Mannan chains of Kluyveromyces lactis mannanproteins are similar to those of Saccharomyces cerevisiae except that they have terminal α1–2-linked N-acetylglucosamine and lack mannose phosphate. In a previous study, Douglas and Ballou (1) characterized a mutant, mnn2-2, which lacked terminal N-acetylglucosamine in its mannanproteins. The mutant had normal levels of N-acetylglucosaminyltransferase activity, and the partially purified enzyme from wild-type and mutant cells had the same apparent size, heat stability, affinity for substrates, metal requirement, and subcellular location. No qualitative or quantitative differences were found between mutant and wild-type cells in endogenous mannan acceptors and pools of UDP-GlcNAc.

Chitin was synthesized at similar rates in wild-type and mutant cells, indicating that the above transport defect is specific. In addition to their previously reported lack of GlcNAc transferase or a hexosaminidase which could remove N-acetylglucosamine from mannanproteins, together, the above observations led Douglas and Ballou (2) to study the biochemical defect underlying this latter phenotype. They found that wild-type and mutant cells had similar pool sizes of UDP-GlcNAc, synthesized chitin at similar rates, and that endogenous mannan acceptors were the same in both cell types. Highly purified preparations of N-acetylglucosaminyltransferase were obtained from both cells and were found to have the same apparent size, heat stability, apparent Km values for substrates, Mn2+ requirements, Vmax, and subcellular location. No evidence was found for an inactive enzyme precursor in mutant cells nor did these cells contain a soluble inhibitor of the transferase or a hexosaminidase which could remove N-acetylglucosamine from the mannanproteins during their biosynthesis and secretion. These observations led the above authors to postulate, among other possibilities, that the mutant might have a defect in compartmentation of substrates thereby preventing the normal biosynthesis of the mannanproteins.

**MATERIALS AND METHODS**

Radioactive Substrates—UDP-N-acetyl[6-3H]glucosamine, 29 Ci/mmol; GDP-[2-3H]mannose, 19 Ci/mmol; and [3H]sodium acetate, 135 mCi/mmol were purchased from DuPont NEN.

Yeast Strains and Growth Conditions—K. lactis Y 43:MATα, his3 were obtained from Dr. Clinton Ballou, University of California, Berkeley, CA.

Yeast strains were grown at 30 °C in shake liquid culture consisting of 1% yeast extract, 2% bactopeptone, containing 2% glucose (YPD) unless otherwise specified. Cultures were maintained on solid YPD media containing 2% Bacto-agar.

GDPase Assay—GDPase was determined essentially as described
Purity and integrity of Golgi-enriched vesicles from wild type, mnn2–2, and mnn2–1 mutants of K. lactis

Results are average of three independent determinations with a coefficient of variation of less than 5%.

| Yeast strain | Specific activity units/mg protein | GDPase | Latency % | Enrichment over homogenate | α1,2-GlcnAc transferase | Enrichment over homogenate |
|--------------|----------------------------------|--------|-----------|---------------------------|------------------------|--------------------------|
|              | Triton                            | +Triton|           |                           |                        |                          |
| Wild-type    | 0.08                              | 1.65   | 95        | 3.6                       | 2.9                    | 4.5                      |
| mnn2–2       | 0.06                              | 1.48   | 96        | 3.4                       | 3.3                    | 4.7                      |
| mnn2–1       | 0.07                              | 1.53   | 95        | 3.4                       | 0.0                    |                          |

a One unit of activity is defined as 1 μmol of inorganic phosphate released per min under standard assay conditions described under "Materials and Methods.

b One unit of activity catalyzes the formation of 1 pmol of product found per min under standard assay conditions described under "Materials and Methods.

Previously (4), briefly, incubation mixtures in a final volume of 0.1 ml, contained 10–50 μg of P2 fraction enzyme protein (4) or 40–250 μg of total homogenate protein, CaCl2 (1 μmol), Triton X-100 (100 μg), GDP or ADP (0.2 μmol), and imidazole buffer, pH 7.6 (20 μmol). Incubations were done for 5 min at 30°C. The reaction was stopped by adding 10 μl of 10% SDS. Inorganic phosphate was determined by the Ames method (5). GDPase activity was calculated as the difference between GDP and ADP hydrolysis.

**Theoretical basis for the translocation assay of nucleotide derivatives into vesicles** has been described previously (6). Briefly, it consists of (i) determining the total radioactive solutes associated with the vesicle pellet (S2) after incubation of vesicles with radioactive substrate and (ii) subtracting the radioactive solutes outside the vesicles in the pellet (S1). This yields the total radioactive solutes within vesicles (S0). The (S0) volume outside the vesicles in the pellet was determined using [3H]acetate as a non-penetrator and was found to be 1.6 μg/mg protein for wild-type as well as for mnn2–2 and mnn2–1 mutant-derived vesicle fractions. Incubations were done in 1 ml of buffer A: 0.25 mM sucrose, 20 mM Tris-HCl, pH 7.5, 5 mM MnCl2. Centrifugation and counting were carried out as described previously (6).

Nudeotide Sugar Translocation Assay—The theoretical basis for the translocation assay of nucleotide derivatives into vesicles has been described previously (6). Briefly, it consists of (i) determining the total radioactive solutes associated with the vesicle pellet (S2) after incubation of vesicles with radioactive substrate and (ii) subtracting the radioactive solutes outside the vesicles in the pellet (S1). This yields the total radioactive solutes within vesicles (S0). The (S0) volume outside the vesicles in the pellet was determined using [3H]acetate as a non-penetrator and was found to be 1.6 μg/mg protein for wild-type as well as for mnn2–2 and mnn2–1 mutant-derived vesicle fractions. Incubations were done in 1 ml of buffer A: 0.25 mM sucrose, 20 mM Tris-HCl, pH 7.5, 5 mM MnCl2. Centrifugation and counting were carried out as described previously (6).

Subcellular FRACTIONATION—Cells were grown in YPD medium to an OD600 of 4. The cell culture was chilled, centrifuged, and converted to spheroplasts as described previously (7) using a total of 20 ml of Zymolyase 100T (ICN). This spheroplast suspension was then centrifuged at 450 g for 10 min. Cells were broken by suspending the pellet in 40 ml of 10 mM triethanolamine, pH 7.2, 0.8 M sorbitol, 1 mM EDTA and drawing the cells rapidly several times into a narrow bore serological pipette. Cell breakage was incomplete, but vesicle integrity was well maintained. The suspension was centrifuged successively at 450 g, 10,000 × g, and 100,000 × g to prepare, respectively, the P2, P3, and P4 fractions of Goud et al. (8). The P3 fraction was enriched in Golgi markers such as GDPase (9, 10), 3,5-fold) and α1,2-GlcNAc transferase (4,5-fold) for both wild-type and mnn2-2-derived vesicles and was used for all translocation or sugar transport experiments. The P3 fraction from mutant mnn2-1 was enriched 3,4-fold in GDPase and had no measurable α1,2-GlcNAc transferase activity.

In Vivo Labeling of Radioactive Glucosamine Containing Lipids—For in vivo labeling of glucosamine containing lipids, exponentially growing cultures (OD600) in YEP, 2% sucrose, were used. Thirty min prior to labeling, 4 OD600 of cells were harvested and suspended in 2 ml of YEP, 0.5% sucrose, [6-3H]glucosamine (1 M cm/m; 60 Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO) was added for 60 min. Following the incubation, the cell culture was centrifuged, the supernatant removed, and the cells washed four times each with water containing 10 mM sodium azide. Cells, suspended in 0.1 ml of 10 mM aqueous NaOH, were broken by vortexing with glass beads, 3 times for 1 min each. 10 μl were used to determine protein concentration and total incorporation of radioactive glucosamine. Chloroform/methanol (1:1) was added to the remaining suspension to achieve a final concentration of chloroform/methanol/aqueous cell suspension of 10:3 (v/v/v). Following centrifugation, the pellet was extracted two more times with 0.6 ml of chloroform/methanol/water (10:10:3) (v/v/v). The pooled lipid extracts were dried under a stream of nitrogen and desalted by butanol extraction (11).

Lipids were analyzed by ascending thin layer chromatography on 0.2-mm Silica Gel 60 plates (EM Science) in a solvent system of chloroform/methanol, 0.2% aqueous KCl (55:45:10). Approximately 40,000 cpm were applied per sample. The developed plates were sprayed with EN HANCE (DuPont NEN) and fluorograms were exposed on Reflection™ film (DuPont NEN) for 3 weeks at ~80°C. To determine sensitivity to mild alkaline hydrolysis, an aliquot of the lipid extract was dried under nitrogen and resuspended in ethanol/water/diethyl ether/pyridine (15:15:5:1) (v/v/v). 100 μl of 0.25 M KOH in methanol was added. Following 1 h incubation at room temperature, 20 μl of 1 N aq. acid was added to neutralize the reaction. The mixture was evaporated under nitrogen, desalted, and separated on thin layer chromatography as described above.

**RESULTS**

Differential Translocation of UDP-GlcNAc into Golgi Vesicles from Wild-type, mnn2-1, and mnn2-2 Mutant K. lactis—Before transport of nucleotide sugars could be measured into Golgi vesicles from the above cells, it was important to determine that these vesicles were sealed, of the same membrane orientation as in vivo, and of comparable purity. A Golgi vesicle-enriched fraction was obtained by a procedure similar to that previously described for vesicles from S. cerevisiae. α2-N- Acetylglucosaminyltransferase activity and guanosine diphosphate-β[methyl-3H]transferase were used to determine their purity, topography, and intactness. As can be seen in Table I, vesicles from wild-type and mutant cells were enriched 4–5-fold over the homogenate for these two marker enzymes (mutant mnn2-1 had no measurable α1,2-GlcNAc transferase activity). In addition, based on the specific activity differences of the GDPase activity with or without Triton X-100, vesicle preparations were found to be 95% latent. Thus, the above vesicles, from wild-type and mutants mnn2-1 and mnn2-2, which were of similar purity, integrity, and membrane topographical orientation as in vivo, could be used for measurements of transport of UDP-GlcNAc and GDP-mannose into a luminal compartment in an assay in vitro.

As can be seen in Table I, while transport of UDP-GlcNAc into Golgi vesicles from wild-type cells was concentration dependent, transport into Golgi vesicles from mnn2-2 mutants was virtually zero and did not change with a 10-fold increase in nucleotide sugar concentration. Vesicles from the mnn2-2 mutant translocate UDP-GlcNAc at rates comparable to vesicles from wild-type cells.

It was important to determine that the above defect in UDP-GlcNAc transport was specific and not a general defect of the Golgi membrane of mutant mnn2-2 cells. We therefore measured the ability of vesicles to transport GDP-mannose. As can be seen in Table II, this nucleotide sugar was translocated into all vesicles with a similar velocity, demonstrating that the...
above defect of UDP-GlcNAc transport was specific. The $V_{\text{max}}$ of GDP-mannose transport into *K. lactis* vesicles was similar to that previously found with vesicles from *S. cerevisiae*; this was expected as both yeasts have similar mannan chains.

Transport of UDP-GlcNAc into Golgi Vesicles from Wild-type *K. lactis* Is Saturable and Temperature Dependent—Because transport of UDP-GlcNAc into Golgi vesicles from yeast has never been previously characterized, it was important to determine whether it had the characteristics of protein carrier mediated transport. Transport was temperature dependent; at 0°C it was 30% of that at 30°C. Transport was linear with time up to 4 min and with protein between 0.7 and 1.9 mg. Transport was found to be saturable with an apparent $K_m$ of 5.5 $\mu$M and a $V_{\text{max}}$ of 8.2 pmol/mg/3 min (Fig. 1).

**Table II**

| Cell type | Substrate | $S_m$ | $S_i$ | $S_o$ | $S_t$ |
|-----------|-----------|------|------|------|------|
| Wt        | UDP-GlcNAc| 0.1  | 0.34 | 0.18 | 0.16 |
| Wt        | UDP-GlcNAc| 1.0  | 3.22 | 1.80 | 1.42 |
| mnn2–2    | UDP-GlcNAc| 0.1  | 0.20 | 0.18 | 0.02 |
| mnn2–2    | UDP-GlcNAc| 1.0  | 2.00 | 1.80 | 0.02 |
| mnn2–1    | UDP-GlcNAc| 1.0  | 3.38 | 1.80 | 1.58 |
| Wt        | GDP-mannose| 1.0 | 10.51 | 1.80 | 8.71 |
| mnn2–2    | GDP-mannose| 1.0 | 7.84 | 1.80 | 6.04 |
| mnn2–1    | GDP-mannose| 1.0 | 9.20 | 1.80 | 7.40 |

**Fig. 1.** Rate of solute accumulation within wild-type *K. lactis* Golgi vesicles versus UDP-GlcNAc concentration in the incubation medium. A P$_3$ vesicle fraction (1.2 mg of protein) was incubated at 30°C for 3 min with different concentrations of UDP-[^3H]GlcNAc (600 dpm/pmol) in a final volume of 1 ml. Results are the mean of two separate determinations.

**Fig. 2.**[^3H]Glucosamine-containing lipids of wild-type and mutant mnn2-2 *K. lactis*. Wild-type and mutant cells were grown in the presence of [^3H]glucosamine as described under "Materials and Methods." Total lipids (lanes 1 and 4) and lipids following mild alkaline hydrolysis (lanes 2 and 3) were separated by thin layer chromatography in chloroform, methanol, 0.22% aqueous KCl (55:45:10) and visualized by fluorography after 27 days.
not secondary to a defect in glycoprotein biosynthesis. The latter organelle presumably uses UDP-GlcNAc for the biosynthesis of Golgi-containing glycoproteins. Other yeast strains, such as Schizosaccharomyces pombe, contain galactose in their outer chains which presumably is transported into the Golgi lumen via UDP-galactose.

The antiporter for yeast Golgi transport of GDP-mannose has been characterized as being GMP; this molecule arises by the action of a Golgi lumenal GDPase. Biochemical, cell biological, and genetic studies have shown that the GDPase plays a pivotal role in the entry of GDP-mannose into the Golgi and subsequent mannosylation of proteins and lipids in vivo (10, 16, 21). Previous studies in vitro (4) have also shown that this GDPase has significant UDPase activity; thus, it is possible that the same GDPase protein may give rise to UMP, the putative antiporter for the UDP-GlcNAc transporter in K. lactis. At this time, it is not clear whether this yeast has a UDPase protein separate from the GDPase or whether the latter protein also mediates the in vivo degradation of UDP to UMP.

The availability of the yeast mutant described here makes this strain an attractive candidate for the cloning of the corresponding transporter. This in turn should allow determination of whether the transporter of UDP-GlcNAc for K. lactis is homologous to other yeast and mammalian transporters.

Acknowledgments—We thank K. Welch and A. Stratton for excellent secretarial assistance.

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