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Localization of Dolichyl Phosphate- and Pyrophosphate-Dependent Glycosyl Transfer Reactions in *Saccharomyces cerevisiae*

M. MARRIOTT AND W. TANNER*

Fachbereich Biologie und Vorklinische Medizin der Universität Regensburg, 84 Regensburg, West Germany

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Membranes from *Saccharomyces cerevisiae* protoplasts were fractionated on a continuous sucrose gradient. Six bands were obtained, which contained altogether about 15% of the total cell protein. From their densities, their behavior in the presence and absence of Mg^{2+} ions, and the distribution of marker enzymes, it was possible to identify fractions enriched in rough and smooth endoplasmic reticulum and in mitochondria. All glycosyl transfer reactions investigated where dolichyl phosphates served as glycosyl acceptors or where dolichyl phosphate- and pyrophosphate-activated sugars served as glycosyl donors showed the highest specific activity and up to 75% of the total activity in the endoplasmic reticulum. This was the case for the reactions involved in the formation of *O*-glycosidic as well as *N*-glycosidic linkages in yeast glycoprotein biosynthesis. Membrane fractions enriched in plasmalemma contained less than 3% of the corresponding activities.

Yeast cells are known to contain a number of extra- and intracellular mannan proteins (2) with carbohydrate moieties attached via *O*-glycosidic and *N*-glycosidic linkages (21, 22). Several reports from this and other laboratories have demonstrated the role of lipid (dolichol)-linked sugars as intermediates in the biosynthesis of these glycoproteins (1, 13, 28). Although all of the enzymes involved in the various reactions appear to be membrane bound (12), little information is available on their subcellular locations.

Previous reports on the fractionation of yeast cells have tended to concentrate on the isolation of particular organelles, e.g. plasma membrane (7), vacuoles (5, 19), or mitochondria (27, 29). To study the intracellular location of enzymes, it is necessary to fractionate total homogenates so that estimates of the recovery of enzyme activity can be made (3). Numinen et al. (23, 24) have done this by using a combination of sucrose and urograffin density gradients and rate zonal centrifugation to investigate the location of possible marker enzymes in *Saccharomyces cerevisiae*. For animal tissues it has been shown that dolichol-mediated mannosyl transfer is localized in a rough endoplasmic reticulum fraction (6). Also, for yeast some information concerning the reactions mentioned above has been obtained by using urograffin gradients (10); unfortunately these gradients have the disadvantage that divalent cations cannot be included in the separation procedure. Hence, we attempted to study the subcellular location of the enzymes involved in mannan protein biosynthesis by using a sucrose density gradient technique. To minimize possible artifacts due to centrifugation and resuspension, the total homogenate was fractionated. This procedure was facilitated by using protoplasts as starting material.

MATERIALS AND METHODS

Production and breakage of protoplasts. *S. cerevisiae* (Fleischmann) was grown as described previously (12) to an absorbance at 270 nm of 2.0. Protoplasts were produced from 1 liter of cells by using crude β-glucuronidase from *Helix pomatia* (Sigma, Munich, West Germany). Before digestion, cells were pretreated with 0.1 M NH_4HCO_3 containing 50 mM mercaptoethanol for 10 min at 30°C, washed, and resuspended in 0.1 M phosphate buffer, pH 6.5, containing 1 M (final concentration) sorbitol. β-Glucuronidase was added at approximately 450 Fishman units per mg (dry weight) of yeast.

Protoplast release was monitored microscopically and after 30 min at 30°C; 80 to 90% of the cells were osmotically sensitive. These protoplasts were washed and resuspended in a minimal volume of 0.1 M Tris-hydrochloride (pH 7.2) containing 10 mM $MgCl_2$ and 0.6 M sorbitol. Breakage was achieved by blending in a Vortex mixer for 1 min with glass beads (diameter, 0.17 to 0.18 mm), using a glass rod to agitate the thick suspension. Unbroken cells and cell wall fragments were removed by centrifugation for 10 min at 1,000 × *g*.

Fractionation procedure. Before fractionation

the protoplast homogenate was treated for 30 min at 30°C in the presence of DNase and the protease inhibitor phenylmethylsulfonyl fluoride as described by Duran et al. (7), which improved subsequent separations. The total homogenate was then diluted and loaded onto continous sucrose gradients (20 to 55% [wt/wt] sucrose in 0.1 M Tris-hydrochloride, pH 7.2, containing 10 mM $MgCl_2$; total volume, 34 ml). The gradients were centrifuged for 3.5 h in an SW27 rotor at 100,000 × *g* (rotor temperature, 1°C). For the majority of the experiments the fractionation was improved by collecting the various bands, pelleting the membranes, and rerunning them on similar sucrose gradients (volume, 17 ml). The second ultracentrifugation was for 13 h under the same conditions.

Tests of marker enzyme activity. Mg^{2+}-adenosine triphosphatase (Mg^{2+}-ATPase; EC 3.6.1.4) was assayed by the method of Emmelot et al. (8). Inorganic phosphate released during the assay was measured by the method of Chen et al. (4). The effect of oligomycin at 5 μg/ml was estimated simultaneously.

Cytochrome *c* oxidase (EC 1.9.3.1) was assayed by measuring the reoxidation of cytochome *c* at 550 nm (31). Reduction of cytochrome *c* was performed as follows. A 11.4-mg amount cytochrome *c* was dissolved in 1 ml of buffer and 60 μl of sodium dithionite (17.4 mg/ml) was added. The mixture was cooled to 0°C, and air was bubbled through until the $E_{550/560}$ ratio was aproximately 10. NADPH:cytochome *c* oxidoreductase (EC 1.6.2.3) was assayed by measuring the reduction of cytochrome *c* at 550 mm (18).

For all marker enzyme assays, time courses and appropriate controls were carried out to determine enzyme activity.

GDP-mannose-mannosyl transfer reactions. The mannosylation of endogenous lipid and protein acceptors was measured as described elsewhere (12). Incorporation into dolichyl monophosphate mannose (DolP-Man) was measured by thin-layer chromatography of the lipid fraction in solvent system A (see below).

The transfer of mannose to endogenous polymer was further investigated by using β-elimination (0.1 M NaOH, 24 h at 20°C) of the radioactive product. Oligomannose residues released by β-elimination were chromatographed by using Whatman no. 1 paper in solvent system B (see below). Fractions M_1 through M_4 were located as described previously (28) and assayed for radioactivity.

Endoglucosidase H (Miles Laboratories) was used to determine the transfer of mannose to non-β-eliminable (inner core) positions. This enzyme hydrolyzes between the two *N*-acetylglucosamine (GlcNAc) residues (32). Radioactively labeled polymer was resuspended in 60 μl of 0.1 M sodium citrate buffer (pH 5.0), and 30 μl of endoglucosidase H (0.2 U/ml) was added. After 20 h at 20°C, the reaction mixture was diluted with 0.05 M acetic acid and applied to a Sephadex G-50 column with dextran blue, stachyose, and mannose as indicators of molecular weight. The column was eluted with 0.05 M acetic acid, and 1-ml fractions were collected and assayed for radioactivity.

DolP-Man-mannosyl transfer reactions. The transfer of [^{14}C]mannose from DolP-[^{14}C]Man was assayed by the method of Sharma et al. (28) in the presence of 1% (final concentration) Triton X-100. Since under these conditions 90% of the mannose is transferred to serine or threonine (1, 28), no further analysis of the product was carried out.

Effect of Mg^{2+} ions on membrane fractionation. The effect of Mg^{2+} ions on the distribution of protein and enzyme activity within the various membrane fractions was measured by using 0.1 M Tris-hydrochloride (pH 7.2) containing 1 mM EDTA as the buffer during the fractionation procedure.

UDP-GlcNAc-*N*-acetylglucosaminyl transfer reactions. The transfer of [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc was measured as described previously (14, 15). The incorporation into lipid was analyzed further by thin-layer chromatography in solvent system A (see below). In some experiments exogenous dolichyl monophosphate was added.

Analytical procedures. Protein was assayed by the method of Lowry et al. (17). Nucleic acid was extracted by using 0.5 M perchloric acid (three times for 30 min at 70°C) and assayed by the method of Mejbaum (20).

The following solvent systems were used for chromatography. Solvent A was $CHCl_3$-methanol-water (65:25:4, vol/vol), and solvent B was butan-1-ol-ethyl acetate-acetic acid-water (40:30:25:40, vol/vol).

Radioactive materials. GDP-[^{14}C]mannose and UDP-[^{14}C]GlcNAc were obtained from The Radiochemical Centre, Amersham, England. Yeast DolP-[^{14}C]Man was prepared as described elsewhere (28).

RESULTS

Fractionation and identification of membranes. Figure 1 shows the distribution of material along the sucrose gradient obtained in the presence of Mg^{2+} ions. Six bands can be seen, and their mean buoyant densities are given in Table 1. This table also indicates the distribution of protein in the six bands, in both the presence and the absence of Mg^{2+} ions. Approximately 10% (7 to 15%) of total homogenate protein was found in these bands; the remainder (soluble proteins) could be recovered from the top of the gradient. In early experiments (Tables 2 and 3) a pellet was also observed; however, treatment of the broken protoplasts with DNase almost completely eliminated this and improved overall separation (7).

Except for band 1, which did not enter the gradient and probably represented denatured proteins, all bands showed a shift in buoyant density in response to Mg^{2+} ions. This is analogous to studies in higher plants (11, 16).

Associated with this shift was a redistribution of protein between bands 3 and 4, which contained approximately 50% of the membrane protein. In the presence of Mg^{2+} ions (which are known to stabilize attachment of ribosomes to rough endoplasmic reticulum [16]), the majority of protein was in band 4, whereas the reverse was true in the absence of Mg^{2+}. Nucleic acid

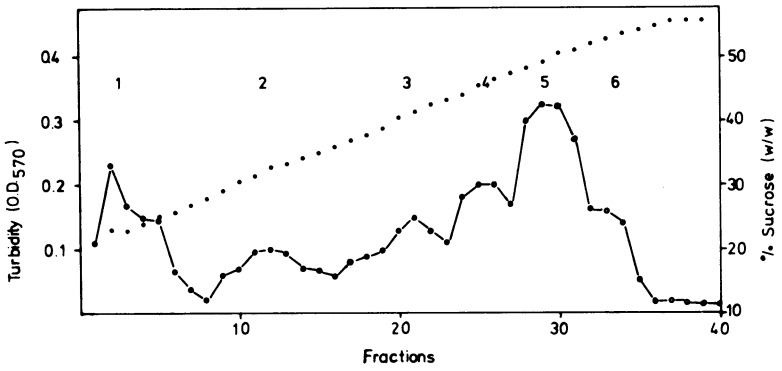

FIG. 1. *Positions of the various membrane bands on the continuous sucrose gradient. Drops were collected along the gradient, and turbidity at 570 nm and sucrose content were determined. One fraction was equivalent to 40 drops. O.D.$_{570}$, Optical density at 570 nm.*

TABLE 1. *Protein and RNA contents of the different membrane bands*[a]

Fraction	With Mg^{2+}			Without Mg^{2+}		
	Protein distribution (%)	Amt (μg) of RNA/mg of protein	Buoyant density (g/ml)	Protein distribution (%)	Amt (μg) of RNA/mg of protein	Buoyant density (g/ml)
Band 1	5	17	1.097	7	20	1.096
Band 2	10	12	1.130	11	15	1.122
Band 3	15	18	1.171	45	7	1.135
Band 4	34	173	1.196	12	12	1.174
Band 5	32	105	1.215	19	100	1.191
Band 6	4	6	1.229	6	8	1.216
Homogenate		115			102	

[a] The membranes from protoplasts were obtained as described in the text; the bands from the first sucrose gradient were rerun individually on a second gradient. Membranes were separated in the presence or absence of Mg^{2+}.

TABLE 2. *Mg^{2+}-ATPase activity in the various membrane bands isolated in the presence of Mg^{2+}*

Fraction	Sp act (μmol/min per mg)	Total activity (μmol/min)[a]	Oligomycin inhibition (%)
Band 1	0.36 ± 0.18[b]	0.17	0
Band 2	0.33 ± 0.07	0.10	0
Band 3	0.56 ± 0.16	0.15	11 ± 8
Band 4	0.61 ± 0.16	0.22	5 ± 1
Band 5	1.11 ± 0.35	0.30	44 ± 10
Band 6	0.15 ± 0.00	0.08	0
Homogenate	0.23 ± 0.03	ND[c]	ND

[a] Total activity per band corresponds to 1 liter of cells with an optical density at 570 nm of 2.0.
[b] Mean ± standard deviation (three experiments).
[c] ND, Not determined.

TABLE 3. *Cytochrome c oxidase activity in the various membrane bands isolated in the presence of Mg^{2+}*

Fraction	Sp act (μmol/min per mg)	Total activity (μmol/min)[a]	% Distribution
Band 1	3.9 ± 2.4[b]	0.4	2
Band 2	10.8 ± 2.9	1.2	5
Band 3	28.0 ± 5.1	3.6	14
Band 4	17.5 ± 4.3	4.2	17
Band 5	52.6 ± 9.5	15.3	62
Band 6	0	0	0
Homogenate	4.1 ± 1.8	133.0	

[a] Typical experiment. Recovery of protein was 10%, and recovery of activity was 20%.
[b] Mean ± standard deviation (three experiments).

analysis showed that band 4 contained appreciable amounts of nucleic acid (likely to be RNA since DNase treatment was incorporated in the fractionation procedure) in the presence of Mg^{2+}; band 5 contained nucleic acid in the presence and absence of Mg^{2+}. These results suggest that band 3 and 4 may be enriched in smooth and rough endoplasmic reticulum, whereas band 5 may contain mitochondria.

Further evidence for this comes from studies

with possible marker enzymes. Mg^{2+}-ATPase activity is shown in Table 2. Consistently high activity was found in band 5, which was sensitive to oligomycin. This is characteristic of mitochondrial ATPase. Similarly, cytochrome *c* oxidase, another mitochondrial enzyme, had the highest specific activity in band 5, which also contained 60% of the membrane-associated activity (Table 3). NADPH:cytochrome *c* oxidoreductase has been reported as a microsomal enzyme in yeasts and higher plants (11, 30). In the presence of Mg^{2+}, the highest specific and total activities of this enzyme were found in band 4 (Table 4). However, significant amounts were also present in bands 3 and 5. In the absence of Mg^{2+} ions, the maximum specific activity was found in band 3, which now had more total activity than band 4. The redistribution in response to Mg^{2+} ions tends to confirm the evidence in Table 1, namely that bands 3 and 4 are enriched in smooth and rough endoplasmic reticulum, respectively.

GDP-mannose-mannosyl transfer. The transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to endogenous dolichyl monophosphate is shown in Table 5. High specific activity was found in bands 3 and 4 when membranes were prepared in the presence of Mg^{2+} ions and in band 3 in the absence of Mg^{2+}. Since a rate of incorporation could not be measured under these conditions, due to the rapid saturation of the endogenous dolichol, the results represent a measure of dolichol content rather than enzyme activity. Table 6 shows the transfer of [^{14}C]mannose from GDP-[^{14}C]mannose to endogenous polymer (mannan protein). When protoplasts were fractionated in the presence of Mg^{2+} ions, the highest specific and total activities were found in band 4. However, a shift of activity to band 3 was observed when Mg^{2+} was omitted from the separation procedure.

The incorporation of mannose was further investigated by using β-elimination, which releases only those residues attached to the protein via an *O*-glycosidic linkage (2, 21). Table 7 shows that, after fractionation in the presence of Mg^{2+}, band 4 had the highest specific and total activities for the transfer to fractions M_1 (first *O*-linked mannose) and M_2. If protoplasts were fractionated in the absence of Mg^{2+}, then maximum specific and total activities for the transfer into these two positions were in band 3 (data not shown). Less clear-cut results were obtained for the mannose residues more distant from the

TABLE 4. *NADPH:cytochrome c reductase activity in the various membrane bands*[a]

Fraction	With Mg^{2+}			Without Mg^{2+}		
	Sp act (nmol/min per mg)	Total activity (nmol/min)	% Distribution	Sp act (nmol/min per mg)	Total activity (nmol/min)	% Distribution
Band 1	0	0	0	1.02	0.46	8
Band 2	1.82	1.25	15	1.75	0.66	12
Band 3	2.52	2.12	25	4.51	1.96	35
Band 4	4.02	3.26	37	3.27	0.78	14
Band 5	2.42	1.97	23	3.48	1.73	31
Band 6	0	0	0	0	0	0
Homogenate	0.77	30.1		0.98	27.9	

[a] In the presence of Mg^{2+} the recovery of protein was 10% and the recovery of activity was 35%. In the absence of Mg^{2+} these values were 7 and 20%, respectively.

TABLE 5. *Distribution of mannosyl transfer activity from GDP-mannose to endogenous dolichyl phosphate in the various membrane bands*[a]

Fraction	With Mg^{2+}			Without Mg^{2+}		
	Sp act (cpm [$\times 10^3$]/15 min per mg)	Total activity (cpm [$\times 10^3$]/15 min)	% Distribution	Sp act (cpm [$\times 10^3$]/15 min per mg)	Total activity (cpm [$\times 10^3$]/15 min)	% Distribution
Band 1	8.4	1.83	2	4.8	1.2	3
Band 2	5.9	1.53	1	5.5	1.8	4
Band 3	21.0	6.68	16	23.5	27.4	67
Band 4	25.2	19.70	48	11.3	3.1	8
Band 5	12.9	9.39	23	10.4	5.8	14
Band 6	9.5	3.65	9	13.5	1.9	4
Homogenate	1.6	65.20		1.5	59.4	

[a] In the presence of Mg^{2+} the recovery of protein was 12% and the recovery of activity was 62%. In the absence of Mg^{2+} these values were 7 and 79%, respectively.

TABLE 6. *Mannosyl transfer activity from GDP-mannose to endogenous protein acceptor in the various membrane bands*[a]

Fraction	With Mg^{2+}			Without Mg^{2+}		
	Sp act (cpm [$\times 10^3$]/min per mg)	Total activity (cpm [$\times 10^3$]/mg)	% Distribution	Sp act (cpm [$\times 10^3$]/min per mg)	Total activity (cpm [$\times 10^3$]/mg)	% Distribution
Band 1	6.5	2.93	3	8.6	2.27	7
Band 2	7.4	9.55	9	8.6	2.75	8
Band 3	11.9	19.75	18	18.7	21.84	64
Band 4	20.0	53.0	48	9.7	2.62	8
Band 5	10.7	23.97	22	5.6	3.15	9
Band 6	6.7	1.14	1	10.7	1.49	4
Homogenate	4.7	268.80		3.3	130.7	

[a] In the presence of Mg^{2+} the recovery of protein was 15% and the recovery of activity was 41%. In the absence of Mg^{2+} these values were 7 and 26%, respectively.

TABLE 7. *β-Elimination pattern of the ^{14}C-mannoproteins formed by the various membrane bands isolated in the presence of Mg^{2+}*[a]

Fraction	Sp act (cpm [$\times 10^3$]/min per mg of protein)[b]				
	M_1	M_2	M_3	M_4	Non-β-eliminable
Band 3	0.51 ± 0.13 (14)[c]	1.87 ± 0.15 (17)	0.31 ± 0.08 (16)	0.09 ± 0.01 (23)	0.81 ± 0.02 (16)
Band 4	1.37 ± 0.20 (68)	3.07 ± 0.30 (53)	0.46 ± 0.02 (49)	0.14 ± 0.00 (69)	1.48 ± 0.23 (55)
Band 5	0.24 ± 0.06 (9)	1.61 ± 0.50 (20)	0.34 ± 0.13 (24)	— (0)	0.54 ± 0.21 (14)
Homogenate	0.17 ± 0.01	0.35 ± 0.02	0.17 ± 0.04	0.15 ± 0.03	0.47 ± 0.18

[a] The recoveries of activity were 48, 70, 24, 6, and 28% for M_1, M_2, M_3, M_4, and non-β-eliminable, respectively.

[b] M_1, Mannose directly linked to serine-threonine; M_2, M_3, M_4, di-, tri-, and tetramannose oligosaccharides, respectively.

[c] Mean ± standard deviation (three experiments). Numbers in parenthesis are distributions of total activity. Bands 1, 2, and 6 showed very low activity; the percentages missing to give 100% were present in these three bands.

protein (M_3 and M_4). In this case the specific activities were only slightly higher in the endoplasmic reticulum bands, as compared, for example, to that of band 5 (Table 7). The recovery of enzyme activity was also low, particularly for incorporation into M_4, suggesting that these enzymes may not be so tightly membrane bound.

The transfer of mannose to inner core positions is shown in Fig. 2 and Table 8. A peak of radioactivity with a molecular weight greater than that of stachyose was observed on treatment with endoglucosidase H (Fig. 2). It is likely that this corresponds to an inner core fragment (10 to 12 mannose residues).

DolP-Man-mannosyl transfer. DolP-Man serves as an intermediate in the attachment of the first *O*-glycosidic-linked mannose residue (M_1) (28). The highest specific activity for the enzyme involved in this reaction was found in band 4 (Table 9). This band also contained approximately 50% of the total membrane-associated activity.

UDP-GlcNAc-*N*-acetylglucosaminyl transfer. A total of 85 to 90% of the activity for the transfer of [^{14}C]GlcNAc from UDP-[^{14}C]GlcNAc was found in bands 3, 4, and 5; hence, only these were studied in detail. Maximum specific activity for the transfer to endogenous lipid and polymer occurred in band 4, although bands 3 and 5 were also reasonably active (Table 10).

Transfer of [^{14}C]GlcNAc to dolichyl pyrophosphate intermediates is shown in Table 11. These reactions could only be measured in the presence of exogenous dolichyl monophosphate.

DISCUSSION

The fractionation procedure described here allows the separation of *S. cerevisiae* protoplast homogenates into six membranous bands. In terms of the biosynthesis of mannan protein and its lipid-linked intermediates, bands 3 through 5 (Fig. 1) are of most interest, since they contain over 70% of the membrane-associated activity. Bands 1 and 2 consist of very light membrane fragments and probably some denatured soluble protein. Rigorous studies to identify the origin of these bands have not been attempted in view of the low activity. However, it may be noted that, after only one centrifugation on a sucrose gradient, bands 1 and 2 are contaminated with

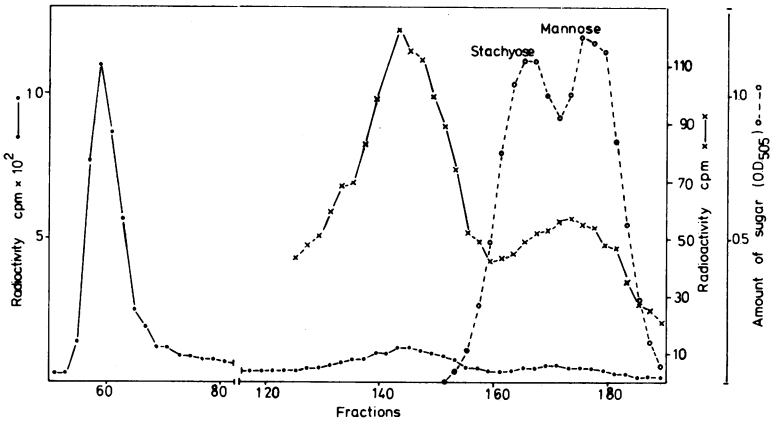

FIG. 2. *Separation of the inner core glyco portion of yeast mannoproteins on Sephadex G-50. ^{14}C-mannoproteins obtained from incubation of GDP-[^{14}C]mannose with band 4 membranes before β-elimination were treated with endoglucosaminidase H, and the products were separated on Sephadex G-50. A control sample not treated with the enzyme did not show a radioactive peak larger than that of stachyose, but contained the same amount of radioactivity in the stachyose-mannose region. Fractions of 0.5 ml were collected. Sugars were determined by the orcine-sulfuric acid method. O.D.$_{505}$, Optical density at 505 nm.*

TABLE 8. *Mannosyl transfer from GDP-mannose to the inner core of endogenous mannoproteins in the various membrane bands isolated in the presence of Mg^{2+}*[a]

Fraction[b]	Sp act (cpm [×10³]/30 min per mg)	% Distribution
Band 3	4.6	10
Band 4	12.6	56
Band 5	7.5	32
Band 6	2.9	2

[a] After β-elimination the residual protein-bound radioactivity was solubilized with endoglucosaminidase H and separated on a Sephadex G-50 column (see Fig. 2).

[b] The radioactivity in bands 1 and 2 was negligible.

TABLE 9. *Mannosyl transfer from DolP-[^{14}C]Man to endogenous protein in the various membrane bands isolated in the presence of Mg^{2+}*[a]

Fraction	Sp act (cpm [×10³]/min per mg)	% Distribution of total activity
Band 1	0.40 ± 0.05[b]	3 ± 1
Band 2	0.46 ± 0.10	6 ± 2
Band 3	1.37 ± 0.20	24 ± 6
Band 4	1.62 ± 0.07	52 ± 4
Band 5	0.74 ± 0.25	16 ± 4
Band 6	0.11 ± 0.01	1
Homogenate	0.18 ± 0.04	

[a] The recovery of protein was 15 ± 3%, and the recovery of activity was 73 ± 5%.

[b] Mean ± standard deviation of three experiments.

more dense material.

Band 6 has also not been identified with certainty. Comparison with the work of Fuhrman et al. (9) and Nurminen et al. (23, 24), in terms of buoyant density, suggests that this material may contain plasma membrane. The activity of band 6 and that of plasma membranes prepared by the method of Fuhrman et al. (9) yield similar results with regard to recovery of protein, enzyme activity, and β-elimination pattern (unpublished data). Attempts to label protoplasts by using ^{125}I iodination or [^{3}H]concanavalin A were unsuccessful, probably due to the lytic activity of the protoplasting enzyme. Although it appears that the plasma membrane is still capable of mannan protein biosynthesis, the majority of the enzyme activity is bound to intracellular membranes.

Bands 3, 4, and 5 have been identified as being enriched in smooth and rough endoplasmic reticulum and mitochondria, respectively; band 5 (buoyant densities, 1.215 and 1.191 in the presence and absence of Mg^{2+} ions, respectively) contains an oligomycin-sensitive Mg^{2+}-ATPase (Table 2), cytochrome *c* oxidase (Table 3), NADH:cytochrome *c* oxidoreductase (unpublished data) and to a certain extent NADPH: cytochrome *c* oxidoreductase (Table 4). All of these are characteristic mitochondrial enzymes.

NADPH:cytochrome *c* oxidoreductase has been reported as a marker enzyme for endoplasmic reticulum in animal and plant cells (11). The majority of this enzyme is located in band 4 when protoplasts are fractionated in the presence of Mg^{2+} ions, but in band 3 in the absence of Mg^{2+}. Associated with this shift in activity when Mg^{2+} ions are omitted from the fractionation procedure is a shift in protein between

TABLE 10. *Transfer of GlcNAc from UDP-[^{14}C]GlcNAc to endogenous dolichyl phosphate and protein in the various membrane bands isolated in the presence of Mg^{2+}*[a]

Fraction	Endogenous lipid[b]		Endogenous polymer[b]	
	Sp act (cpm/min per mg)	% Distribution	Sp act (cpm/min per mg)	% Distribution
Band 3	65.9 ± 11.7[c]	28.5 ± 3.0	83.6 ± 13.4	25.0 ± 1.0
Band 4	84.7 ± 14.6	47.5 ± 3.5	134.0 ± 27.6	51.5 ± 3.5
Band 5	63.9 ± 14.2	24.0 ± 0.0	93.4 ± 23.3	23.5 ± 2.5
Homogenate	11.9 ± 2.0		27.5 ± 5.5	

[a] For this experiment the membrane bands were not centrifuged a second time, since enzyme activities were unstable.

[b] For endogenous lipid the recovery of protein was 8.5 ± 0.6% and the recovery of activity was 52 ± 5%. For endogenous polymer the recovery of activity was 35 ± 3%.

[c] Mean ± standard deviation of three experiments.

TABLE 11. *Formation of dolichyl pyrophosphate-GlcNAc and dolichyl pyrophosphate-(GlcNAc)$_2$ from UDP-[^{14}C]GlcNAc in the various membrane bands*[a]

Fraction[b]	Dolichyl pyrophosphate-GlcNAc formation		Dolichyl pyrophosphate-(GlcNAc)$_2$ formation	
	Sp act (cpm/min per mg)	% Distribution	Sp act (cpm/min per mg)	% Distribution
Band 2	24.7	2	36.2	1
Band 3	86.4	18	188.0	20
Band 4	129.0	41	310.0	50
Band 5	98.5	39	141.0	28
Homogenate	19.2		32.9	

[a] Exogenous DolP was added as acceptor (5-μg incubation). Membrane bands were tested after the first gradient centrifugation (see also Table 10).

[b] In bands 1 and 6 no activity could be detected.

bands 3 and 4 and a loss of nucleic acid from band 4 (Table 1). As Fig. 1 shows, bands 3 through 6 run very close to each other, and a certain amount of cross-contamination is inevitable. Although bands 3 and 4 are enriched in smooth and rough endoplasmic reticulum, it cannot be assumed even after two ultracentrifugations that they are pure membrane fractions. Nuclear membranes, by the way, which have been reported to catalyze lipid-mediated glycosylations (25), might also contribute to bands 3 and 4. Nevertheless, some important conclusions can be drawn from the data.

Considering initially the biosynthesis of *O*-glycosidic mannose residues, the first mannose (fraction M_1) is attached via a lipid-linked intermediate (DolP-Man) (2, 28). The maximum specific and total activities for the biosynthesis of DolP-Man and M_1 occur in band 4 when protoplasts are fractionated in the presence of Mg^{2+} ions (Tables 5 and 7). If Mg^{2+} is not included in the fractionation procedure, maximum specific and total activities occur in band 3. The same result was obtained for the biosynthesis of M_2 (Table 7). Less clear-cut results were obtained for the biosynthesis of M_3 and M_4, and, especially for the latter, the recovery of enzyme activity was low, suggesting that the enzyme may not be so tightly membrane bound.

The remaining mannose residues are attached to the protein via a chitobiose unit, and the biosynthesis of this *N*-glycosidic linkage occurs via a lipid intermediate (14, 15, 26). The endoplasmic reticulum also appears to be the site of synthesis of this component (Tables 10 and 11) and its subsequent transfer to the protein (Table 10). Further mannosylation reactions involved in biosynthesis of the inner core (Table 8 and Fig. 2) also occur with maximum activity in the endoplasmic reticulum. The synthesis of the outer chain has not yet been studied in detail. Of obvious interest is the relative size of the non-β-eliminable moiety synthesized by the endoplasmic reticulum as compared with that synthesized by other membranes.

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