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# Lipid-Mediated Glycosylation of Endogenous Proteins in Isolated Plasma Membrane of *Saccharomyces cerevisiae*

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A highly purified plasma membrane fraction from *Saccharomyces cerevisiae* was obtained by centrifugation on discontinuous sucrose and Urografin gradients. This plasma membrane fraction was capable of glycosylating endogenous proteins. It is shown that glycolipids play an intermediate role in these glycosylation reactions; with uridine 5'-diphosphate-*N*-acetylglucosamine as sugar donor the intermediate lipids possessed stability towards alkali and chromatographic mobilities similar to polyprenyl diphosphate *N*-acetylglucosamine and polyprenyl diphosphate di-*N*-acetylchitobiose.

In eucaryotic cells glycosylation of proteins is assumed to take place mainly in the endoplasmic reticulum and the Golgi structure (13). In yeast, however, there are also indications for the involvement of the plasma membrane in the formation of certain glycoproteins, namely the extracellular mannoprotein enzymes acid phosphatase (EC 3.1.3.2) and invertase (EC 3.2.1.26). These enzymes are most probably located in the periplasmic space between the plasma membrane and the yeast cell wall. Linnemans et al. (9) have observed that in the maturing yeast cell acid phosphatase is formed in invaginations of the plasma membrane and deposited against the inside of the cell wall. Meyer and Matile (12) have suggested that glycosylation of invertase could occur in the plasma membrane; this suggestion is supported by the results of Holbein et al. (7). However, it is not clear at this moment whether this glycosylation includes the first steps of the process, namely the attachment of the first sugar residues of the side chain to the polypeptide via linkage to an asparagine-*N* ("initial" glycosylation), or whether it is only a "further" glycosylation of partly synthesized carbohydrate side chains.

Earlier work in our laboratory has shown that a particulate fraction from yeast contains transferases which catalyze the transfer of *N*-acetylglucosamine (GlcNAc) from UDP-*N*-acetylglucosamine to endogenous lipids and endogenous proteins (21). In this process the glycosylated lipid polyprenyldiphosphate-di-*N*-acetylchitobiose [$(GlcNAc)_2$-PP-dol] acts as an intermediate, transferring di-*N*-acetylchitobiose to protein. These two GlcNAc residues are always the first sugar residues found in carbohydrate side chains linked *N*-glycosidically to polypeptides.

To establish whether a similar transfer of GlcNAc to endogenous proteins can also take place in the plasma membrane of yeast cells, we have now isolated a plasma membrane fraction of high purity and examined it for the presence of the components required for such an initial glycosylation.

## MATERIALS AND METHODS

**Materials.** All chemicals used were analytical grade. UDP-[$^3$H]GlcNAc (specific activity, 6.6 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, United Kingdom. Tunicamycin was a gift of G. Tamura, University of Tokyo, Japan. *Saccharomyces cerevisiae* X2180-1A wild type was a gift of C. E. Ballou, University of California, Berkeley. Urografin 76 was purchased from Schering A. G., Berlin, Federal Republic of Germany. For measurement of radioactivity, Instagel from Packard-Becker, Brussels, Belgium, was used as a scintillation solution.

**Preparation of a plasma membrane fraction.** *S. cerevisiae* was cultured continuously in a Bio-Flo (model $C_{30}$, New Brunswick Scientific Co., Inc., New Brunswick, N.J.) at 30°C in a medium described previously (27). Plasma membrane vesicles were prepared by a modification of the method of Fuhrmann (5). Yeast (15 g) was suspended in 20 ml of an osmotic stabilizer (450 mM KCl, 20 mM tri-ethanolamine, 10 mM $MgCl_2$, pH 6), mixed with 35 ml of ice-cold Ballotini glass beads (0.25 to 0.30 mm diameter), and disrupted by shaking for 1 min in a cell homogenizer MSK (Braun, Melsungen, Federal Republic of Germany). Whole cells and cell debris were removed by centrifugation (5 min, 2,000 × *g*, 4°C), and the supernatant was recentrifuged (10 min, 5,000 × *g*, 4°C). The pellet was washed three times in osmotic stabilizer. This sediment, suspended in 1 ml of 10% (wt/wt) sucrose in osmotic stabilizer, was centrifuged in a discontinuous sucrose density gradient (2.2 ml of 60-50-40-30-20% sucrose in osmotic stabilizer [wt/wt], 1 h, 94,500 × *g*, 4°C) in a Spinco SW 41 rotor. The material resting upon 50% sucrose (density 1.21 to 1.26 $g \cdot ml^{-1}$) was isolated and centrifuged in a discontin-

uous Urografin gradient (16, 17). Urografin 76 was diluted with osmotic stabilizer to 38-32.35-28.5-23.75-19% (vol/vol). After centrifugation in an SW 41 rotor (1 h, 94,500 × *g*, 4°C) the material of density 1.17 to 1.20 g·ml$^{-1}$ was isolated.

**Analytical methods.** Protein was estimated by the Lowry method (10). The activity of the $Mg^{2+}$-dependent ATPase (EC 3.6.1.3) was determined by suspending about 0.2 mg of protein in 50 mM Tris-10 mM $MgCl_2$-1mM ATP (pH 6.5) to a final volume of 0.6 ml and incubating for 10 min at 25°C. The reaction was stopped, and inorganic phosphate was determined by the method of Ohnishi et al. (18). To test the sensitivity to oligomycin, this inhibitor was added to the incubation mixture in a concentration of 10 μg·ml$^{-1}$. The activity of NADPH-cytochrome *c* oxidoreductase (EC 1.6.2.4) was determined by the method of Green and Ziegler (6). α-Mannosidase (EC 3.2.1.14) activity was measured by the method of Levvy and Conchie (8) at pH 7.2 as recommended by Nurminen et al. (16). One unit of each enzyme is the amount that will catalyze the transformation of 1 μmol of substrate per min at the recommended temperature.

Total nucleic acid was determined by the method of Spirin (23). The DNA content was measured with a diphenylamine reagent by the method of Burton (2).

**Electron microscopy.** For thin sectioning, membranes were fixed in 3% glutaraldehyde, 2% formaldehyde, 2.5% dimethylsulfoxide, and 1% acrolein (vol/vol) in 0.08 M $KH_2PO_4$ (pH 7.0) during 30 min, and postfixed in a solution of 1% $OsO_4$ (wt/vol) in the same buffer. The fixed membranes were dehydrated in graded acetone, to which in the last step a few drops of 2,2-dimethoxypropane were added, and embedded in Araldite. Ultrathin sections were poststained in lead citrate by the method of Reynolds (22) and next in a saturated solution of uranyl acetate in distilled water during 5 min. For freeze-fracturing, material was fixed in the buffered trialdehyde solution mentioned above and subsequently soaked in 25% glycerol (vol/vol). Specimens were quickly frozen from about 5°C in a mixture of solid and liquid nitrogen and transferred to a Denton freeze-etch machine. The material was fractured at −110°C. After replication by Pt/C, the biological material adhering to the replica was removed with 70% sulfuric acid and a hypochlorite solution.

**Assay procedure for incorporation of [$^3$H]-GlcNAc.** To measure the transfer of GlcNAc, the following incubation mixture was used: 50 mM Tris (pH 7.0), 5 mM $MnSO_4$, 450 mM KCl, 0.25 μCi of UDP-[$^3$H]GlcNAc and a plasma membrane fraction containing 0.5 to 1.0 mg of protein in a total volume of 200 μl. After incubation for 30 min at 30°C, the reaction was stopped by adding 1.5 ml of $CH_3OH$ and 3.0 ml of $CHCl_3$ by the method of Reuvers et al. (21). Isolation of an alkali-stable lipid fraction, chromatography of the glycolipids, washing of residual material, β-elimination, hydrazinolysis, and Pronase digestion of endogenous proteins, as well as measurement of radioactivity, were performed as described by Reuvers et al. (21).

When [$^3$H](GlcNAc)$_2$-PP-dol was used as a substrate in the transfer of GlcNAc to endogenous protein, the same procedure was followed, except that Triton X-100 (final concentration, 0.4%) was added to the reaction mixture. The residual material was washed as described by Reuvers et al. (21).

## RESULTS

**Isolation of plasma membranes.** During isolation and purification of the fraction containing plasma membranes from a yeast homogenate by density gradient centrifugation, we have identified the plasma membranes by their specific marker enzyme, the oligomycin-insensitive, $Mg^{2+}$-dependent ATPase with a pH optimum of 6.5. A similar enzyme is also present in mitochondria (pH optimum, 8 to 9), but it differs from the plasma membrane ATPase in its sensitivity to inhibition by oligomycin (3, 16, 17). Because data concerning the distribution of membranes during centrifugation on sucrose and Urografin gradients indicate that the buoyant density of mitochondrial membranes is close to that of plasma membranes (16, 17), the most important contaminant to be reckoned with would seem to be mitochondrial membrane fragments. We have therefore determined the activity of the $Mg^{2+}$-ATPase at pH 6.5 in fractions from density gradients in the presence and absence of oligomycin. (The more obvious mitochondrial marker, cytochrome *c* oxidase, cannot be used because this enzyme activity is absent from our anaerobically grown yeast (16). However, the not yet fully developed mitochondria already contain the oligomycin-sensitive ATPase.)

When the 2,000 to 5,000 × *g* fraction of a yeast homogenate was centrifuged on a sucrose gradient (20 to 60%), it separated into three main bands: $S_0$, $S_1$, and $S_2$. As the figures in Table 1 show, fraction $S_1$ seems to consist mainly of mitochondrial material, which is in agreement

TABLE 1. *Oligomycin-insensitive $Mg^{2+}$-ATPase activity in yeast membrane fractions of varying density*[a]

| Gradient fraction | Density range (g·ml$^{-1}$) | $Mg^{2+}$-ATPase (pH 6.5) (U/mg of protein) | Oligomycin insensitivity (%) |
|---|---|---|---|
| $S_0$ | 1.12 | ND | ND |
| $S_1$ | 1.16-1.21 | $44 \times 10^{-3}$ | 27 |
| $S_2$ | 1.21-1.26 | $41 \times 10^{-3}$ | 60 |
| $U_{2a}$ | 1.15-1.17 | $12 \times 10^{-3}$ | 42 |
| $U_{2b}$ | 1.17-1.120 | $29 \times 10^{-3}$ | 94-100 |

[a] $S_0$, $S_1$, $S_2$: fractions obtained by centrifugation of 2,000- to 5,000 × *g*-membrane fraction on a buffered sucrose gradient. $U_{2a}$, $U_{2b}$: subfractions of $S_2$ obtained by centrifugation on a buffered Urografin gradient. One enzyme unit (U) is defined as the quantity of enzyme that sets free 1 μmol of inorganic phosphate per min. Oligomycin insensitivity is calculated as (activity with oligomycin/activity without oligomycin) × 100%. ND, Not determined.

with the results of Fuhrmann et al. (5). Fraction $S_2$ seems to be more plasma membrane-like in character than fraction $S_1$. Therefore, fraction $S_2$ (3 to 5 mg of protein) was further purified by centrifugation on an Urografin density gradient (19 to 38% vol/vol). It separated into two subfractions: $U_{2a}$ and $U_{2b}$. Fraction $U_{2a}$ seems to consist mainly of contaminating mitochondrial material, whereas the practically "mitochondria-free" plasma membranes gathered in fraction $U_{2b}$ (Table 1). When fraction $U_{2b}$ was recentrifuged on an Urografin gradient, it again formed a single band at the same density of 1.17 to 1.20 $g \cdot ml^{-1}$. This density range is in accordance with the data for yeast plasma membranes presented by Matile et al. (11).

**Examination of the purity of the plasma membrane fraction.** Although the plasma membrane fraction obtained as described above seems to be largely free from contamination by mitochondrial membranes, it is conceivable that it still contains some membrane fragments of a different cellular origin: endoplasmic reticulum, vacuolar membrane, and nuclear membrane. Judging by their buoyant density, microsomal material ($\rho \sim 1.10$) and vacuolar membrane ($\rho \sim$ 1.09 to 1.13) would seem to be too light (16); nuclear membranes might be a possibility if membrane fragments would remain attached to DNA during the homogenization of yeast cells, in which case their buoyant density would be raised. To make sure, we have examined our preparation for the presence of NADPH-cytochrome *c* oxidoreductase (marker for microsomal membranes) (16), α-mannosidase (marker for vacuolar membrane) (26), RNA and DNA (marker for nuclear material). Table 2 shows the results, in comparison with figures for the whole homogenate and the 2,000 to 5,000 × *g* fraction from which the plasma membrane fraction was obtained.

We conclude that our plasma membrane preparation is free from contamination with microsomal material and only contains negligible traces of vacuolar and nuclear membrane fragments. The latter is particularly reassuring, because recently Palamarczyk and Janczura have reported on the occurrence of lipid-mediated glycosylation in yeast nuclear membrane (19).

The low RNA content of fraction $U_{2b}$ also indicates that very little material from rough endoplasmic reticulum or other ribosomal material can be present.

The plasma membrane preparation was also examined by electron microscopy. In ultrathin section electron micrographs vesicles of various sizes were seen, with a triple-layered membrane structure (Fig. 1C and D). A morphological characteristic of yeast plasmalemma is the presence of regions of hexagonally arrayed membrane particles (5, 11). When fraction $U_{2b}$ was examined by the freeze-fracturing technique, this pattern can be recognized on many vesicles (Fig. 1A and B).

**Incorporation of GlcNAc into glycolipids and into residual material.** When a plasmalemma fraction from yeast was incubated with UDP-[$^3$H]GlcNAc in the manner described in Material and Methods, radioactive GlcNAc was incorporated in a crude lipid fraction and into residual material. During the first 15 min, incorporation into lipid was rapid (15,000 $dpm \cdot mg^{-1}$ of membrane protein); thereafter, it slowed down considerably. Incorporation into residual material was much slower (2,500 $dpm \cdot mg^{-1}$ of membrane protein) and proceeded linearly for at least 45 min. Eighty-five to ninety percent of the labeled crude lipids were resistant to hydrolysis by mild alkali when heated for 20 min at 60°C in 0.2 M NaOH by the method of Reuvers et al. (21). Such an alkali-stability is a property of the polyprenoid-sugar intermediates of glycoprotein synthesis.

**Characterization of the alkali-stable lipids.** Upon thin-layer chromatography of the crude lipid fraction in the solvent chloroform-

TABLE 2. *Testing the purity of the plasmalemma fraction ($U_{2b}$)*[a]

| Fraction | NADPH-cytochrome *c* reductase | | α-mannosidase | | RNA | | DNA | |
|---|---|---|---|---|---|---|---|---|
| | mU/mg of protein | Total mU | mU/mg of protein | Total mU | μg/mg of protein | Total μg | μg/mg of protein | Total μg |
| Homogenate | 8 | 5,400 | 11.2 | 3,700 | 131 | $35 \times 10^3$ | 41 | $11 \times 10^3$ |
| 2,000–5,000 × *g* | 0 | 0 | 8.3 | 150 | ND | ND | 6 | 110 |
| Plasmalemma | 0 | 0 | 1.2 | 5 | 29 | 117 | 3 | 12 |

[a] The elimination of other membranous material during the preparation of a plasmalemma fraction was ascertained by determining the content of several markers. Microsomal membranes, NADPH-cytochrome *c* oxidoreductase; vacuolar membranes, α-mannosidase; RER, RNA; nuclear membrane, DNA. Details are described in the text. ND, Not determined.

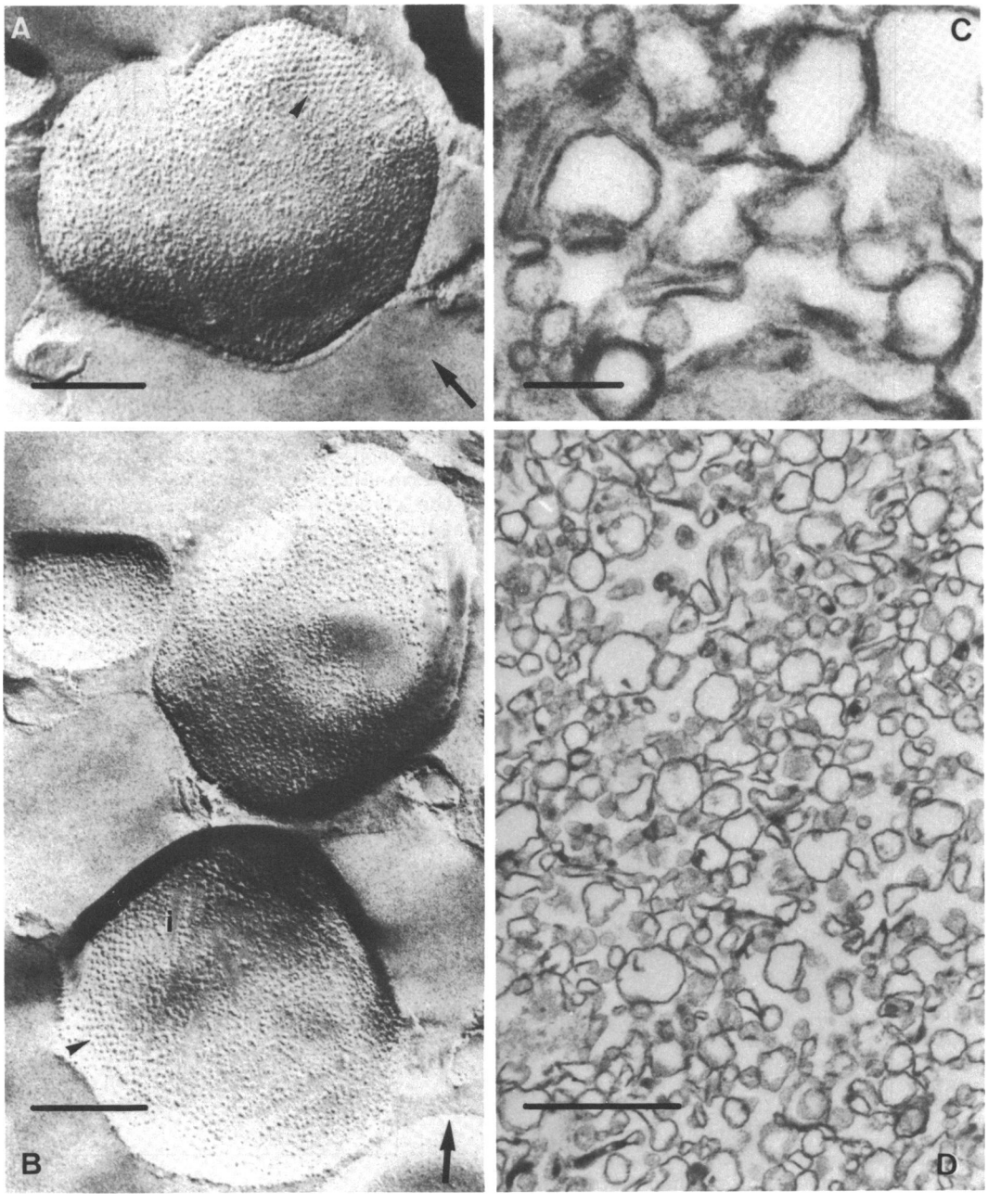


FIG. 1. *Electron microscopy of plasmalemma fraction. (A, B) Replicas of freeze-etched, fractured plasma membrane vesicles (fraction $U_{2b}$). The characteristic hexagonal arrangement of membrane particles (arrow) and remnants of invaginations (i) are clearly visible. Bar represents 0.2 μm. (C, D) Micrograph of a thin section of the isolated plasma membrane fraction $U_{2b}$. (C) Bar represents 0.2 μm. (D) Bar represents 1.0 μm.*

methanol-water (60:35:6, vol/vol) two peaks of radioactivity appeared: their mobilities ($R_f$ = 0.18 and 0.26) coincided with those of authentic samples of GlcNAc-PP-dol and $(GlcNAc)_2$-PP-dol run simultaneously on the same plates (Fig. 2).

**Examination of the residual material.** Evidence that the labeled sugar in the residual

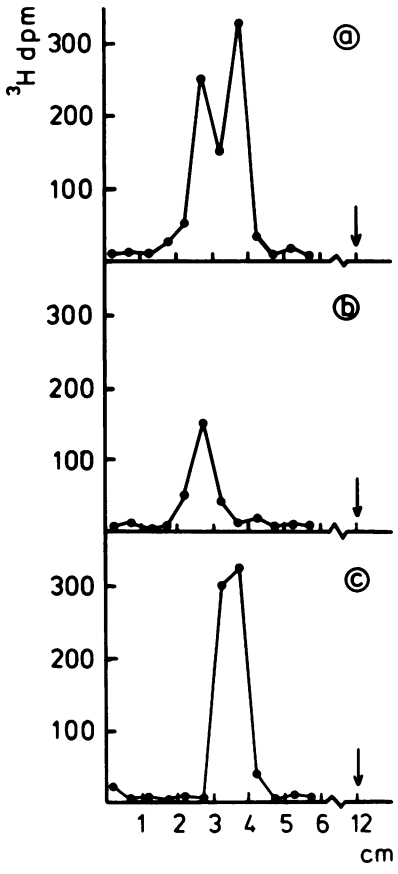


FIG. 2. *Thin-layer chromatography of crude lipid fraction extracted from plasma membranes and standards. Plasma membranes (0.7 mg of protein) incubated with 0.25 μCi of UDP-[$^3$H]GlcNAc for 30 min at 30°C. Reaction stopped with 22.5 volumes $CHCl_3$-$CH_3OH$ (2:1, vol/vol). The chloroform-methanol extract was washed with 0.2 volumes 0.9% NaCl, and lower phase was then washed with Folch's theoretical upper phase. Solvent: chloroform-methanol-water, 60:35:6. (a) crude lipid fraction (1,000 dpm); (b) authentic sample of [$^3$H](GlcNAc)$_2$-PP-dol (400 dpm); (c) authentic sample of [$^3$H]GlcNAc-PP-dol (800 dpm). Arrow indicates solvent front.*

material is attached to protein was obtained by incubating the material with pronase. Gel filtration on Bio-Gel P4 of the residual material before and after treatment with pronase shows that before treatment the radioactivity mainly eluted in one peak of high molecular weight, whereas after treatment the radioactivity was found in a broad range of lower molecular weights (Fig. 3).

After treatment of the residual material with 0.1 M NaOH for 24 h at room temperature ("β-elimination," a method to remove *O*-glycosidically linked sugars from proteins; see reference 1), 85 to 93% of the radioactivity was still indiffusible upon dialysis. This suggests that practically all the radioactivity is *N*-glycosidically linked to protein.

To determine the size of the carbohydrate moiety containing the labeled GlcNAc, we removed it intact from the protein by hydrazinolysis (14). During this treatment, GlcNAc residues were deacetylated. After hydrazinolysis practically all the radioactivity appeared in the supernatant. On a column of Bio-Gel P2 nearly all the label in the supernatant moved in one peak, with a mobility expected for chitobiose (Fig. 4a) (21).

From these experiments, we conclude that in plasma membranes, just as in a crude particulate fraction from yeast (21), after incubation with UDP-GlcNAc two GlcNAc residues are attached to endogenous protein via an *N*-glycosidic bond.

**Intermediary role of polyprenoid-sugars in the glycosylation of proteins.** Evidence that the polyprenoid derivatives containing labeled GlcNAc in the alkali-stable lipid fraction are indeed intermediates in the glycosylation of endogenous protein was obtained in the following experiments. (i) Plasma membranes were incubated with UDP-[$^3$H]GlcNAc in the presence and absence of tunicamycin (final concen-

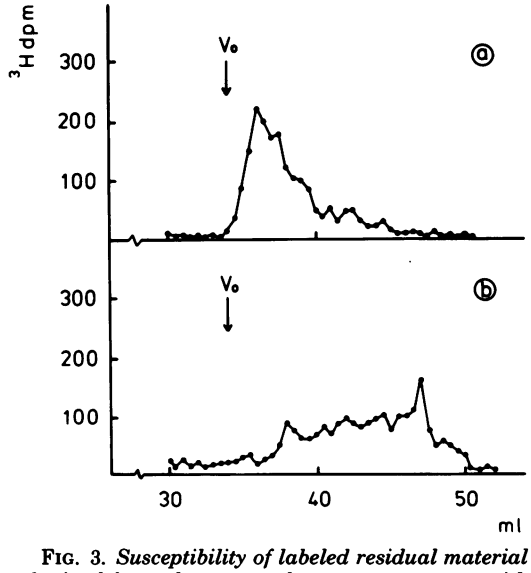


FIG. 3. *Susceptibility of labeled residual material obtained from plasma membranes to treatment with pronase. Plasma membrane fraction was incubated with UDP-[$^3$H]GlcNAc as described in the text. Residual material (2,000 dpm) was filtered over Bio-Gel P4 (column 90 by 1 cm) before (a) and after (b) digestion with 2 mg of pronase for 5 h at 30°C, followed by 1 h at 50°C. Additional pronase added after 1 h (2 mg), 2 h (2 mg), and 3 h (4 mg). $V_o$, Void volume measured with Dextran Blue.*

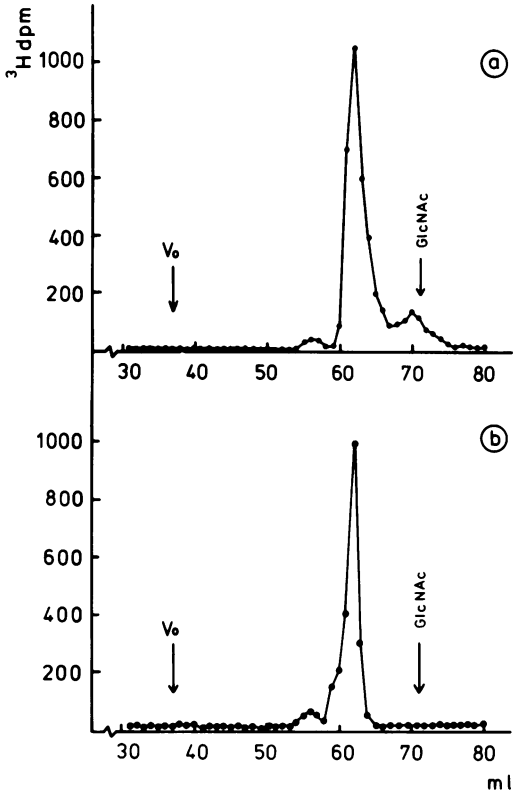


FIG. 4. *Filtration on Bio-Gel P2 of the saccharide moiety released from the residual material by hydrazinolysis. Residual material was heated with 0.5 ml of anhydrous hydrazine in a sealed tube for 30 h at 100°C, the hydrazine was evaporated under a stream of $N_2$, the residue was suspended in 1.0 ml of 15% (vol/vol) acetic acid and centrifuged. The supernatant was filtered on a column of Bio-Gel P2, 90 by 1 cm. (a) Plasma membranes incubated with UDP-[$^3$H]GlcNAc, supernatant 4,000 dpm. (b) Plasma membranes incubated with [$^3$H](GlcNAc)$_2$-PP-dol, supernatant 2,000 dpm. $V_o$, void volume, measured with Dextran Blue.*

tration, 12 μM). This antibiotic is known to inhibit the formation of GlcNAc-PP-dol (24, 25). Its addition to the standard incubation mixture reduced the incorporation of radioactivity into alkali-stable lipids to 10% of the control value; in residual material the incorporation of radioactive GlcNAc was also inhibited, albeit to a lesser extent (Table 3). (ii) According to the reaction: UDP-GlcNAc + dolichyl-P ⇌ UMP + GlcNAc-PP-dol, addition of UMP to the incubation mixture should diminish the incorporation of [$^3$H]GlcNAc into both alkali-stable lipids and residual material. This is indeed the case (Table 3). (iii) When plasma membranes were incubated with [$^3$H](GlcNAc)$_2$-PP-dol instead of with UDP-[$^3$H]GlcNAc (in this case, the standard reaction mixture also contained 0.4% Triton X-100), there was a small but distinct transfer of radioactive label to residual material: 6% of the 15,000 dpm added as GlcNAc-lipid. After hydrazinolysis of the residual material and filtration of the radioactive supernatant on Bio-Gel P2, the radioactivity again mainly moved as a single peak with the mobility of chitobiose (Fig. 4b). This indicates that di-*N*-acetylchitobiose can be transferred from glycolipid to protein. To establish in more detail the sequence of events when UDP-GlcNAc is the substrate, plasma membranes were incubated with [$^3$H]GlcNAc-PP-dol and unlabeled UDP-GlcNAc. After 30 min the lipids were extracted and examined by thin-layer chromatography. As Fig. 5 shows, about 65% of the label added as [$^3$H]GlcNAc-PP-dol was recovered as (GlcNAc)$_2$-PP-Dol. This experiment suggests that a second GlcNAc residue is added to the first glycolipid intermediate, whereupon di-*N*-acetylchitobiose is transferred from glycolipid to protein (see also reference 21).

## DISCUSSION

This paper provides evidence that a purified plasma membrane fraction from yeast can transfer GlcNAc from UDP-GlcNAc to polyprenoid lipids and from glycolipids to endogenous protein.

Careful examination of the final plasmalemma preparation obtained for the presence of marker enzymes specific for other membranes and of RNA and DNA showed that it indeed has a high state of purity. The plasma membranes were

TABLE 3. *Effect of tunicamycin (TM) and UMP upon incorporation of [$^3$H]GlcNAc in lipid and protein fractions of plasmalemma*[a]

| Expt no. | Conditions | Lipid fraction | | Residual material | |
|---|---|---|---|---|---|
| | | dpm | % | dpm | % |
| 1 | Control-TM | 22,125 | 100 | 2,161 | 100 |
| | (12 μM) | 1,775 | 8 | 1,026 | 47 |
| 2 | Control-TM | 7,204 | 100 | 1,283 | 100 |
| | (12 μM) | 875 | 12 | 716 | 56 |
| 3 | Control-UMP | 9,131 | 100 | 1,607 | 100 |
| | (5 mM) | 1,120 | 12 | 268 | 16 |
| 4 | Control-UMP | 9,919 | 100 | 1,578 | 100 |
| | (5 mM) | 844 | 9 | 496 | 31 |

[a] Plasma membrane fraction was incubated with UDP-[$^3$H]GlcNAc in standard reaction mixture, with additions as indicated. For details consult the text. Each figure is the average of closely agreeing duplicate determinations. In experiment 1, the amount of plasma membranes used (milligram of protein) was twice that used in the other three experiments.

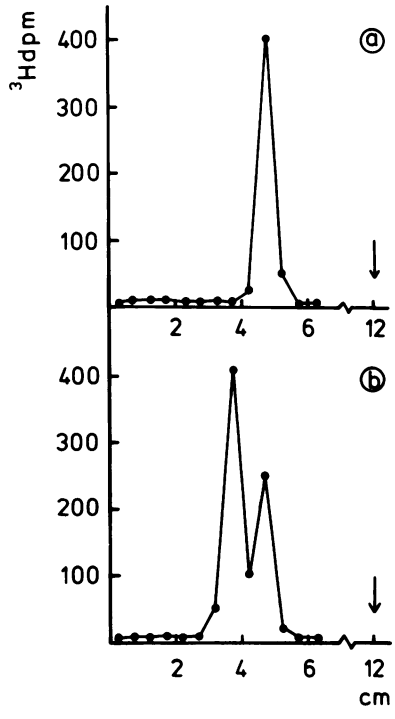


FIG. 5. *Thin-layer chromatography of crude lipid fraction extracted from plasma membranes after incubation with [$^3$H]GlcNAc-PP-dol and unlabeled UDP-GlcNAc. Solvent: chloroform-methanol-water, 60:35:6. (a) Authentic sample of [$^3$H]GlcNAc-PP-dol (500 dpm). (b) Crude lipid fraction (800 dpm). The reaction mixture contained 15,000 dpm [$^3$H]GlcNAc-PP-dol, 1 mM UDP-GlcNAc, and 0.3% Triton X-100. Incubation time, 30 min.*

free of fragments of mitochondrial membranes and endoplasmic reticulum, and practically free of fragments of vacuolar membranes, ribosomes, and nuclear material.

Our conclusion that yeast plasmalemma is capable of initial glycosylation of proteins via glycolipid intermediates is based on the following observations. (i) Incorporation of [$^3$H]-GlcNAc into endogenous protein decreases when the transfer of GlcNAc-phosphate from UDP-GlcNAc to dolichyl phosphate is diminished by adding tunicamycin or UMP (Table 3). (ii) Most of the label in [$^3$H]GlcNAc-PP-dol is transferred to $(GlcNAc)_2$-PP-dol by plasma membranes in the presence of unlabeled UDP-GlcNAc (Fig. 5). (iii) $(GlcNAc)_2$-PP-dol can act as a direct donor of di-*N*-acetyl-chitobiose to endogenous membrane protein (Fig. 4b). The most likely route for the transfer of GlcNAc seems to be UDP-GlcNAc → GlcNAc-PP-dol → $(GlcNAc)_2$-PP-dol → $(GlcNAc)_2$-protein. We have earlier established the same sequence of events for a crude preparation of yeast membranes (21).

Plasma membranes incorporate [$^3$H]GlcNAc from UDP-[$^3$H]GlcNAc into endogenous lipids at about the same rate as this crude membrane preparation (1,000 to 40,000 × *g*), but the rate of incorporation of [$^3$H]GlcNAc into endogenous protein is about three to four times slower per milligram of membrane protein. Possibly the plasmalemma preparation has relatively less suitable acceptor proteins.

It is possible that some GlcNAc can be transferred directly from UDP-GlcNAc to protein bypassing the glycolipids, because the incorporation of [$^3$H]GlcNAc into protein is less depressed by adding tunicamycin or UMP to the reaction mixture than the incorporation into lipid (Table 3).

This study suggests that, in a simple eucaryote like yeast at least, glycosylation of proteins need not take place exclusively on the nascent polypeptide chain in the endoplasmic reticulum (13). Interestingly, initial glycosylation of proteins has also been reported to occur in plasma membranes of rabbit reticulocytes (20). Our results support the hypothesis of Linnemans et al. (9) that the glycoenzyme acid phosphatase can be formed in invaginations of the yeast plasma membrane.

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