INVOLVEMENT OF DOLICHOLMONOPHOSPHATE IN THE FORMATION OF SPECIFIC MANNOSYL-LINKAGES IN YEAST GLYCOPROTEINS

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SUMMARY: A membrane fraction from Saccharomyces cerevisiae catalyzes the transfer of mannosyl residues from GDP-Man partly via dolicholmonophosphate into a heterogenous glycoprotein fraction. The pattern of radioactive products obtained after mannosylation with GDP-[^{14}C]Man is similar to that obtained with dolicholmonophosphate-[^{14}C]mannose. In each case more than 70% of the radioactivity can be released by ß-elimination. Evidence is presented, that only the mannosyl residue directly linked to protein is incorporated via dolicholmonophosphate.

Polyprenols have first been shown to act as glycosyltransfer intermediates in the biosynthesis of bacterial cell wall components (1). In addition considerable evidence has accumulated that lipophilic intermediates are of importance in glycoprotein synthesis in various eucaryotic cells (2, 3, 4). In yeast the active lipid fraction has been identified as dolichols-14 to -18 (5) and also in liver it seems certain that the lipids are dolichols (6, 7). Less is known, however, about the exact nature of the glycoproteins.

In the case of S. cerevisiae a particulate in vitro system mannosylates dolicholmonophosphate from GDP-Man (5, 8). The DMP-Man[1)] thus formed has been suggested to be an intermediate in cell wall mannan biosynthesis (8, 9, 10). Yeast mannan is a glycoprotein containing a large molecular weight polymannose portion and oligomannose units which can be released by ß-elimination (11). It will be shown here that mannose from DMP-Man is specifically incorporated into polymeric components. More than 70% of the mannose incorporated is linked to glycoproteins of which only a minor fraction is cell wall mannan.

[1)] DMP=dolicholmonophosphate; DMP-Man=dolicholmonophosphate-mannose

Mannosyl-Donor	% Radioactivity released by ß-Elimination	Pronase
GDP-[^{14}C] Man	74	62
DMP-[^{14}C] Man	73	79 [1]

[1] Average value of two determinations. All other values are average values of 4 experiments.

Table 1

Release of Polymer-Bound Radioactivity by ß-Elimination and by Treatment with Pronase. Mannosylation of polymer with GDP-[^{14}C] Man: particulate fraction from yeast (0.9 mg protein) was incubated with 0.23 µC GDP-[^{14}C] Man (s.A. 154) in the presence of 2 mM $MnCl_2$, 2.3 mM $MgCl_2$, 50 mM Tris/HCl pH 7.4 in a total volume of 180 µl for 30 min at 21°C. The reaction was stopped by adding 1.8 ml of chloroform:methanol (3:2). The precipitate was washed with methanol, 80% methanol/water (KCl saturated) and 80% methanol/water. 90% of the radioactivity added was incorporated into the precipitate. Mannosylation of polymer with DMP-[^{14}C] Man : 215 000 cpm of DMP-[^{14}C] Man prepared as described in Materials and Methods was incubated with particulate fraction from yeast (900 µg protein) in the presence of 5 mM $MnCl_2$ and 2.5 mM $MgCl_2$ in a total volume of 400 µl; other conditions as above. 12% of the radioactivity was incorporated into the precipitate. For ß-elimination the precipitate was incubated with 0.1 N NaOH for 24 h at 21°C. The sample was then dialyzed, the external solution concentrated, de-ionized with Dowex 50-H^+, and an aliquot counted in a Betaszint 5000. For pronase digestion the precipitate was incubated with 1 mg pronase in 2 ml NH_4HCO_3 (0.5%, pH 7.9) at 37° for 24 h under a drop of toluene. Extended incubation with further addition of pronase did not release additional radioactivity. After dialysis the external solution was concentrated again and an aliquot counted.

MATERIAL AND METHODS:

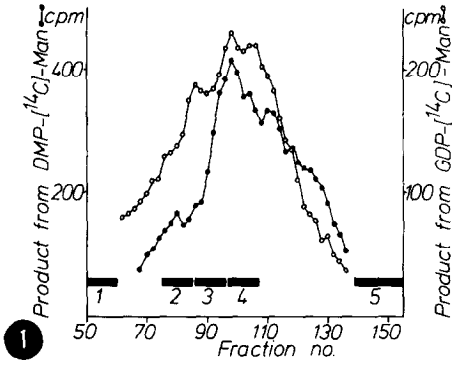
The particulate membrane fraction of S. cerevisiae (strain 66·24, Fleischmann Lab.) was prepared as described previously (8, 9). Dolichol from yeast was isolated according to Burgos et al. (12) and chemically phosphorylated according to Popják et al. (13). Mannosylation of dolicholmonophosphate: 60 µg DMP (in 120 µl acetone) were mixed with 80 µl 0.1 M MgEDTA and dried under N_2 . After mixing with 40 µl 0.6 % Triton and 160 µl Tris-HCl (0.05 M, pH 7.4, containing 5 mM $MgCl_2$) , 0.2 µC GDP-[^{14}C] Man (s.A. 154) and 0.6 mg protein (membrane fraction) were added to give a total volume of 300 µl. After 15 min at 21°C the reaction was

stopped by the addition of 3 ml chloroform/methanol (3:2), the precipitate was removed and the organic phase containing up to 60% of the radioactivity was washed as described (8). The radioactivity was found exclusively to be DMP-[^{14}C] Man (5).

RESULTS AND DISCUSSION:

Evidence for the glycoprotein nature of the alcohol insoluble radioactive material, obtained when a yeast membrane fraction is incubated with GDP-[^{14}C] Man or with DMP-[^{14}C] Man (3, 8), has been summarized in table 1. Whereas the radioactivity remained polymer-bound, when the samples were treated with 0.01 N HCl for 30 min at 100°C, 70 to 80% of the radioactivity became dialyzable in the presence of 0.1 N NaOH (21°C; 24 h). This treatment is considered to be fairly specific for breaking 0-glycosidic linkages to serine or threonine (14). Pronase treatment and subsequent dialysis resulted in 60% release of radioactivity when GDP-Man and a somewhat higher percentage when DMP-Man was the mannosyl donor (table 1).

To characterize and compare the glycoprotein fractions mannosylated in vitro from GDP-[^{14}C] Man and DMP-[^{14}C] Man, they were solubilized and run through standardized Sephadex G-200 columns and in addition applied to disc-electrophoresis. Fig. 1 shows that with both mannosyl donors a similar pattern of rather heterogenous products is obtained. The molecular weight of the main component in each case is approximately 75 000, which corresponds to the molecular weight reported for the mannan glycoprotein from yeast (11). Similarly the disc gel pattern shows that more than one component gets mannosylated from both the mannosyl donors used (fig. 2); again the pattern of radioactive products is fairly similar. In addition it can be seen, however, that only a small portion of the total radioactivity corresponds to a mannan standard (fraction A prepared according to Sentandreu and Northcote, 11). The latter observation has been confirmed independently in the following way: to an incubation mixture with GDP-[^{14}C] Man and to one with DMP-[^{14}C] Man non-radioactive fraction A was added as carrier after the termination of the reaction. Then fraction A was re-isolated following exactly the procedure of Sentandreu and Northcote (11). Although the cold carrier material has been fully recovered only



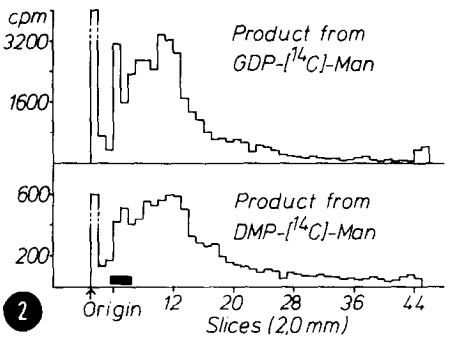


Fig. 1
Determination of the Size Distribution of the Glycoproteins Mannosylated via GDP-[^{14}C]Man or DMP-[^{14}C]Man on Sephadex G-200. The alcohol precipitable material was prepared as in Table 1 . It was then solubilized in ethylenediamine and subsequently dialyzed against 2 l of water. The Sephadex column had been standardized with dextran blue (1), aldolase (2), ovine serum albumin, dimer (3), bovine serum albumin (4), and ovine serum albumin (5). Fractions of 2 ml were collected and the radioactivity determined in aliquots.

Fig. 2
Separation of the Glycoprotein Fractions by Polyacrylamide Disc-Gel-Electrophoresis. The glycoprotein fraction was obtained as described in Fig. 1 . Electrophoresis was carried out according to Davis (15). Gels were stained for Fraction A with the Alcian Blue reagent (16). Gel slicing and scintillation counting was carried out according to Moss and Ingram (19). Barr indicates position of fraction A.

36% of the radioactivity were regained when GDP-Man and 12% when DMP-Man was the mannosyl donor.

Whereas in general the products mannosylated by GDP-Man seem to be identical to a large extent to those mannosylated via DMP-Man, one important difference has been noticed. When the dialyzable radioactivity after ß-elimination was chromatographed on paper, a di- and trisaccharide were observed besides radioactive mannose only when GDP-[^{14}C]Man had served as mannosyl donor (fig. 3 top and middle). The disaccharide has been identified as dimannose by the following criteria: a) acid hydrolysis (1 N HCl, 1 hr, 100°C) yielded only radioactive mannose; b) the compound was split by jack bean α-mannosidase (kindly supplied by Dr. Lehle); [^{14}C]mannose was the only product obtained. After reduction with $NaBH_4$ [^{14}C]mannose and [^{14}C]mannitol were obtained.

The difference in the products of ß-elimination can be explained by the

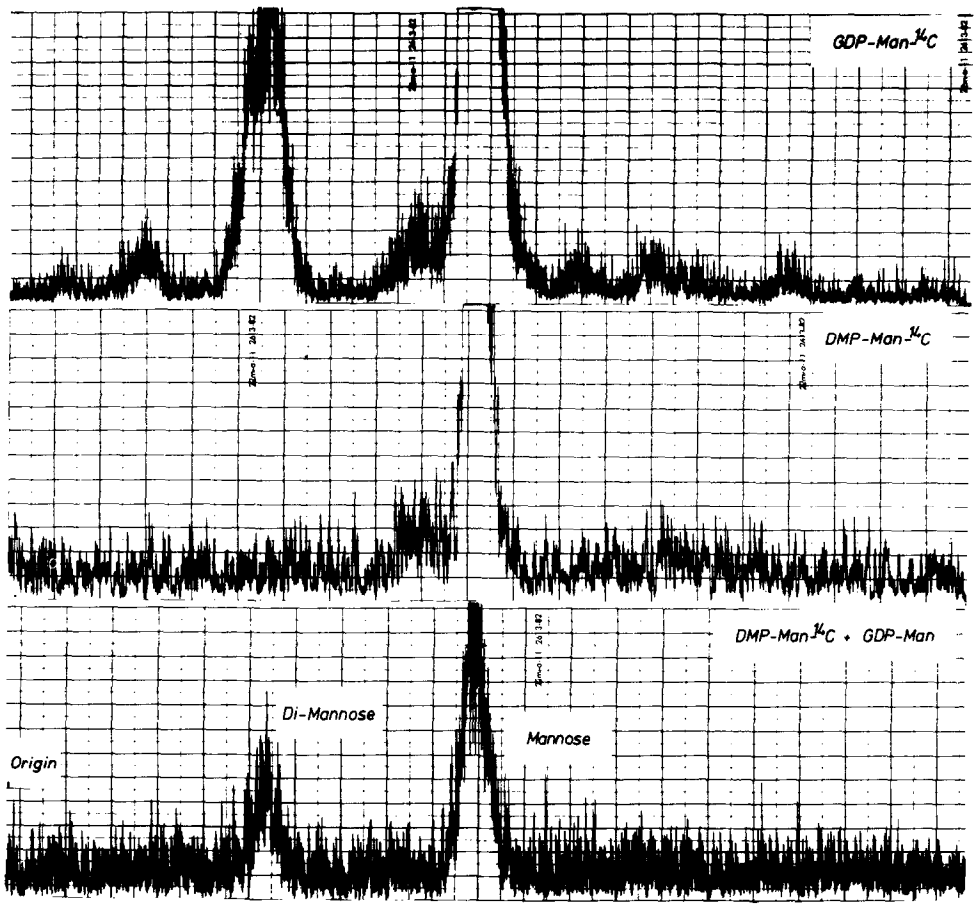


Fig. 3
Chromatographic Separation of the Dialyzable Products Obtained after ß-Elimination. Mannosylation of the glycoprotein fraction was carried out with GDP-[^{14}C] Man (top), with DMP-[^{14}C] Man (middle) or with DMP-[^{14}C] Man plus 10 µM non-radioactive GDP-Man. The products were separated on paper in ethylacetate:butanol:acetic acid: H_2O = 30:40:25:40 . For other conditions see table 1 .

assumption that DMP-Man serves as mannosyl donor only for the first mannose, which is directly linked to serine or threonine, but cannot serve this function for subsequent mannoses. These would have to be transferred directly from GDP-Man. In the presence of GDP-[^{14}C]Man the direct mannosylation of the protein would have to take place via the endogenous DMP. Evidence for this interpretation was obtained by the experiment of fig. 3/bottom. In this case the glycoprotein biosynthesis was carried out in parallel to that of fig. 3/middle, with the only exception that in addition to DMP-[^{14}C]Man also non-radioactive GDP-Man was included in the incu-

bation. When this glycoprotein was taken through ß-elimination and the products were chromatographed, the radioactive disaccharide was obtained again.

These results thus show that a membrane preparation from S. cerevisiae transfers mannose from GDP as well as from DMP to various glycoproteins. More than 70% of the mannose incorporated under the conditions described here are linked 0-glycosidically (serine, threonine). Glycoproteins containing either mannose or mannose oligomers linked in such a way are known to occur in yeast. In addition to cell wall mannan (11) this has been reported for invertase and for acid phosphatase (17, 18).

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