Synthesis and Possible Role of Carbohydrate Moieties of Yeast Glycoproteins

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Synthesis and possible role of carbohydrate moieties of yeast glycoproteins

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The pathways for protein N- and O-glycosylation in yeast cells are summarized. Evidence is presented that the terminal glucosyl residues of the dolichyl-PP-oligo-saccharide intermediate are responsible for decreasing the K_m for the peptide to be N-glycosylated.

A liposomal model system is introduced that allows the study of a dolichyl phosphate (Dol-P) dependent transmembrane transport of mannosyl residues. The results obtained so far suggest that the mannosylation of Dol-P and the transmembrane translocation of Dol-P-Man are catalysed by the enzyme more or less simultaneously. However, only about 8–10 % of the enzyme molecules incorporated into the liposomes seem to carry out the 'coupled' reaction.

The glycosylation of carboxypeptidase Y is not required for this protein to reach the vacuole, its target organelle. In the presence of low concentrations of tunicamycin, however, yeast cells do stop growth. This does not seem to be due to the inhibition of secretion of glycoproteins like external invertase. It is postulated that protein glycosylation is crucial for a cell cycle event during the G1 phase.

Introduction

The participation of dolichyl phosphate activated sugars in glycoprotein synthesis has long been studied in yeast (Tanner 1969) and it is now clear that at least for N-glycosylation the pathway worked out for animal cells (Robbins *et al.* 1977; Li *et al.* 1978) also proceeds in the yeast *Saccharomyces cerevisiae* (Lehle 1981; Parodi 1981).

The reactions of the dolichol pathway are always catalysed by membrane-bound enzymes, which in all organisms are localized in the rough endoplasmic reticulum (Czichi & Lennarz 1977; Marriott & Tanner 1979, 1980).

Two major questions, however, have not yet been answered satisfactorily. Firstly, *why* are dolichyl phosphates as 'lipid intermediates' involved in this biosynthetic process? Secondly, what is the crucial general importance of protein glycosylation, evident from the fact that the dolichol pathway has obviously been preserved in a very conservative way during evolution (Lehle *et al.* 1980)? Experiments related to both these questions will mainly be discussed in this paper.

The pathway of O- and N-glycosylation in yeast

O-glycosylation

A variety of fungal glycoproteins contain short oligomannose side chains linked to serine or threonine (Sentandreu & Northcote 1969; Yen & Ballou 1973). In their synthesis dolichyl phosphorylmannose (Dol-P-Man) constitutes the mannosyl donor for the first mannosyl residue linked to protein (figure 1); subsequent mannose residues are transferred directly from

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GDP-Man (Babczinski & Tanner 1973; Sharma *et al.* 1974). This reaction sequence has been described for a number of fungal cells (for references see Lehle 1981), whereas in animal cells dolichyl intermediates do not seem to participate in O-glycosylations (Babczinski 1980).

N-glycosylation

The statement that the dolichol pathway of N-glycosylation in fungal cells most probably proceeds in an identical way to that in animal cells is based on the following observations.

(*a*) The existence of dolichyl-PP-oligosaccharides containing GlcNAc, Man and Glc have been described (Parodi 1977, 1981; Lehle 1980, 1981) and the oligosaccharides synthesized *in vitro* and *in vivo* have been shown to be of the same size as the mammalian $GlcNAc_2Man_9Glc_3$ (Lehle *et al.* 1980).
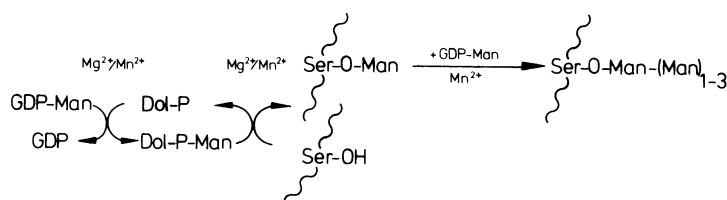

FIGURE 1. Pathway of O-glycosylation in yeasts.

(*b*) Dol-P-Man, GDP-Man and Dol-P-Glc that act as sugar donors for the Dol-PP-oligosaccharide seem to correspond to those of animal cells (Chapman *et al.* 1980; Parodi 1981; Lehle 1981).

(*c*) Three glucosyl residues are trimmed off the oligosaccharide after this has been transferred to the protein acceptor (Parodi 1979; Lehle 1980).

(*d*) Tunicamycin specifically blocks the GlcNAc transfer from UDP-GlcNAc to dolichyl-P (Lehle & Tanner 1976).

It is not yet clear whether the pathway ends with the glucose-trimming reactions or whether some of the mannosyl residues are also hydrolysed off, as in animal cells. Because the core structure proposed for yeast mannoproteins (Ballou 1976; Hashimoto *et al.* 1981) is not identical to the $Dol-PP-GlcNAc_2Man_9$ structure published by Li *et al.* (1978), it seems likely that 1 to 3 mannosyl residues have to be removed and subsequently added again to different hydroxyl groups.

In connection with the function of the transiently present glucose moieties it has been shown that the rate of the transfer of the oligosaccharide *en bloc* to the protein is much slower if the glucoses are missing (Turco *et al.* 1977; Spiro *et al.* 1979). By using the solubilized oligosaccharyl transferase from yeast and the hexapeptide Tyr-Asn-Leu-Thr-Ser-Val as carbohydrate-accepting molecule, this reaction was studied in greater detail (Sharma *et al.* 1981). As shown in table 1 the enzyme uses $Dol-PP-(GlcNAc)_2$ and $Dol-PP-(GlcNAc)_2Man$ almost as efficiently as $Dol-PP-(GlcNAc)_2-Man_9Glc_3$, but it does not transfer $(GlcNAc)_2Man_9$ at all to the hexapeptide. The K_m values for $Dol-PP-(GlcNAc)_2$ and $Dol-PP-(GlcNAc)_2Man_9Glc_3$ did not differ significantly (table 1). Surprisingly, however, the apparent affinity of the enzyme for the hexapeptide changed drastically with the dolichyl-PP donor molecule and was more than tenfold greater with $Dol-PP-(GlcNAc)_2-Man_9Glc_3$ than with $Dol-PP-(GlcNAc)_2$ (figure 2). This could mean that the presence of the glucosyl residues on the Dol-PP-oligosaccharide positively affects the enzyme's affinity for the protein to be glycosylated.

Does dolichol have a 'carrier' function in transmembrane transport?

As mentioned in the introduction, most of the available evidence indicates that dolichol-dependent glycosylations proceed at the endoplasmic reticulum. In fungal cells this is also true for the initial reactions of *O*-glycosylation (Lehle *et al.* 1977; Esmon *et al.* 1981). All these glycosylations of protein occur during the synthesis of the polypeptide chain and in all likelihood on the luminal side of the reticulum membrane (Larriba *et al.* 1976; Rothman & Lodish 1977;

TABLE 1. DONOR SUBSTRATE SPECIFICITY OF THE SOLUBILIZED

OLIGOSACCHARYL TRANSFERASE

(A radioactive count rate of 8000 min^{-1} of each of the donors was used.)

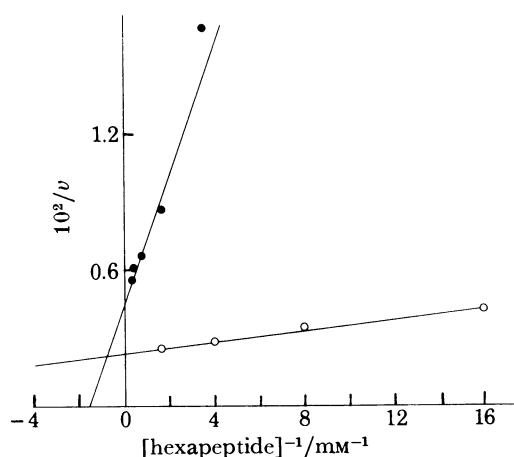| donor substrate	transfer to the hexapeptide in 15 min		apparent K_m for the donor substrate
	count min^{-1}	percentage	μM
Dol-PP-(GlcNAc)$_2$(Man)$_9$(Glc)$_3$	2667	33	0.63
Dol-PP-(GlcNAc)$_2$(Man)$_9$	51	0.6	—
Dol-PP-(GlcNAc)$_2$(Man)$_1$	2415	30	—
Dol-PP-(GlcNAc)$_2$	2089	26	1.2
Dol-PP-GlcNAc	94	1.1	—

FIGURE 2. Dependence of the K_m for the hexapeptide on the glycosyl donor substrate. Standard enzyme assays were carried out for 10 min as described in Sharma *et al.* (1981) with a radioactive count rate of 8000 min^{-1} of Dol-PP-(GlcNAc)$_2$Man$_9$Glc$_3$ (○) and of Dol-PP-(GlcNAc)$_2$ (●); v is expressed as the count min^{-1} per minute of incubation.

Glabe *et al.* 1980). Because the sugar nucleotides, the ultimate precursors, are produced in the cytoplasm the problem arises how and in what form the activated sugars cross the membrane. These aspects have recently been thoroughly discussed by Hanover & Lennarz (1981) and Lennarz (this symposium). Although the dolichol-bound monosaccharides and oligosaccharides have long been suspected to represent the transmembrane transport species, this has never been demonstrated. On the contrary, neither free polyprenyl phosphates (McCloskey & Troy 1980) nor Dol-PP-(GlcNAc)$_2$ (Hanover & Lennarz 1978) move at a measurable rate across artificial or natural membranes. It has therefore been suggested that the synthesis and the transmembrane

movement of the dolichol-bound saccharide might occur at the same time, i.e. the glycosyl transferase also catalysing the translocation step (Haselbeck & Tanner 1982; Hanover & Lennarz 1982).

A liposomal system for transmembrane transport

A system to test dolichyl phosphate-mediated mannosyl transfer through a lipid bilayer was set up in the following way: the mannosyltransferase of yeast cells catalysing the reversible reaction

$$\text{GDP-Man} + \text{Dol-P} \underset{}{\overset{\text{Mg}^{2+} \text{ or } \text{Mn}^{2+}}{\rightleftharpoons}} \text{Dol-P-Man} + \text{GDP}$$
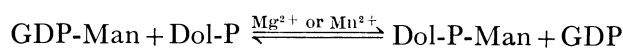
was solubilized and purified approximately a thousandfold (Babczinski 1980; Haselbeck & Tanner 1982). This enzyme was incorporated into soybean lecithin liposomes containing
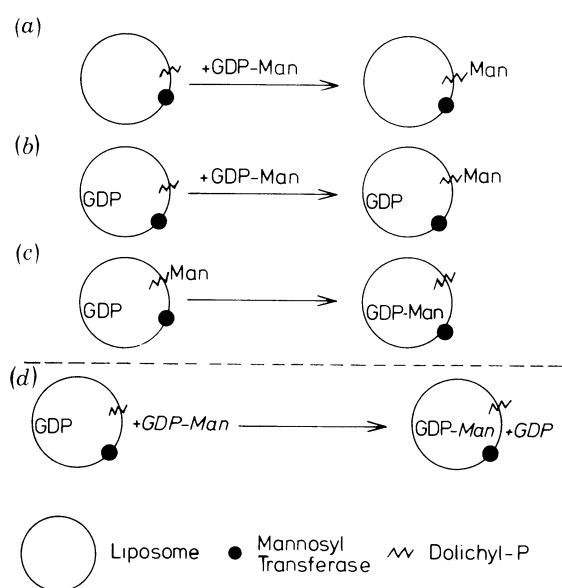

FIGURE 3. Dolichyl phosphate-mediated mannosyl transfer into liposomes: schematic reaction sequence.

Dol-P (Dol-P:lecithin ratio 1:17). When GDP-[^{14}C]Man was added, the liposomal Dol-P was mannosylated (figure 3a), and this was also observed when the liposomes were preloaded with 7 mM GDP (figure 3b). However, only in the latter case did the liposomes, which were separated from the excess external GDP-[^{14}C]Man by a Sepharose 4B column, also contain a water-soluble radioactive compound (figure 4). This was identified as GDP-[^{14}C]Man (Haselbeck & Tanner 1982) and the result has therefore been interpreted as a transmembrane transfer of a mannosyl residue from external GDP-Man to internal GDP (figure 3d). That Dol-P is indeed required in the reaction sequence shown in figure 3d, and that Dol-P-Man is therefore most probably a transport intermediate, as shown in figure 3c, is indicated by the data of table 2.

From the results so far presented it is not possible to distinguish between a mannosylation of liposomal Dol-P and a subsequent transmembrane movement of Dol-P-Man ('flip-flop') and the second possibility of a simultaneous mannosylation and translocation; in the latter case both events would be catalysed by the enzyme. Two observations are in favour of the second possibility.

(a) The time course of formation of internal GDP-Man and liposomal Dol-P-Man in liposomes either preloaded or not preloaded with GDP is shown in figure 5. In GDP-preloaded liposomes the internal GDP-[^{14}C]Man formed always amounts to about 8% of the total liposomal Dol-P-[^{14}C]Man synthesized. This can be interpreted to mean that only a small fraction of the enzyme carries out the whole reaction, i.e. mannosylation plus translocation,
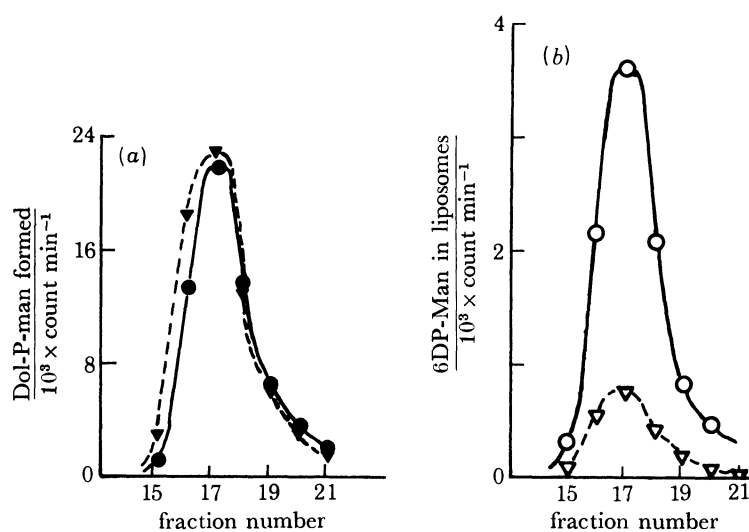

FIGURE 4. Radioactivity from GDP-[^{14}C]Man incorporated into (a) the lipid fraction and (b) the water-soluble fraction of Dol-P-containing liposomes. The liposomes were separated from the incubation medium on a Sepharose 4B column; they appeared in the void volume. —, Liposomes with GDP; – – –, liposomes without GDP. For other experimental details see Haselbeck & Tanner (1982).

TABLE 2. MANNOSYL TRANSFER FROM EXTERNAL GDP-[^{14}C]MAN TO INTERNAL GDP: DEPENDENCE ON DOL-P AND ON MANNOSYL TRANSFERASE

conditions	radioactive count min^{-1} in:	
	Dol-P-Man	GDP-Man in liposomes
complete liposomes	28 911	1491
minus Dol-P	222	36
minus enzyme	24	20
complete liposomes with amphomycin (75 µg)	11	109

whereas most of it only catalyses the mannosylation step. In case of a rate limiting 'flip-flop' of Dol-P-[^{14}C]Man, an initial lag in the formation of internal GDP-[^{14}C]Man and a subsequent steady increase in its percentage of the total liposomal radioactivity would have been expected. That most of the liposomal Dol-P-Man formed is indeed oriented with its mannosyl residue to the outside is inferred from the observation that approximately 75% of its radioactivity is rapidly lost when 1 mM GDP is added to the external medium (Haselbeck 1982).

(b) When liposomes were prepared with polyprenyl phosphates of differing chain lengths, the rate of formation of internal GDP-[^{14}C]Man was more or less independent of the kind of polyprenyl phosphate used, whereas the rate of polyprenyl-P-Man formation differed significantly (table 3). For a Dol-P-Man 'flip-flop', a translocation rate (measured as internal GDP-Man formed with time) proportional to the radioactivity present in Dol-P-Man would again have been expected.

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Comparison of activities in vitro *and* in vivo

Assuming, then, that the mannosyl transferase catalyses simultaneously both the manno-sylation of Dol-P as well as the transmembrane translocation of Dol-P-Man – whereas in the liposomal system both these steps seem to be 'uncoupled' to a large extent – the 'internalized' mannosyl residue might subsequently undergo two types of reactions. Either it gets transferred directly from membrane-bound Dol-P-Man to the intraluminally growing Dol-PP-(GlcNAc)$_2$-Man$_5$ to form Dol-PP-(GlcNAc)$_2$Man$_9$ or it gets transferred back to GDP present in the e.r.
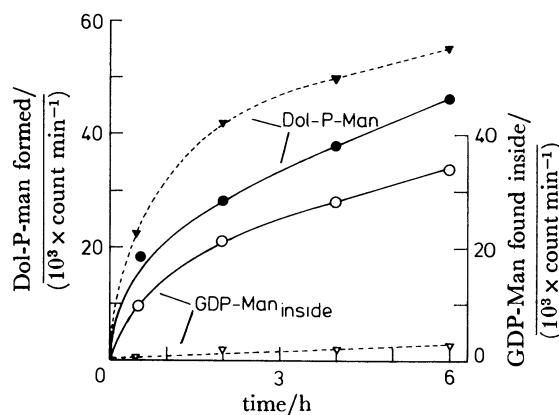

Figure 5. Time course of [^{14}C]mannose transfer from GDP-[^{14}C]Man to dolichyl phosphate and to internal GDP. —, Liposomes with GDP; – – –, liposomes without GDP.

TABLE 3. DEPENDENCE OF MANNOSYL TRANSFER ON POLYPRENYL CHAIN LENGTH

	Dol-P-Man formed (count min^{-1})	mannosyl residue transported into liposome	
		(count min^{-1})	percentage of Dol-P-Man
C$_{35}$–Dol-P	170 294	4100	2.4
C$_{60}$–Dol-P	79 447	3382	4.2
C$_{80-100}$–Dol-P	40 225	4134	10.3

lumen analogous to the situation in the liposomal system. The internal GDP-Man could then serve as mannosyl donor for all those reactions that have been shown *in vitro* to require GDP-Man directly. In the yeast cell the latter reaction would be involved in the formation of the *O*-glycosidic side chains (except for the first mannosyl residue) as well as in the extension of the outer chains of mannoproteins (Parodi 1981; Lehle 1981), which can amount to more than two-thirds of the carbohydrate content of yeast mannoproteins (Ballou 1976). Thus if all mannose residues of yeast mannoproteins require the formation of Dol-P-Man for their trans-membrane transport, the mannosyl transferase activity *in vivo* would have to be considerably greater than that required for reactions directly dependent on Dol-P-Man. For a mannose content of 10 % of yeast dry mass (Sentandreu & Northcote 1968) and a generation time of 2 h, a Dol-P-Man forming activity of 310 μmol h^{-1} g^{-1} yeast dry mass would be required. This has to be compared with a maximal activity measured *in vitro* of 165 μmol h^{-1} g^{-1} yeast dry mass at saturating GDP-Man and optimal Dol-P concentrations. Although substrate

saturation possible does not exist *in vivo*, when all the possible fallacies of work *in vitro* are considered, the two values do not differ to an extent that would exclude Dol-P-Man as a general transmembrane transport vehicle for mannose in *S. cerevisiae*.

Why are yeast proteins glycosylated anyway?

In recent years an increasing number of examples concerning the functional importance of carbohydrate moieties of glycoproteins became known. Certainly among the best documented are those of receptor-mediated pinocytosis (Neufeld & Ashwell 1980). Since yeast cells contain

TABLE 4. DISTRIBUTION OF GLYCOSYLATED AND NON-GLYCOSYLATED (CARBOHYDRATE-FREE) CARBOXYPEPTIDASE Y IN CELL FRACTIONS

	carboxypeptidase Y		carbohydrate-free carboxypeptidase Y	
	count min^{-1}	percentage	count min^{-1}	percentage
cytosol	768	18.3	612	16.9
vacuoles	2598	62.1	2126	58.7
pellet	894	21.4	854	23.4

a lysosome-like vacuole, with a number of enzymically active glycoproteins it was natural to speculate that in this organism too the carbohydrate moieties are responsible for trageting the enzymes to the vacuole. Carboxypeptidase Y has been studied in more detail. It was shown that the enzyme contains four asparagine-linked oligosaccharides (Trimble & Maley 1977; Hasilik & Tanner 1978*b*) and that phosphomonoester and phosphodiester groups are indeed present within a larger oligomannose moiety (Hashimoto *et al.* 1981; Schwaiger *et al.* 1982). In the presence of tunicamycin, carbohydrate-free carboxypeptidase Y is synthesized, although in a reduced amount (Hasilik & Tanner 1978*b*). Is this carbohydrate-free protein still transported into the vacuole? Cells treated with tunicamycin contained approximately equal amounts of [^{14}C]phenylalanine-labelled intact carboxypeptidase Y and the carbohydrate-free form (Schwaiger *et al.* 1982), and both forms were found to be present in vacuoles isolated from these cells to the same extent (table 4). Thus, obviously neither mannose 6-phosphate residues nor any other part of the carboxypeptidase Y carbohydrate moiety is required as sorting signal for the protein to reach the vacuole. Evidence emerges that also in mammalian cells an additional sorting signal besides mannose 6-phosphate has to exist (Owada & Neufeld 1982; Waheed *et al.* 1982; Jessup & Dean 1982).

Because yeast carboxypeptidase, like other lysosomal enzymes, is synthesized via a 'pro' form with an extension of the peptide chain (Hasilik & Tanner 1976, 1978*a*), the sorting information could of course be located in this extra peptide piece (M_r 6000). A mutant deficient in carboxypeptidase Y processing (Hemmings *et al.* 1981) still transports the procarboxypeptidase Y into the vacuole (Schwaiger, unpublished). Thus the conversion of the pro form into the final carboxypeptidase Y form is not required for the protein to be put into the vacuole. This does not, however, rule out the possible role of the extra peptide piece as a target signal.

Nevertheless, the functional importance of the carbohydrate moiety of carboxypeptidase Y is again completely open and so is the question posed in the introduction about a crucial role of protein glycosylation in general. For yeast cells, and for many others, it has been shown that the secretion of proteins is inhibited when glycosylation is prevented, for example with

tunicamycin (Kuo & Lampen 1974). The important question in this context is, however, whether yeast cells stop growing, when N-glycosylation is inhibited by tunicamycin. So far this has not been observed when reasonably low inhibitor concentrations have been used (Hasilik & Tanner 1976); these cells were in the late logarithmic phase. Their growth is, however, completely inhibited with tunicamycin at 4–10 μg ml^{-1} when low-density logarithmically growing cultures
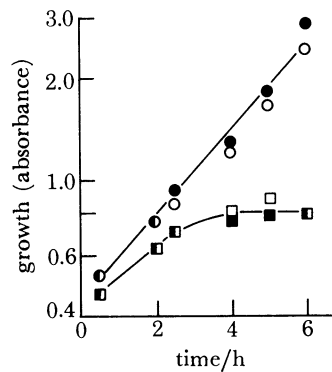

FIGURE 6. Growth inhibition of yeast cells (*S. cerevisiae* LBC H 1022 aα) by tunicamycin (8 μg ml^{-1}). Open symbols, glucose; filled symbols, sucrose; circles, without tunicamycin; squares, with tunicamycin.

are used (figure 6). Since yeast cells growing on sucrose require the presence of external invertase for growth, the effect of tunicamycin under these conditions was expected. The inhibition is identical, however, when the cells are grown on glucose (figure 6). This indicates that the complete cessation of growth after about 3 h is not due to the interference of tunicamycin with the synthesis and secretion of invertase. An analysis of the inhibited cells showed that they all stopped growth in the G1 phase of the cell cycle (Arnold & Tanner 1982). It is therefore tempting to speculate that protein glycosylation is crucial for a cell cycle event during the G1 and before the S phase. This hypothesis will have to be carefully checked in the future.

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