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Dolichyl phosphate-mediated mannosyl transfer through liposomal membranes

(reversible dolichyl phosphate-mannose formation/transport of activated sugar)

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ABSTRACT **A partially purified (up to 1000-fold) mannosyl transferase that catalyzed the reversible reaction GDP-Man + Dol-*P* ⇌ Dol-*P*-Man + GDP was incorporated into liposomes consisting of soybean lecithin and dolichyl phosphate (Dol-*P*). The enzyme transferred the mannosyl moiety from external GDP-Man to liposome-associated Dol-*P*. However, when the liposomes were preloaded with GDP, mannosyl residues were also transferred to the inside, giving rise to internal GDP-Man by the reverse reaction. This transfer of an activated sugar through a membrane required the presence of Dol-*P* and the enzyme in the liposome. Mannosyl residues were not transferred to the inside when the liposomes were preloaded with ADP or GMP. Amphomycin completely inhibited the formation of Dol-*P*-Man as well as the transfer of mannose into the liposomes. The results are taken as evidence for the often postulated role of dolichols in sugar translocation through membranes. The data are discussed in relation to glycoprotein synthesis at the endoplasmic reticulum.**

Dolichol-linked sugars are involved in protein glycosylation in all eukaryotic organisms. *N*-Glycosylation proceeds via the dolichol pathway (1–3) whereby the dolichyl pyrophosphate-bound oligosaccharide $(GlcNAc)_2Man_9Glc_3$ is transferred to the asparagine amide of animal (1–3), fungal (4–7), and plant proteins (8, 9). The involvement of dolichyl phosphate (Dol-*P*)-activated sugars in *O*-glycosylation has only been reported for fungi (10–13).

Although the dolichols in all these reactions were assumed to be associated with the transport of sugars through membranes (thus, the designation "carrier lipid"), such a function has never been demonstrated. On the other hand, neither free polyprenyl phosphates (14) nor Dol-*PP*-$(GlcNAc)_2$ (15) move at a measurable rate across the lipid bilayer of artificial or natural membranes. Because the sugar-nucleotides for glycoprotein synthesis are supplied from the cytoplasm whereas the glycosylated proteins are segregated into the endoplasmic reticulum (ER) lumen (16), the sugars must certainly traverse the membrane. If dolichol-linked sugars *per se* cannot permeate membranes, transport of sugar might be mediated by the specific glycosyltransferase responsible for their formation.

To test this possibility, a solubilized and partially purified mannosyltransferase from yeast (17) that catalyzes the reversible reaction

$$\text{GDP-Man} + \text{Dol-}P \xrightleftharpoons{Mg^{2+} \text{ or } Mn^{2+}} \text{Dol-}P\text{-Man} + \text{GDP}$$

was incorporated into liposomes. The liposomes were prepared from soybean lecithin and Dol-*P*. When these liposomes were preloaded with GDP, the transfer of radioactive mannosyl residues from GDP-[^{14}C]Man outside to GDP inside could be demonstrated. In order for the reaction to occur, the incorporation of Dol-*P* and the mannosyltransferase into the liposomes and the presence of divalent ions within the lipsome were obligatory.

MATERIAL AND METHODS

Materials. GDP-[^{14}C]mannose (199 Ci/mol; 1 Ci = 3.7×10^{10} becquerels) was obtained from New England Nuclear; soybean lecithin (L-α-phosphatidylcholine, commercial grade) was from Sigma. Dolichol was isolated from yeast and chemically phosphorylated as described (18). All other chemicals were obtained commercially and were of highest purity available.

Enzyme Preparation. The growth of yeast (*Saccharomyces cerevisiae*, strain 66.24, Fleischmann Laboratories), the preparation of the membranes, and the solubilization of the mannosyltransferase were as described (17). The purification procedure up to the third column (i.e., the first column run on hydroxylapatite and the second one on DEAE-cellulose) followed exactly the published purification scheme (17). After the second column, sodium deoxycholate was added to the active fraction (peak 1) to give a final concentration of 0.1%. This fraction was then put once more through a DEAE-cellulose column; all the enzyme activity and about half the protein was eluted with 10 mM potassium phosphate, pH 6.8/0.5% Triton X-100. The active fraction was put onto a hydroxylapatite column (3.5 × 2.3 cm), washed with 40 ml of 10 mM potassium phosphate buffer (pH 6.8), and then eluted with 60 ml of a linear 10–150 mM potassium phosphate (pH 6.8) gradient containing 0.5% Triton X-100. One-milliliter fractions were collected and tested for protein and enzyme activity. The four fractions with the highest specific activity (fractions 40–43) were pooled; the specific activity of the mannosyltransferase obtained was 280-fold higher than that of intact membranes (Table 1) and the purification based on total cell protein was 1040-fold. On $NaDodSO_4$ gels, this protein fraction contained one major band with a molecular weight of 31,000 (about 40% of the protein) and a number of minor bands.

Enzyme Assay. With detergent-solubilized enzyme, the transfer of radioactivity from GDP-[^{14}C]Man to exogenous Dol-*P* was measured as described (17). The 70-μl reaction mixture contained 7 mM $MgCl_2$, 0.05 μCi of GDP-[^{14}C]Man, 5 mM Tris·HCl (pH 7.4), 5 μg of Dol-*P*, 0.35% Triton X-100, and 0.1–15 μg of protein.

Enzyme integrated into liposomes was assayed in a mixture of 25 μl of liposomes (215 μg of phospholipid) in 10 mM potassium phosphate, pH 6.8/100 mM KCl plus 45 μl of a solution containing 12.5 mM Tris·HCl (pH 7.4), 7 mM $MgCl_2$, and 0.05 μCi of GDP-[^{14}C]Man. The liposomes contained 1.12 μg of protein (enzyme after column 2) or 0.12 μg of protein (enzyme after column 4) and 3 μg of Dol-*P*.

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Abbreviations: Dol-*P*, dolichyl phosphate; ER, endoplasmic reticulum.

Table 1. Purification of GDP-Man-Dol-*P* mannosyltransferase

	Total protein, mg	Total activity, nmol/min	Specific activity, nmol/min/mg	Purification*
Membranes	390	400	1.0	1
Triton extract	129	397	3.1	3
First hydroxylapatite column	38	384	10.0	10
First DEAE-cellulose column	13.8	236	17.1	17
Second DEAE-cellulose column	8.2	195	23.7	24
Second hydroxylapatite column (gradient)	0.12	34.7	289.1	289

* In this experiment the membrane protein amounted to 27% of the whole cell protein. Because all transfer activity was membrane bound, the purification factor has to be multiplied by 3.7 to relate the values to total cell protein.

Preparation of Liposomes. In principal the procedure of Orlich and Hauska (19) was followed. Soybean lecithin was dissolved in chloroform/methanol, 2:1 (vol/vol), at 6 mg/150 μl. When Dol-*P* was to be incorporated, 150 μg of Dol-*P* was dissolved in addition. After drying, 150 μl of the corresponding enzyme fraction in 10 mM potassium phosphate, pH 6.8/0.5% Triton X-100 was added along with 7 mM $MgCl_2$ and, where indicated, 7 mM GDP, GMP, or ADP. Then the solution was sonicated for 4 min at 40 W at 0°C. The liposomes formed were separated on a Sepharose 4B column (1.2 × 17 cm). They appeared in about 700 μl as sharp peak in the void volume when eluted with 10 mM potassium phosphate, pH 6.8/100 mM KCl.

When liposomes containing Dol-*P* and the enzyme were prepared in the presence of [^{14}C]sucrose, 2% of the radioactivity was associated with the liposomal peak corresponding to 0.5 μl of internal volume per mg of phospholipid. These [^{14}C]sucrose-containing liposomes were pooled after the column step, kept at room temperature for 3 hr, and then run again through the Sepharose 4B column; they still contained about 90% of their original radioactivity (Fig. 1). When the same experiment was carried out with GDP-[^{14}C]Man of low specific activity (1.6 Ci/mol) a condition in which only 40% of the radioactivity was converted to Dol-*P*-Man and 60% was left as internal GDP-Man, 100% of the radioactivity stayed with the liposomes after 3 hr—i.e., the liposomes remain completely tight for GDP-Man.

RESULTS

Incorporation of Dol-*P* and of Mannosyltransferase into Liposomes. Both Dol-*P* and mannosyltransferase could be incorporated into lecithin vesicles prepared according to the method of Orlich and Hauska (19). Sedimentation of freshly prepared, unfractionated vesicles at 150,000 × *g* revealed that 54% of the total Dol-*P* (150 μg) was found in the pellet, representing 5% of the total sample volume. The amounts of Dol-*P* in the supernatant solution and pellet were determined by the ability of these two fractions to function as the sole source of acceptor in the transferase assay. Similarly, when 60 μg of protein from the fraction obtained after the second column in the purification (Table 1) was used, 34 μg of the protein (56%) and 60–70% of the mannosyltransferase activity were associated with the liposomes. The protein/phospholipid ratio of the vesicles was 5.7 μg/mg, and the Dol-*P*/phospholipid ratio was 13.5 μg/mg.

Mannosyl Transfer Activity of the Liposomes. Liposomes prepared in the presence of mannosyltransferase and Dol-*P* were separated from the residual volume on a Sepharose 4B column. When these liposomes were incubated with GDP-[^{14}C]Man and subsequently separated again from unreacted sugar-nucleotide on a column, the liposomes retained radioactivity and all the radioactivity was present in Dol-*P*-Man (Fig. 2). Control liposomes prepared in the absence of Dol-*P* did not incorporate radioactivity. The incorporation of mannosyl residues was dependent on time and on the amount of liposomes incubated (Fig. 3).

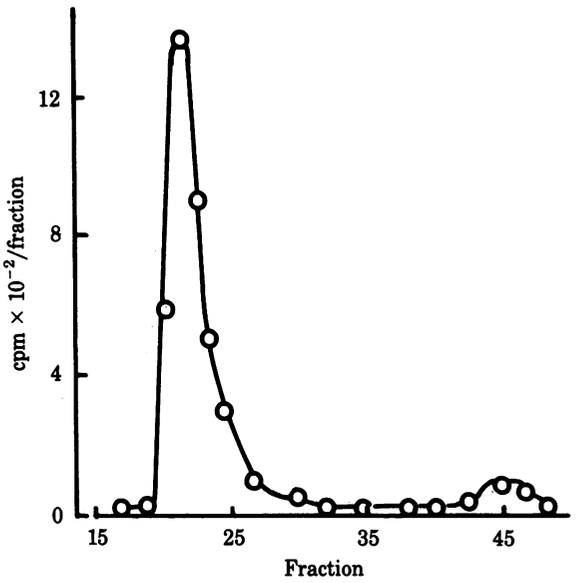

FIG. 1. Separation of liposomes loaded with [^{14}C]-sucrose on a Sepharose 4B column. Liposomes were prepared from soybean lecithin, Dol-*P* phosphate, and mannosyltransferase in Triton X-100; 0.1 μCi of [^{14}C]sucrose (394 Ci/mol) was included in the preparation medium. The liposomes appearing in the void volume after separation on Sepharose 4B were pooled, kept at room temperature for 3 hr, and then rerun on Sepharose 4B to determine the amount of [^{14}C]sucrose that had leaked out (radioactivity past fraction 40).

Transfer of Mannosyl Residues to Internal GDP. When liposomes containing Dol-*P* and mannosyltransferase were preloaded with GDP and then incubated with GDP-[^{14}C]Man, radioactivity was found associated with the liposomes separated by the column, but now about 5% of this radioactivity was found in a water-soluble compound (Fig. 4). This compound was iden-

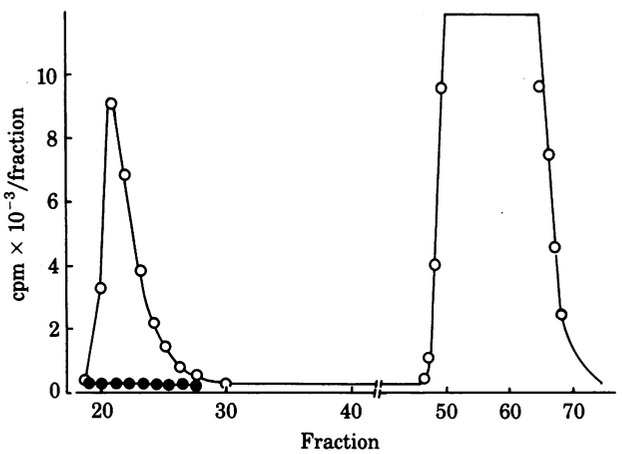

FIG. 2. Activity of GDP-Man-Dol-*P* mannosyltransferase integrated into liposomes. Liposomes were prepared in the presence (○) and absence (●) of Dol-*P*. Incubation of these liposomes in the standard assay scaled up 6-fold for 40 min with GDP-[^{14}C]Man and subsequent chromatography of the incubation medium on Sepharose 4B yielded radioactivity in the void volume (Dol-*P*-[^{14}C]Man) only with liposomes containing Dol-*P*.

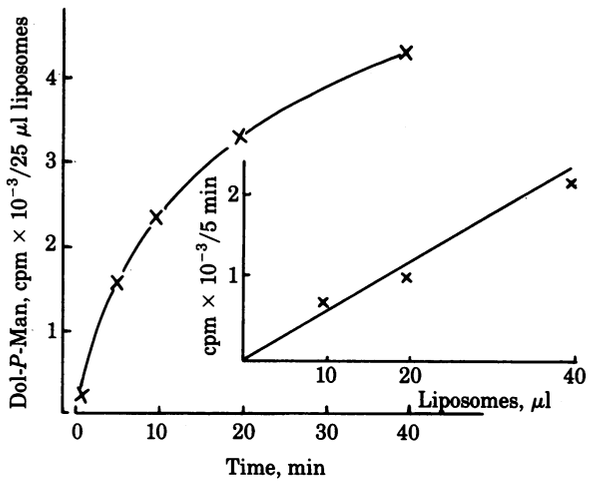

FIG. 3. Time and concentration dependence of liposome-integrated mannosyltransferase.

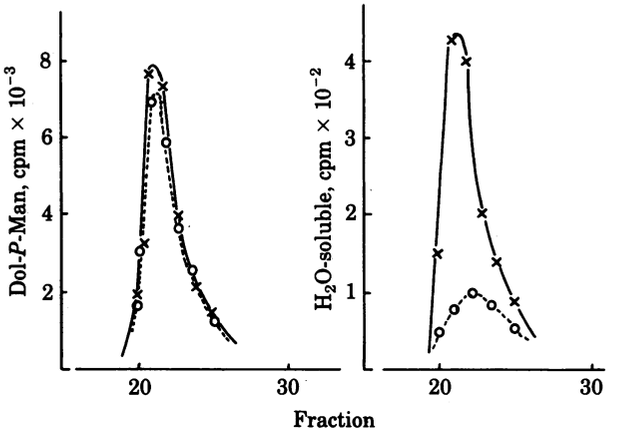

FIG. 4. Formation of Dol-*P*-[^{14}C]Man and internal GDP-[^{14}C]Man with liposomes. The experimental conditions were as in Fig. 2, except that one batch of liposomes was preloaded with nonradioactive GDP (x). ○, ● Control liposomes. (*Left*) Radioactivity in the chloroform/methanol phase (Dol-*P*-[^{14}C]Man) in the various void volume fractions of the Sepharose column. (*Right*) Radioactivity in the water-soluble fraction of the liposomes.

tified as GDP-Man by paper chromatography (Fig. 5).

Radioactivity >1.8% of that in Dol-*P*-Man was observed in GDP-Man only when the liposomes were preloaded with GDP (Table 2). Optimal conditions included the preloading of Mg^{2+} into the liposomes. Mannosyl residues apparently were transferred from external GDP-[^{14}C]Man to internal GDP via the formation of Dol-*P*-Man. Because the mannosyltransferase requires divalent metal ions (11, 17), Mg^{2+} within the liposomes stimulated the transfer of mannose from Dol-*P*-Man to internal GDP.

That Dol-*P*-Man is an obligatory intermediate in the formation of internal GDP-Man is seen from the data of Table 3. Liposomes prepared in the absence of Dol-*P* produced neither radioactive Dol-*P*-Man nor intravesicular water-soluble radioactivity when incubated with GDP-[^{14}C]Man. The same was true in complementary experiments in which only the mannosyltransferase was missing. In the complete system, however, neither Dol-*P*-Man nor internal GDP-Man was formed in the presence of amphomycin, an inhibitor of mannose transfer (20).

Because the reactions described so far were carried out with a mannosyltransferase purified approximately 80-fold with respect to total protein (fraction after second column of Table 1), the ability of a more highly purified fraction of the mannosyltransferase to catalyze the above reactions was ascertained. The fraction with the highest specific activity from the gradient (after fourth column, Table 1) was incorporated into liposomes. The purification factor of the transferase in this fraction was 1040-fold with respect to total cell protein, and on a $NaDodSO_4$ gel this fraction consisted of one major protein (approximately 40%) and a number of minor bands. The major protein band was the only one for which a good correlation existed between the amount of protein and the enzyme activity in the various gradient fractions from the column. Therefore, this protein with a molecular weight of 31,000 most likely is the mannosyltrans-

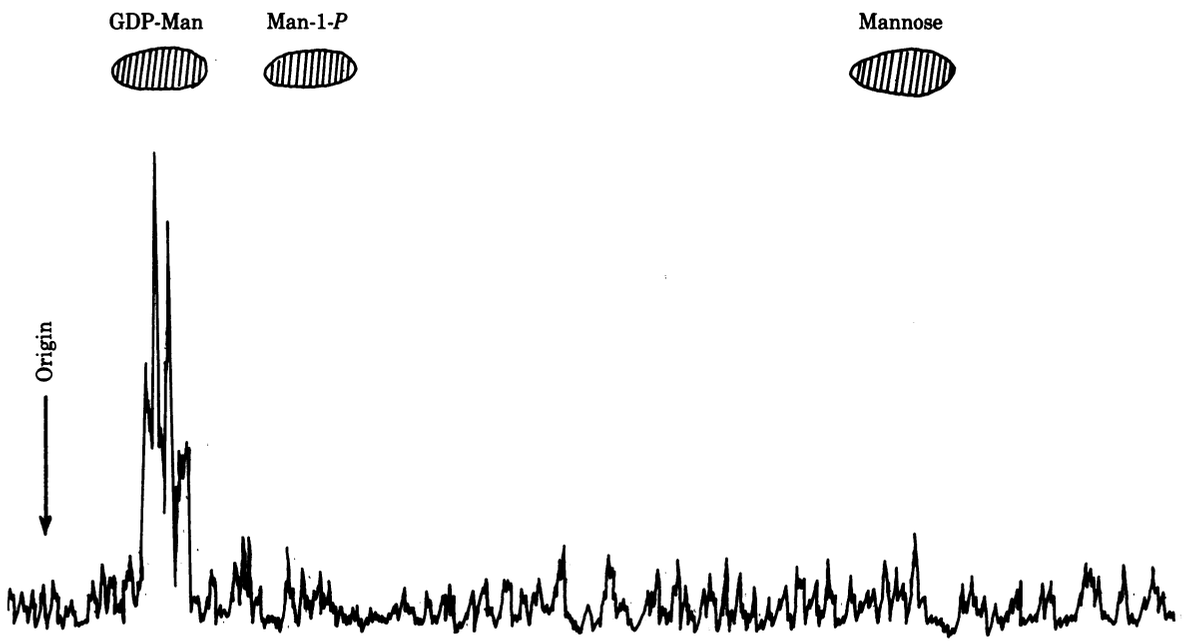

FIG. 5. Scan of a paper chromatogram developed in ethyl acetate/butanol/acetic acid/water, 3:4:2.5:4 (vol/vol), to identify the water-soluble product associated with liposomes that were preloaded with GDP (see Fig. 4). Dol-*P*-Man runs with the front (not on the scan); there was no radioactivity.

Table 2. Transfer of [^{14}C]mannose from external GDP[^{14}C]Man to liposomal Dol-*P* and to the water-soluble fraction associated with liposomes

Liposomes preloaded with	Radioactivity: Dol-*P*-Man, cpm	Water-soluble fraction: cpm	Water-soluble fraction: %*
Nothing (control)	16,578	247	1.5
GDP	24,128	1311	5.4
GMP	28,880	484	1.6
ADP	20,979	396	1.8
GDP (no Mg^{2+})	16,340	449	2.7

Liposomes containing Dol-*P* and the mannosyltransferase after the first DEAE-cellulose column were prepared. The incubations were carried out with 6 times the amounts (150 μl liposomes) of the standard assay for 140 min at room temperature. The liposomes were preloaded with the corresponding nucleoside mono- and diphosphates at 7 mM. At the end of the incubation time the liposomes were separated from the residual incubation medium on Sepharose 4B. The liposome peak was extracted with chloroform/methanol, 3:2, and the radioactivity in the water and the chloroform phase was determined (see also Fig. 4).

* The various incubations had to be carried out with liposomes prepared at different days; because it turned out that the amount of Dol-*P*-[^{14}C]Man formed is a good measure of the amount of liposomes recovered from the Sepharose column and actually incubated in each experiment, the percentage values probably are most valid for direct comparison.

ferase. When this protein fraction (1/10th the amount of that used in previous experiments) was incorporated into liposomes, the mannosylation of integrated Dol-*P* and the formation of internal GDP-Man was also observed (Table 3).

DISCUSSION

Although the results reported do not definitely exclude a GDP/GDP-Man exchange, such a reaction seems highly unlikely because the obligatory role of Dol-*P* in the membrane would be puzzling (Table 3). The rate of mannosyl transfer to GDP in the inside of liposomes is fairly slow when compared to the rate of Dol-*P*-Man formation (<5%). However, the slow rate is most likely not determined by the translocation step but rather by the back reaction Dol-*P*-Man + GDP → GDP-Man + Dol-*P*. It was observed that even low amounts of Triton X-100 strongly inhibit this back reaction. In the presence of 0.05% Triton, an amount likely to be present within the liposomes (checked with [^{3}H]Triton X-100), the reverse reaction in the presence of 1 mM GDP was strongly inhibited. This is most likely the reason for the requirement of the high GDP concentration (7 mM) inside the liposomes; at 1 mM GDP, the mannosyl transfer to the inside amounted to about 2.5% of the radioactivity found in Dol-*P*-Man and thus was only 60% above the control value without GDP preloading.

Table 3. Dependence of transfer of mannose from external GDP-[^{14}C]Man to internal GDP on Dol-*P* and on mannosyltransferase

	Radioactivity: Dol-*P*-Man, cpm	GDP-Man inside: cpm	GDP-Man inside: %
Complete liposomes*	28,911	1491	5.2
No Dol-*P*	222	36	
No enzyme	24	20	
Complete liposomes† plus amphomycin (75 μg)	11	109	
Complete liposomes with enzyme after fourth column†	14,318	917	6.4

* The liposomes (150 μl) containing 6.7 μg of protein (mannosyltransferase after second column) were incubated for 140 min at room temperature (other conditions: 6 times the standard assay). Further treatment as in Table 2.

† The liposomes (150 μl) containing 0.7 μg of protein (mannosyltransferase after fourth column), other conditions as above.

The mechanism of monosaccharide and oligosaccharide transfer across intracellular membranes like those of the ER or the Golgi apparatus is not understood. The possibility that these membranes might be freely permeable to sugar-nucleotides has repeatedly been discussed (21); however, this seems unlikely because they exclude even hexose-6-phosphates (22). The sugars almost certainly have to cross the membranes in an activated form, which could be achieved either by a transport system exchanging XDP-sugar and XDP or by a reaction sequence analogous to the one described here. The cytoplasmic glycosylation of a Dol-*P* molecule located within the membrane and the subsequent transfer of the sugar moiety to a free nucleotide diphosphate on the *trans* side of this membrane exactly fulfills the requirement for transferring glycosyl units and at the same time retaining their high group transfer potential.

On the other hand, the glycosylated Dol-*P* could also donate the sugar moiety to a growing Dol-*PP*-oligosaccharide chain which might be assembled in the ER lumen. The observation by Chapman *et al.* (23) that the formation of Dol-*PP*-$(GlcNAc)_2$ Man_5 proceeds in a mutated cell line that is able to synthesize Dol-*P*-Man only at a very low rate could indicate that the heptasaccharide is assembled at the cytoplasmic side and transferred as such to the ER luminal side—for example, by the oligosaccharyltransferase. This would agree with the observation by Snider *et al.* (24) that the early steps of Dol-*PP*-oligosaccharide formation proceed at the cytoplasmic side; however, because the enzymes catalyzing the final steps were also reported to be destroyed by Pronase attack from the cytoplasmic side (24), the exact topological arrangement of the enzymatic components of the system may be more complicated.

Whereas in mammalian cells Dol-*P*-Man solely serves the function of supplying some of the mannosyl residues in the synthesis of Dol-*PP*-$(GlcNAc)_2Man_9Glc_3$ (1–3), in yeast cells, Dol-*P*-Man additionally is involved in the synthesis of *O*-glycosidically linked mannose tetrasaccharides (10, 11). The corresponding reactions are localized in the ER (25, 26), and the mannoproteins formed are secreted into the cell wall space (27). *In vitro*, Dol-*P*-Man has been shown to be the donor only for the first mannosyl residue linked to the serine/threonine hydroxyl, whereas the additional three mannosyl residues are directly transferred from GDP-Man (10–12). This *O*-glycosylation pathway has always been difficult to understand, especially in regard to the unclarified yet probable role of Dol-*P* as transport vehicles through membranes. From the results reported here it seems possible, however, that *in vivo* all mannosyl residues cross the ER membrane via Dol-*P*-Man but, whereas the enzyme transferring the first mannosyl residue to the protein requires Dol-*P*-Man directly as donor, the other mannosyl residues must be retransformed, by use of GDP in the ER lumen, into GDP-Man which is then used by the following mannosyltransferases. It will be important in the future to find out whether this Dol-*P*-dependent transport step indeed occurs *in vivo*.

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