts

Chromatography on Bio-Gel P-4 of the labelled

Fig. 3. Appearance of labelled Dol-*P* oligosaccharide in MAP after DEAE-cellulose chromatography

DEAE-cellulose chromatography of the MAP fraction obtained from microsomes incubated with (●---●) GDP-[^{14}C]mannose and (○---○) [^3H]Dol-*P*, [^3H]Dol-*P* phosphate and unlabelled GDP-mannose (○---○), or unlabelled Dol-*P* and GDP-[^{14}C]mannose (●---●). The labelled peaks of Dol-*P*-oligo from the three experiments were eluted in tubes 4–8. The assay and chromatography conditions are described in the Materials and methods section.

oligosaccharide obtained by mild acid hydrolysis from the purified lipid oligosaccharide from MAP and CMW fractions yielded a single component in both cases (Fig. 4). The component obtained from the MAP fraction had a higher M_r compared with that of the component obtained from the CMW fraction. These results also suggest that the components obtained from MAP and CMW fractions by filtration on Bio-Gel P-4 represent oligosaccharides that, in the native state, were attached to a lipid moiety by an acid-labile bond.

Transfer of labelled oligosaccharide from lipid oligosaccharide to protein

In order to investigate the possible role of the lipid oligosaccharide obtained from the MAP fraction as an intermediate in glycoprotein biosynthesis, microsomes were incubated with this lipid oligosaccharide labelled with [^{14}C]mannose as oligosaccharide donor. Fig. 5 shows the time-dependent transfer of oligosaccharide from the lipid oligosaccharide to endogenous protein. The reaction reached a maximum rate at 5 min. With a further increase of the period of incubation, there was a decrease in the amount of radioactivity recovered in the protein. This decline may represent the beginning of processing reactions for this high- M_r lipid oligosaccharide.

DISCUSSION

The lipid oligosaccharide product extracted in CMW fractions in our studies has a similar solubility, acid-lability and chromatographic properties of the lipid oligosaccharides synthesized in various tissues reported by others (Behrens *et al.*, 1973; Spiro *et al.* 1976). The labelled lipid oligosaccharide obtained in MAP fractions shows identical acid-lability and chromatographic properties, but the Bio-Gel P-4 chromatographic results show that

Fig. 4. Bio-Gel P-4 separation of oligosaccharide obtained from MAP and CMW extracts

The profile obtained after filtration on a Bio-Gel P-4 column (1.8 cm $\times$ 80 cm) of the labelled oligosaccharide released by mild acid hydrolysis from DEAE-cellulose-purified lipid oligosaccharide of MAP ($\circ$) and CMW extract ($\bullet$) is shown. The column was equilibrated and eluted with 0.1 M-pyridine/acetate buffer, pH 5.0. The positions of elution of *p*-nitrophenyl maltoheptaoside (M_r 1274) and *p*-nitrophenyl mannoside (M_r 301.3) are indicated. V_0 refers to the void volume of the column determined with Blue Dextran. Recovery of radioactivity from the column was complete.

Fig. 5. Effect of incubation time on the incorporation of labelled oligosaccharide from oligosaccharide lipid into endogenous protein

Incubations were carried out with rat liver microsomes with 80000 c.p.m. of labelled lipid oligosaccharide under the standard assay conditions as described in the Materials and methods section, and incorporation into endogenous protein was measured.

the oligosaccharide obtained from the fraction MAP is larger in molecular size than that obtained from CMW fraction. The larger number of glycosyl residues present in this oligosaccharide probably makes it soluble in the polar MAP fraction.

The labelled lipid oligosaccharide recovered from the

MAP fraction accounted for about 60% of the labelled GDP-mannose used in the assay, whereas only 10% of the labelled oligosaccharide was recovered from the CMW fraction. Behrens *et al.* (1971, 1973) and others (Spiro *et al.*, 1976) in the original lipid oligosaccharide extraction procedure had used the same solvent system (chloroform/methanol/water, 3:2:1, by vol.) as we did for the first extraction step. The MAP extract obtained after this step is normally discarded on the assumption that this fraction only contain the water-soluble labelled GDP-mannose and hydrolysed products from the lipid oligosaccharide. The transfer of radioactivity from GDP-[14 C]mannose to the oligosaccharide derivatives soluble in a methanol/water mixture has been previously reported. Some of these results suggest that these oligosaccharide derivatives are neutral and phosphorylated oligosaccharides formed by two consecutive enzymic-hydrolysis steps (Cacan *et al.*, 1980) from Dol-*P-P*-oligo. An oligosaccharide product soluble in water has been also obtained during the transfer of labelled oligosaccharide to endogenous insoluble glycopolymer (Oliver & Hemming, 1975b).

DEAE-cellulose chromatographic separation of lipid oligosaccharide from MAP fraction showed the occurrence of labelled Dol-*P* and oligosaccharide in experiments performed for the synthesis of lipid oligosaccharide either with [3 H]Dol-*P* or with [3 H]Dol-*P* and GDP-[14 C]mannose (Fig. 3). Furthermore, the oligosaccharide obtained from the MAP fraction is also transferred to endogenous protein, suggesting its participation in the synthesis of glycoproteins. The discovery of this high- M_r lipid oligosaccharide in the MAP fraction is expected to stimulate further research on its role in oligosaccharide processing and in glycoprotein synthesis.

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REFERENCES

- Behrens, N. H., Parodi, A. J. & Leloir, L. F. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2857–2860
- Behrens, N. H., Carminatti, H., Staneloni, R. J., Leloir, L. F. & Cantarella, A. I. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3390–3394
- Cacan, R., Hoflack, B. & Verbert, A. (1980) *Eur. J. Biochem.* **106**, 473–479
- Godelaine, D., Beaufay, H. & Wibo, M. (1979) *Eur. J. Biochem.* **96**, 27–34
- Hsu, A. F., Baynes, J. W. & Heath, E. C. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2391–2395
- Hubbard, S. C. & Robbins (1980) *J. Biol. Chem.* **251**, 6400–6408
- Oliver, G. J. A. & Hemming, F. W. (1975a) *Biochem. J.* **152**, 191–199
- Oliver, G. J. A. & Hemming, F. W. (1975b) *Eur. J. Biochem.* **58**, 223–229
- Parodi, A. J., Behrens, N. H., Leloir, L. F. & Carminatti, H. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3268–3272
- Sarkar, M. & Mookerjee, S. (1984) *Biochem. J.* **219**, 429–436
- Spiro, M. J., Spiro, R. G. & Bhoyroo, V. D. (1976) *J. Biol. Chem.* **251**, 6400–6408
- Staneloni, R. J., Ugalde, R. A. & Leloir, L. F. (1980) *Eur. J. Biochem.* **105**, 275–278
- Waechter, C. J., Lucas, J. J. & Lennarz, W. J. (1973) *J. Biol. Chem.* **248**, 7570–7579