PARTICIPATION OF DOLICHOL PHOSPHO-MANNOSE IN THE GLYCOSYLATION OF YEAST WALL MANNO-PROTEINS AT THE POLYSOMAL LEVEL

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1. Introduction

The major yeast wall glycoprotein (yeast mannan) is formed by mannose units linked to polypeptide backbones either through an O–glycosydic bond to hydroxyl groups of serine and threonine or by an N–glycosydic bond conecting a residue of asparagine to a di-*N*-acetyl-chitobiose unit [1,2].

Dolichol-phosphate derivates especially dolichol-phosphomannose (DPM) appear to play an important role [3,6,18] in the transfer of the first mannosyl residue to the hydroxyaminoacids. Guanosine-diphosphate-mannose (GDP-mannose) acts as the intermediate donor of mannose residues for further elongation of the oligosaccharides [7]. Some of the enzymes involved in the synthesis of specific bonds have also been characterized [6,8,21].

Evidence that the initial glycosylation of glycoproteins in higher cells occurs at the level of nascent polypeptide has been reported by different groups [9–12] and studies carried out in our laboratory have shown that glycosylation of the mannoproteins of *Saccharomyces cerevisiae* wall is also initiated at the polysomal level [13].

In this paper we present evidence that lysates of protoplasts incorporated mannose from either GDP-mannose or DPM in non-lipidic high molecular weight material. From the conditions of the reaction, the specific activities of the radioactive polysome and membrane fractions and the release of mannose by alkaline treatment it is suggested that DPM is the intermediate precursor for the mannosylation of hydroxy aminoacids at the polysomal level of the yeast wall glycoproteins.

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2. Materials and methods

S. cerevisiae $^{-136}$*ts* was obtained from L. H. Hartwell and protoplasts were prepared with the lytic complex of *Helix pomatia* [13].

The preparation of ^{14}C-mannolipids was carried out using *S. cerevisiae* LK2G12 cells which were disrupted in a Braun homogenizer. The particulate preparation which sedimented between 2000 × *g* for 10 min and 40 000 × *g* for 40 min was extracted with chloroform/methanol (2:1, v/v) and treated as described elsewhere [4]. The fractions eluted with chloroform/methanol (1:1) from a silicic acid column [4] was rechromatographed on another silicic acid column eluted with a discontinuous gradient of chloroform/methanol (7:1–7:7, v/v). The fractions eluted with chloroform/methanol (7:2, v/v) contained DPM and were used as a donor of [^{14}C]mannose.

Alkaline treatment (β-elimination) was carried out in 0.5 N NaOH at room temperature. The low molecular weight released materials were separated by descending paper chromatography on Whatman No. 1 paper. Chromatograms were developed with ethylacetate/pyridine/water (5:3:2, v/v/v) and spots visualized with the ammonical silver nitrate reagent of Trevelyan et al. [20].

3. Results

In a previous paper we described that mannosylation at the polysomal level could be carried out by cells but not by freshly prepared protoplasts [1]. Additional studies to examine protein synthesis by freshly prepared and regenerating protoplasts showed that protein synthesis in freshly prepared *S. cerevisiae* $^{-136}$*ts* protoplasts was undetectable but increased to significant levels during the regeneration process (fig.1).

In order to determine if the diminished capability of protein synthesis in freshly prepared protoplasts was due to a lack of polysomes, lysates of these protoplasts were fractionated in a sucrose gradient. Polysomes were not found, neither was radioactivity detected at the position expected for polysomes, after a pulse of [^{14}C]mannose. In contrast, when the lysates were obtained from protoplasts after a 150 min regenerating period (regenerating protoplasts) the sucrose gradient exhibited patterns similar to that obtained from growing cells. (fig.2).

In order to identify the mannose donor at the

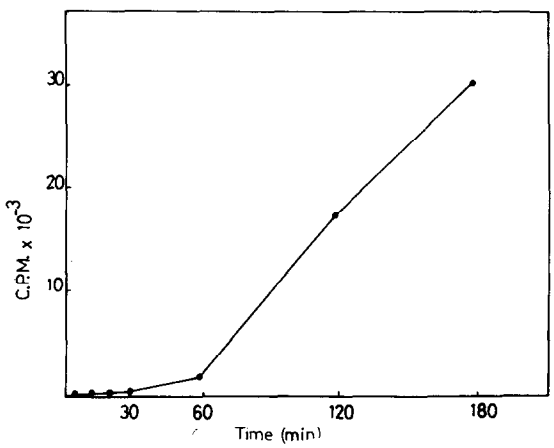

Fig.1. Incorporation of [^{14}C]threonine by *S. cerevisiae* $^{-136}$*ts* protoplasts. Washed protoplasts from 3 × 10^{10} cells were suspended in 40 ml of 0.6 M $MgSO_4$-containing YM-1 medium. The suspension was incubated at 23°C for 5 min, and 1 ml of [^{14}C]threonine (1 μCi/ml; specific activity, 0.25 mCi/mmol) was added. Aliquots (2 ml) were taken at indicated times, precipitated with 1 vol. of ice-cold 10% trichloroacetic acid (TCA), filtered through Whatman GD/C glass filter discs, washed with 50 ml of 5% TCA, dried and counted [13].

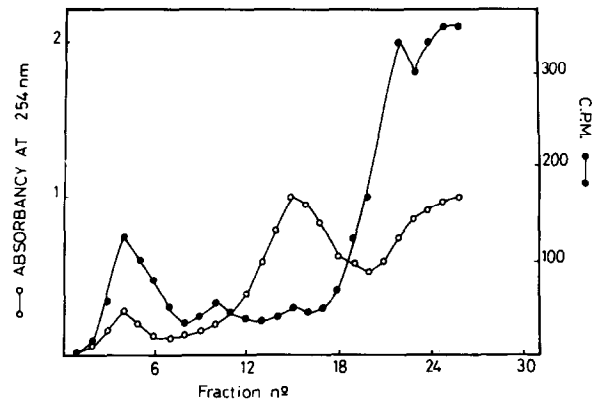

Fig.2. Ribosomal profiles of 'regenerating protoplasts' incubated with [^{14}C]mannose. Cells (10^{10}) were transformed into protoplasts which were incubated for 150 min in stabilized YM-1 medium as in fig.1. After washing, the 'regenerating protoplasts' were suspended in 2 ml of 0.6 M $MgSO_4$-containing YM-1 medium (carbon source was one-tenth of original concentration) prepared in TKM and pulsed for 30 sec with 1 ml of [^{14}C]mannose (2.5 μCi/ml; specific activity 3 mCi/mmol). Five volumes of ice cold hypertonic TKM buffer containing cycloheximide (100 μg/ml) and heparine (0.015%) were then added to stop protein synthesis. Then protoplasts were washed with ice-cold hypertonic TKM buffer containing cycloheximide and heparine and lysed in 1 ml of TKM buffer containing cycloheximide and heparine in the proportion described above. Triton X-100 and sucrose were added to a final concentration of 1% and 0.2 M respectively. Ribosomal profiles were obtained essentially as described by Preisler et al. ([19] see also [13]). Fraction 4–11 correspond to polysomes, fraction 12–16 to ribosomes and fraction 19–22 to membranes.

polysomal level either GDP-[^{14}C]mannose or dolichol-phospho-[^{14}C]mannose was added to lysates of 'regenerating protoplasts'. The lysates were prepared in the presence of cycloheximide to inhibit protein synthesis. As shown in fig.3A and fig.3B both substrates could act as donor of mannose to nascent glycoprotein on the polysomes. The level of incorporation of [^{14}C]mannose into glycoprotein from the glycolipid decreased in the presence of 'cold' GDP-mannose (fig.3C). This result further supports the observation that both substrates can be utilized for mannosylation at the polysome level.

However, some differences could be observed. When GDP-mannose was used as a substrate the specific incorporation (c.p.m./absorbance) at the polysomal level was lower than in the upper part of the gradient

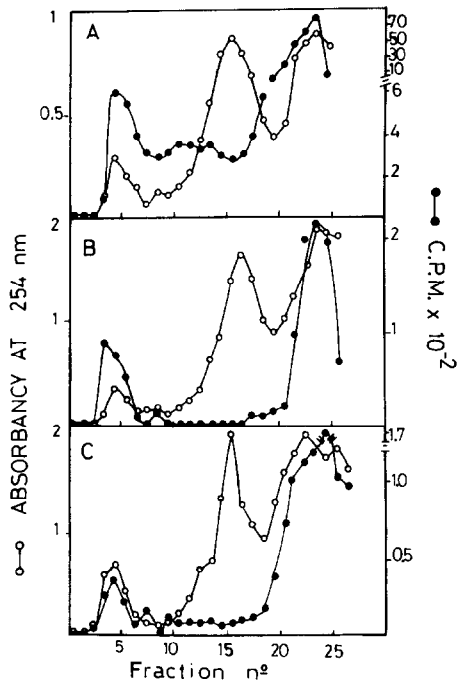

Fig.3. Transfer of [^{14}C]mannose to polysomal fraction 'in vitro'. Washed 'regenerating protoplasts' from 1.9 × 10^{10} cells were divided in three aliquots and lysed in 0.8 ml of TKM buffer containing cycloheximide (100 μg/ml) and heparine (0.015%) in presence of: (A) 0.2 ml of GDP-[^{14}C]-mannose (1 μCi/ml, specific activity 74 mCi/mmol), (B) 0.2 ml of dolichol-phosphate-[^{14}C]mannose (80 000 c.p.m.), (C) 0.2 ml of dolichol-phosphate-[^{14}C]mannose (80 000 c.p.m.), containing 2.5 mM cold GDP-mannose in 0.125% Triton X-100. Samples were incubated for 2 h at 30°C, placed on ice, made 1% Triton X-100 and 2% sucrose, and the ribosomal profiles of the postmitochondrial supernatant (supernatant of 15 000 × *g*) determined. Samples of 32 drops from the gradient were precipitated with 20 vol. of chloroform/methanol (2:1, v/v) filtered on Whatman GF/C discs and washed with 20 ml of the following solvents: choloroform/methanol (2:1, v/v), methanol 80% (KCl saturated), methanol 80% and methanol 50% [17]. Radioactivity in the dried discs was determined as described elsewhere [13]. Fractions 4–11 correspond to polysomes, fractions 12–16 ribosomes and fractions 19–22 membranes.

(fig.3A and table 1). In contrast when the substrate was dolichol-phospho-mannose the specific incorporation was higher at the polysomal level as compared with the membrane level (fig.3B and table 1).

The presence of cycloheximide at the time of substrate addition was necessary so as to obtain glycosylation at the polysomal level 'in vitro'. When protoplasts were obtained in the presence of cycloheximide from cycloheximide pretreated cells they failed to incorporate radioactivity at the polysomal level either from GDP-[^{14}C]mannose or dolichol phospho-[^{14}C]mannose. In addition the radioactivity at the position of membrane material was much less. These results may indicate that after blockage of protein synthesis the glycosylating systems went on catalysing the transfer of mannose, producing a partial saturation of acceptor sites.

The results described indicate that the DPM may act as a donor of mannose at the polysomal level. However the observation that GDP-mannose also acts as a donor raises the question of whether DPM is first converted to GDP-mannose which in turn acts as the intermediate donor. To examine whether or not DPM was the immediate precursor in the mannosylation of nascent polypeptides, a high Mg^{2+} concentration was used in an incubation mixture devoid of Mn^{2+} ions. Sharma et al. [6] have shown that under these conditions only the first O–glycosydically

Table 1
Influence of the substrate in the specific incorporation of [^{14}C]mannose at different subcellular levels

Substrate	Specific incorporation		Ratio
	Polysomes	Membranes	Polysomes/membranes
GDP-[^{14}C]mannose	2601	4550	0.59
Dolichol-phospho-[^{14}C]mannose	130	81	1.7

The specific incorporation was determined as the ratio between the total radioactivity and optical denstiy of polysomes [4–11] and membranes [19–25] fractions from fig.3A and 3B.

Table 2
Nature of the transfer reaction from GDP-mannose and dolichol-phospho-mannose to glycoproteins

Substrate	Subcellular level	Incorporated material			β-eliminated product
		Total (cpm)	β-eliminable (cpm)	(%)	
GDP-[^{14}C]mannose	Polysomes	3200	990	30	Mannose
	Membranes	21 889	2890	13	Mannose
Dolichol-phosphate-[^{14}C]mannose	Polysomes	213	195	91	Mannose
	Membranes	924	880	95	Mannose

Fractions 4–11 (polysomes) and 19–22 (membranes) from fig.3A and 3B were subjected to β-elimination and the nature of the low molecular weight materials released separated by chromatography (see Materials and methods).

linked mannose residue is transferred by a particulate membrane preparation and this process is carried out through DPM.

When the substrate used was GDP-[^{14}C]mannose a small amount (30%) of the mannose bound to the polysomes in the presence of high Mg^{2+} concentrations was incorporated as part of O–glycosidic linkages (table 2).

If DPM was used as a substrate more than 90% of radioactivity transferred to polysomes was released by alkaline treatment. This observation argues against the possibility that exogenous dolichol-phospho-[^{14}C]-mannose could be converted to GDP-[^{14}C]mannose which would then serve as substrate (table 2). Hence it is reasonable to suggest that a significative amount of the mannose transferred at the polysomal level is derived from DPM.

4. Discussion

The ability to isolate polysomes from 'regenerating protoplasts' has allowed studying the glycosylation procedure of glycoproteins 'in vitro' by following the incorporation of mannose at the level of nascent polypeptide.

Evidence is presented which is consistent with the interpretation that DPM is the initial glycosyl donor for the polypeptide chains on the ribosomes. This interpretation is based in the following observations: (I) Incorporation of mannose from exogenous DPM takes place at the polysomal level. (II) Incorporation of mannose from GDP-mannose at this level takes place through DPM. The present data are consistent with previous work carried out at membrane level that showed the involvement of DPM in the biosynthesis of the first O-mannosyl residue [6] and in the present work we show that at least part of it occurs at the polysomal level.

However, our observations do not rule out the possibility that glycosylation mediated by other donors may also occur at the polysomal level. Recently Lehle and Tanner [15] have found a dolichol pyrophospho-oligosaccharide in which the heterosaccharidic portion is composed by two mannose residues linked to a di-*N*-acetyl-chitobiose. This lipid appears to be synthesized from dolichol-pirophospho-di-*N*-acetylchitobiose and GDP-mannose. Such an endogenous glycolipid acting as donor of groups might be responsible for the alkali-resistant radioactivity observed associated with the polysomes when GDP-mannose is used as a substrate.

Intermediate lipid carriers whose heterosaccharidic portion is similar to the inner heterosaccharidic portion of several glycoproteins have also been found in higher organisms [16, 3, 9, 15, 17] and it is supposed that they transfer sugars to glycoproteins. This transfer could take place while the glycoproteins are migrating into the lumen of the rough endoplasmic reticulum to their deposition sites.

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