THE FORMATION OF A MANNOSE-CONTAINING TRISACCHARIDE ON A LIPID AND ITS TRANSFER TO PROTEINS IN YEAST

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1. Introduction

It has been reported that yeast mannan is covalently linked to proteins by two kinds of linkages, *O*-glycosidic bond to hydroxylamino acid and *N*-glycosidic bond to asparagine through di-*N*-acetylchitobiose. The participation of lipid intermediates has been suggested in the biosynthesis of the asparagine-type glycoproteins in mammals [1] fowls [2] and plants [3]. This paper presents evidence that in yeast microsomes a mannose-containing trisaccharide–lipid is formed by a mechanism similar to that in the above organisms and that the saccharide moiety of the trisaccharide–lipid can directly be incorporated into proteins without further mannosylation.

2. Materials and methods

[*Acetate*-^{14}C]UDPGlcNAc (50 Ci/mol) was prepared enzymatically from [^{14}C]acetate [4,5]. [U-*glucose*-^{14}C]UDPGlcNAc (300 Ci/mol) and [U-*mannose*-^{14}C]-GDPMan (160 Ci/mol) were purchased from Amersham Searle. Di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose and tetra-*N*-acetylchitotetraose were prepared from chitin by partial acid hydrolysis. Pronase P was purchased from Kaken Kagaku.

Saccharomyces cerevisiae AHU 3027 cells harvested at the early log phase were disintegrated by X-press. The microsome fraction was collected by centrifugation at 100 000 × *g* for 90 min from the 13 000 × *g* supernatant, suspended in 5 mM Tris-maleate (pH 7.5) containing 0.25 M sucrose and stored at −20°C.

Di-*N*-[^{14}C]acetylchitobiose–lipid was prepared as follows. After a reaction mixture, containing 100 mM Tris–maleate (pH 9.2), 40 mM $MgCl_2$, 0.2% Na-deoxycholate, 100 000 cpm of [^{14}C]UDPGlcNAc and 20 μl of yeast microsomes (0.3–0.5 mg of protein) in a total volume of 50 μl, was incubated for 60 min at 30°C, it was supplemented with 50 μl of another mixture which had the same composition as the first one had, but containing 4 mM unlabeled UDPGlcNAc instead of [U-^{14}C]UDPGlcNAc. After further incubation for 10 min, di-*N*-[^{14}C]acetylchitobiose–lipid was extracted with chloroform–methanol (2:1), washed with water and dried in vacuo. In this preparation, 80% of radioactivity was usually accounted for by di-*N*-[^{14}C]acetylchitobiose–lipid (fig.1a).

The reaction mixture for the assay of [^{14}C]trisaccharide–lipid formation contained 100 mM Tris–maleate (pH 9.4), 5 mM $MgCl_2$, 0.2% Na-deoxycholate, 2 mM GDPMan, di-*N*-[^{14}C]acetylchitobiose–lipid (about 600 cpm), and 1 μl of yeast microsomes (15–25 μg of protein) in a total volume of 50 μl. After incubation for 10 min at 30°C, the lipid fraction was extracted as above and hydrolyzed at 100°C for 15 min in 0.01 N HCl containing 0.1% Triton X-100. After extraction with 1-butanol, the water phase was lyophilized and chromatographed on paper in 1-butanol/pyridine/water (6:4:3). The paper was cut into 0.8 cm pieces and measured for radioactivity in a liquid scintillation counter. [^{14}C]-Labeled trisaccharide–lipid used for the assay of trisaccharide–protein formation was synthesized under the same

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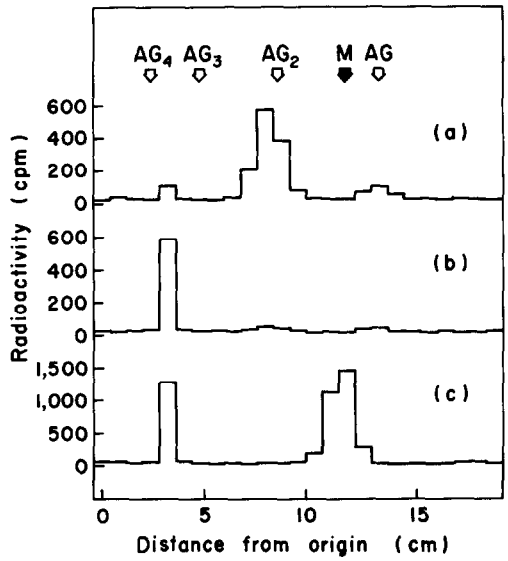

Fig.1. Paper chromatography of the mild acid hydrolysates of the lipid-linked saccharides. (a) Di-*N*-[^{14}C]acetylchitobiose–lipid prepared from 100 000 cpm of [*acetate*-^{14}C]-UDPGlcNAc. (b) [^{14}C]Trisaccharide–lipid prepared from about 700 cpm of di-*N*-[^{14}C]acetylchitobiose–lipid. (c) The products from incubation of 50 000 cpm of [^{14}C]GDPMan with 0.54 mg of yeast microsomes in the presence of 2 mM UDPGlcNAc. The other conditions were essentially the same as in the preparation of [^{14}C]trisaccharide–lipid. Paper chromatography of each hydrolysate was carried out using the solvent described in the text with the standard samples, mannose (M), *N*-acetylglucosamine (AG), di-*N*-acetylchitobiose (AG_2) and so on, running in parallel.

conditions, except that the microsomes added were increased to 20 μl and the incubation time to 20 min.

The reaction mixture for the assay of [^{14}C]-trisaccharide–protein formation contained 100 mM Tris–Cl (pH 7.8), 20 mM $MnCl_2$, 1.6% Triton X-100, [^{14}C]trisaccharide–lipid (about 400 cpm) and 20 μl of yeast microsomes (0.3–0.5 mg of protein) in a total volume of 50 μl. After incubation for 20 min at 30°C, the radioactive substrate was extracted as above. The precipitate remaining after the extraction was washed with acetone and water, and the radioactivity was measured in the system of toluene/Triton (2:1) with a liquid scintillation counter.

3. Results

The incubation of di-*N*-[^{14}C]acetylchitobiose–lipid with the yeast microsomes in the presence of GDPMan resulted in formation of another lipid-linked saccharide which showed a smaller mobility than the substrate lipid on silica gel G thin-layer chromatography in chloroform/methanol/water (65:25:4). The paper chromatographic mobility of the mild acid hydrolysates of the reaction product suggests that in this reaction di-*N*-[^{14}C]acetylchitobiose–lipid converted to a mannose-containing oligosaccharide–lipid, probably trisaccharide–lipid (fig.1b). The optimal pH for this reaction was 9.4, and the optimal concentration of $MgCl_2$ was 5 mM. Figure 2 shows that the formation of the oligosaccharide–lipid was dependent on the concentration of GDPMan. The formation of the oligosaccharide–lipid could also be detected by using [^{14}C]GDPMan as a radioactive substrate. Incorporation of [^{14}C]mannose into the oligosaccharide–lipid was markedly stimulated by UDPGlcNAc, and the saccharide moiety of the mannose-labeled oligosaccharide–lipid coincided on the paper chromatograms with the saccharide moiety of the oligosaccharide–lipid derived from di-*N*-[^{14}C]-acetylchitobiose–lipid (fig.1c). The mannose-labeled oligosaccharide–lipid formation in the presence of 2 mM UDPGlcNAc was not inhibited by 2 mM GDP, while the formation of a Man–lipid, possible dolichol-*P*-Man, was inhibited by 90%. From this effect of GDP, it is most likely that the mannose residue

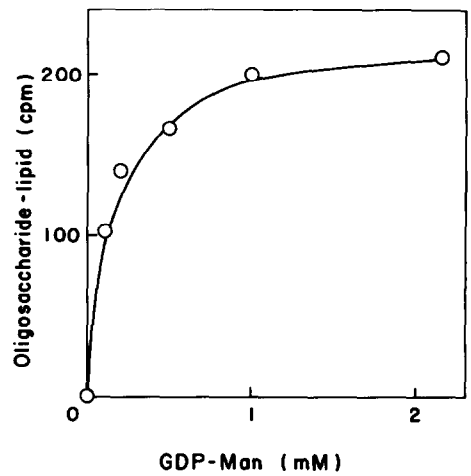

Fig.2. Effect of the concentration of GDPMan on the transfer of mannose to di-*N*-[^{14}C]acetylchitobiose–lipid. For assay procedures, see Materials and methods.

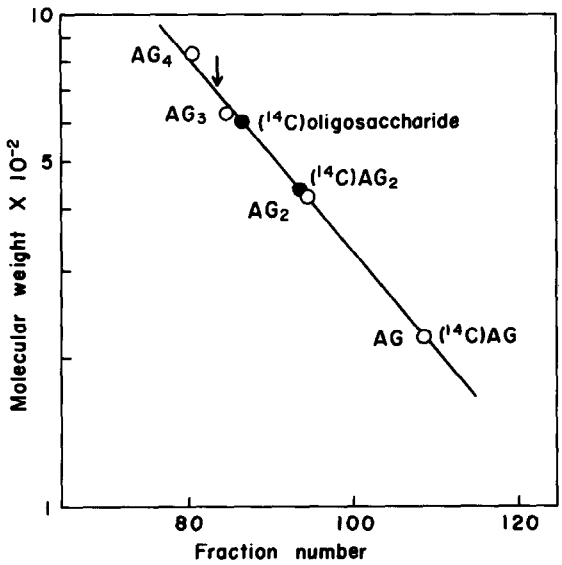

Fig.3. Molecular weights of [^{14}C]oligosaccharide and the radioactive product from pronase digestion of [^{14}C]oligosaccharide–protein. The results of two experiments are summarized. A mixture of hydrolysates composed of [^{14}C]oligosaccharide (1260 cpm), di-*N*-[^{14}C]acetylchitobiose (1210 cpm) and [^{14}C]GlcNAc (1450 cpm) was applied to a Sephadex G-15 column (0.95 × 70 cm) with standard samples, AG to AG_4 (see the legend to fig.1), and eluted with 0.05 M acetic acid (0.28 ml/fraction). The elutions of the standards (○) were determined by reducing power, and those of the hydrolysates (●) by radioactivity. Gel filtration of the pronase digests of the [^{14}C]oligosaccharide–protein was performed by the same procedures as above. The arrow points to the elution of the main radioactive products.

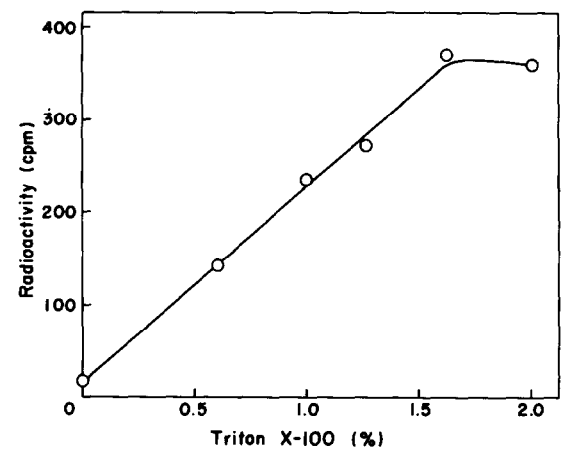

Fig.4. Effect of Triton X-100 on the formation of [^{14}C]-trisaccharide–protein. For assay procedures, see Materials and methods.

was directly transferred from GDPMan to the disaccharide–lipid.

To estimate the molecular weight of the saccharide moiety of the mannose-containing oligosaccharide–lipid, the mild acid hydrolysates of the saccharide–lipids prepared from [U-^{14}C]UDPGlcNAc were subjected to Sephadex G-15 column chromatography (fig.3). The observed molecular weight of the [^{14}C]-oligosaccharide, 600, was in good agreement with the theoretical value of $Man(GlcNAc)_2$, 586.

When the [^{14}C]trisaccharide–lipid was incubated with the microsomes, the saccharide portion was directly transferred to proteins. The activity was not observed unless Triton X-100 was introduced to the incubation mixture (fig.4). The reaction required manganese ion, which could be replaced by magnesium ion. Under the standard incubation conditions, almost all the radioactivity from [^{14}C]trisaccharide–lipid was incorporated into proteins within 30 min. Since the addition of GDPMan showed little effect on the reaction rate, it seems unlikely that the transfer of the radioactivity to proteins occurred after further addition of mannose to the trisaccharide–lipid from endogenous GDPMan or dolichol-*P*-Man. The transfer of radioactivity to proteins was also observed with disaccharide–lipid as a substrate without GDPMan added. Thus, 26% of radioactivity was incorporated into proteins from di-*N*-acetylchitobiose–lipid under the conditions in which 43% of radioactivity was incorporated from trisaccharide–lipid.

The radioactivity incorporated into proteins from [^{14}C]trisaccharide–lipid was not extracted with chloroform/methanol (2:1), chloroform/methanol/water (1:1:0.3) or water. It became soluble in water by pronase treatment, and gel filtration of the digest gave no longer the trisaccharide (fig.3). The molecular weight of the main radioactive material in the digest coincides with that of $Man(GlcNac)_2Asn$, 694. Two minor products with the molecular weights of about 800 and 880 were also obtained. These observations indicate that the radioactive trisaccharide was covalently linked to proteins.

4. Discussion

Since Parodi et al. have reported the formation of a polysaccharide–lipid containing about 20 glycose units and the transfer of its saccharide portion to proteins [6], it seems to be generally believed that the saccharide can not be transferred to proteins till it reaches a considerably large size on the lipid. Our results show that in the yeast microsomal system the mannose-containing trisaccharide can be transferred to proteins without further mannosylation.

On the other hand, we observed that small amounts of mannose-containing oligosaccharide–lipids with greater saccharide chains than trisaccharide were formed in the presence of GDPMan in some cases. Such oligosaccharide–lipids were also detected by using [^{14}C]GDPMan as a substrate. The saccharide portions of these lipids appeared to be greater than the tetrasaccharide reported by Lehle et al. [7]. They could not detect the formation of the trisaccharide–lipid, but we found that under the conditions described here for the mannose transfer, only the [^{14}C]trisaccharide–lipid was accumulated. This permitted easy preparation of the saccharide–lipid, leading to the indication of the transfer of the trisaccharide to proteins. Although it was observed that the presence of Triton X-100 facilitated the transfer of the mannose-containing trisaccharide and perhaps di-*N*-acetylchitobiose to proteins, it is unknown at which stage the glycosylation of proteins occurs in the physiological state. The radioactivity from the di-*N*-[^{14}C]acetylchitobiose–lipid could partially be transferred after addition of mannose from endogenous GDPMan, but the bulk of the transfer can not be explained in this way.

In our preliminary experiment, [*glucose*-^{14}C]-dolichol-*P*-*P*-GlcNAc prepared from pig liver dolichol monophosphate with rate microsomes [8], was found to act aslo as a substrate for the yeast enzyme. Thus, the mannose-containing trisaccharide linked through a pyrophosphate bridge to mammalian dolichol was easily produced in the present system. When this mannose-containing trisaccharide–lipid was incubated with rat liver microsomes in the presence of GDPMan, the lipid intermediate was mannosylated further. Therefore this yeast system may be very useful for the investigation of glycoprotein synthesis in other systems.

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