The VRG4 Gene Is Required for GDP-mannose Transport into the Lumen of the Golgi in the Yeast, Saccharomyces cerevisiae

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In the yeast Saccharomyces cerevisiae, glycoproteins and sphingolipids are modified in the Golgi by the addition of mannose residues. The critical mannosyl donor for these reactions is the nucleotide sugar, GDP-mannose, whose transport into the Golgi from the cytoplasm is required for mannosylation. This transport reaction has been well characterized, but the nucleotide sugar transporter has yet to be identified in yeast. VRG4 is an essential gene whose product is required for a number of Golgi-specific functions, including glycosylation and the organization of the endomembrane system. Here, data are presented that demonstrate that the primary role of Vrg4p is in the transport of GDP-mannose into the Golgi. The vrg4 mutation causes a general impairment in mannosylation, affecting N-linked and O-linked glycoprotein modifications as well as the mannosylation of sphingolipids. By using an in vitro assay, vrg4 mutants were shown to be specifically defective in the transport of GDP-mannose into Golgi vesicles. The Vrg4p protein localizes to the Golgi complex in a pattern that suggests a wide distribution throughout the Golgi. Vrg4p displays homology to other putative nucleotide sugar transporters, suggesting that the VRG4 gene encodes a Golgi GDP-mannose transporter. As Vrg4p is essential, these results suggest that a complete lack of mannosylation of glycoproteins in the Golgi leads to inviability. Alternatively, the essential function of Vrg4p in yeast involves its effect on sphingolipids, which would imply a critical role for mannossylinositol phosphorylceramides or mannosyl diphosphoinositol ceramides on growth and viability.

The Golgi complex is the site where the terminal glycosylation of both proteins and lipids occurs. Unlike mammalian cells, in the yeast Saccharomyces cerevisiae, glycoproteins and sphingolipids are exclusively modified by the addition of mannose residues in the Golgi. Glycoproteins can undergo two types of modifications in which oligosaccharides are linked to either asparagine residues (N-linked) or serine/threonine residues (O-linked) (for review see Refs. 1 and 2). Both of these glycosylation pathways initiate in the endoplasmic reticulum (ER) and terminate in the Golgi. After transport of the protein to the Golgi, most N-linked oligosaccharides are elongated by a series of different mannosyltransferases to form glycoproteins that contain outer chains of 50 or more mannose residues. The α1,6-linked outer chain is highly branched with α1,2- and α1,3-linked mannoses. As in higher eukaryotes, it appears that the various mannosyltransferases that catalyze these sequential reactions are compartmentalized from one another within the individual Golgi cisternae. In the case of O-linked sugars, up to five mannoses are added after the addition of the first mannose in the ER (3, 4). The phosphoinositol-containing sphingolipids in yeast also undergo mannosylation in the Golgi. In S. cerevisiae, there are three major classes of sphingolipids. These include the inositol phosphophorylceramides (IPCs) and the mannosylinositol phosphorylceramides (MIPC and M(IP)2C) (for review, see Ref. 5). MIPC and M(IP)2C contain a single mannose attached to the inositol (6), although little is known about the mannosyltransferase(s) that catalyzes this reaction.

The mannosyl donor for all of these Golgi-localized reactions is the nucleotide sugar GDP-mannose, whose site of synthesis is the cytoplasm. Before it can be utilized by the different luminal mannosyltransferases, GDP-mannose must be transported into the Golgi by a specific nucleotide sugar transporter (7). Once the sugar is donated to luminal mannosyltransferase acceptors, the nucleoside diphosphate GDP is converted to a monophosphate by a nucleoside diphosphatase (7). As in the mammalian Golgi, the transport of the nucleotide sugar into the lumen is coupled to the outward exit of the monophosphate in yeast. The yeast GDPase-encoding gene, GDA1, has been isolated (8). As predicted, a deletion of GDA1 results in the underglycosylation of proteins and lipids, although the null allele has no effect on growth.

Many nucleotide sugar transport activities have been reported, which differ from one another in their substrate specificity and subcellular localization (9). Since the cytoplasm is the sole site at which nucleotide sugars are synthesized, they must be transported into the various organelles in which glycosylation occurs. Mammalian cells require the transport of many different nucleotide sugars due to the diversity of carbohydrate processing in the Golgi. Carbohydrate chains may contain galactose, sialic acid, fucose, xylose, N-acetylgalcosamine, and N-acetylgalactosamine. In contrast, in S. cerevisiae, glycosylation in the Golgi is largely restricted to mannosylation which in principle requires only a single transporter.

The VRG4 gene is an essential gene that is required for a number of different Golgi-specific functions, including N-linked glycosylation (10–12), secretion, protein sorting, and the maintenance of a normal endomembrane system (12). The data in this report demonstrate that the transport of GDP-mannose into the Golgi is the principal function of the VRG4 gene product. A simple system to assay GDP-mannose transport is described, using permeabilized yeast cells. With this system, we demonstrate that vrg4 mutants are specifically defective in...
lumenal GDP-mannose transport in vitro. The protein sorting and membrane defects in vrg4 mutants may be explained by an indirect effect on sphingolipid mannosylation that normally occurs in the Golgi.

**MATERIALS AND METHODS**

**Yeast Strains, Media, and General Genetic Methods—**The yeast strains used in this study are listed in Table I. Media preparation and standard yeast genetic methods used for sporulation, tetrad dissection, and strain constructions have been described (13). YPAD liquid medium was supplemented with 0.5M KCl for the growth of the vrg4 mutant strains (10, 12). Hygromycin B (Boehringer Mannheim) was used to generate a linear fragment containing the Vrg4p TAG with the addition of formaldehyde for 3.7% for 30 min at room temperature. Cells were harvested, resuspended in 10 ml of 3m formaldehyde, 0.1 m KPO4, pH 6.8, and fixed for an additional 1–2 h at room temperature. Fixed cells were washed with 1 m sorbitol, 100 mM HEPES, pH 6.8, 5 mM NaN3, and spheroplasted by the addition of 10% sucrose, 1 m sorbitol, and 0.1% Triton X-100. After washing, cells were overlaid with mounting medium containing 25 ng/ml 4,6-diamidino-2-phenylindole.

**Radiolabeling of Cells and Lipid Analysis—**Cultures were grown to an A600 of 1 in Wickerham’s minimal medium (18), containing 2% glucose and lacking myoinositol (WH-I). Labeling was initiated by the addition of 5 µCi/ml [3H]myoinositol (American Radiolabeled Chemicals, St. Louis, MO). Cells were metabolically labeled for 10 min at 30 °C and chased by the addition of 4 volumes of WH containing 40 µg/ml unlabeled myoinositol. Lipids were extracted by the addition of chloroform/methanol (1:2) containing 1% ice-cold NaN3. Cells were washed once in NaN3, suspended in 100 mM HEPES, pH 6.8, 5 mM NaN3, and spheroplasted by the addition of formaldehyde for 3.7% for 30 min at room temperature. Fixed cells were washed with 1 m sorbitol, 100 mM HEPES, pH 6.8, 5 mM NaN3, and spheroplasted by the addition of 10% sucrose, 1 m sorbitol, and 0.1% Triton X-100. After washing, cells were overlaid with mounting medium containing 25 ng/ml 4,6-diamidino-2-phenylindole.

**Preparation of Permeabilized Yeast Cells—**Permeabilized yeast cells (YPY) were prepared as described (9) with minor modifications. 100–200 ml of cells were grown in YPAD medium containing 0.5 m KCl at 30 °C to an A600 of 1–2. After harvesting, cells were suspended at 50 absorbance units/ml in (0.75 × YPA, 0.5% glucose, 0.7 M sorbitol, 10 mM Tris-HCl, pH 7.5) and 10 units of lyticase/absorbance unit of cells was added to form spheroplasts. After 20 min incubation at 12°C, were used at a 1:10 dilution. Secondary anti-rabbit or anti-mouse antibodies (Amersham Corp.), conjugated to horseradish peroxidase, were used at a 1:3000 dilution and were detected by chemiluminescence (Amersham Corp.) followed by autoradiography.

**Indirect Immunofluorescence—**10 ml of logarithmic cultures (1–3 × 107 cells/ml) of SEY2510 (vrg4::LEU2) or Ty-OCH-HA (15) were fixed by the addition of formaldehyde for 3.7% for 30 min at room temperature. Fixed cells were washed with 1 m sorbitol, 100 mM HEPES, pH 6.8, 5 mM NaN3, and spheroplasted by the addition of 10% sucrose, 1 m sorbitol, and 0.1% Triton X-100. Cells were metabolically labeled for 10 min at 30 °C and chased by the addition of 4 volumes of WH containing 40 µg/ml unlabeled myoinositol. Lipids were extracted by the addition of chloroform/methanol (1:2) containing 1% ice-cold NaN3. Cells were washed once in NaN3, suspended in 100 mM HEPES, pH 6.8, 5 mM NaN3, and broken by vortexing with glass beads.

The lysate was removed from the glass beads, and lipids were extracted by adding 600 µl of chloroform/methanol (1:1) to 90 µl of the cell extract to achieve a final concentration of (10:10:3) chloroform/methanol/aqueous solution. After centrifugation, the pellet was re-extracted for 45 min with chloroform/methanol/H2O (10:10:3). The pooled lipid fractions were dried under N2 gas and desalted by phase separation. After cold and 0.1% Triton X-100, and broken by vortexing with glass beads.

**High performance thin layer chromatography** Silica Gel 60 plates (0.2 mm) were stained (110 °C) for 2 h and then cooled to room temperature prior to using. Samples were applied (150,000 cpm per lane), and ascending chromatography was performed using a chloroform/methanol, 0.22% KCl in H2O (55:45:10) solvent system (in tanks equilibrated with solvent for 1–2 h). After chromatography, plates were air-dried, sprayed with EN3HANCE (NEN Life Science Products) and fluorographed overnight.

**Preparation of Permeabilized Yeast Cells—**Permeabilized yeast cells (YPY) were prepared as described (9) with minor modifications. 100–200 ml of cells were grown in YPAD medium containing 0.5 m KCl at 30 °C to an A600 of 1–2. After harvesting, cells were suspended at 50 absorbance units/ml in (0.75 × YPA, 0.5% glucose, 0.7 M sorbitol, 10 mM Tris-HCl, pH 7.5) and 10 units of lyticase/absorbance unit of cells was added to form spheroplasts. After 20 min incubation at 12°C, were used at a 1:10 dilution. Secondary anti-rabbit or anti-mouse antibodies (Amersham Corp.), conjugated to horseradish peroxidase, were used at a 1:3000 dilution and were detected by chemiluminescence (Amersham Corp.) followed by autoradiography.
30 °C, over 80% of the yeast cells were converted to spheroplasts. Spheroplasts were centrifuged at 1,500 × g for 3 min and resuspended in 0.75 × YPA containing 0.7% sorbitol and 1% glucose. After incubating at 30 °C for 20 min to allow metabolic recovery, cells were washed with lysis buffer (400 mM sorbitol, 20 mM HEPES, pH 6.8, 150 mM potassium acetate, 2 mM magnesium acetate) and resuspended in lysis buffer at 300 absorbance units/ml. Aliquots of PYCs were slowly frozen over liquid nitrogen for 1 h and immediately transferred to −70 °C.

Standard GDP-mannose Transport Assay—GDP-mannose transport was measured in permeabilized cells. Reactions contained 20 mM buffer at 300 absorbance units/ml. Aliquots of PYCs were slowly frozen to one-half the original volume in buffer H. Reactions were initiated by mixing 5 μl of endogenous GDP-mannose. Membranes were concentrated to one-half the volume in buffer H. Reactions were initiated by mixing 5 μl of reaction buffer (buffer H) (20 mM HEPES, pH 6.8, 150 mM potassium acetate, 250 mM sorbitol, 5 mM magnesium acetate) to remove cytosol and endogenous GDP-mannose. Membranes were concentrated to one-half the original volume in buffer H. Reactions were initiated by mixing 5 μl of membranes (containing 10–20 μg of protein) with 20 μl of reaction buffer, bringing the final protein concentration to 0.4–0.8 mg/ml. Protein concentrations were determined using the BCA reagent (Pierce).

After incubating at 30 °C for 6 min, the reaction was stopped by adding 0.5 ml of ice-cold buffer H, and samples were placed on ice. Membranes were pelleted by centrifugation at either 14,000 × g or 100,000 × g in an ultracentrifuge (Beckman Optima TL). Free radioactivity in solutes was removed by washing the membrane pellet three times with 1 ml of ice-cold buffer H. Pellets were resuspended in 100 μl of 0.1% Triton X-100, and 50-μl sample was removed, added to 1 ml of scintillation mix, and radioactivity quantitated in a scintillation counter. The amount of GDP-[3H]mannose that nonspecifically bound to the outside of membrane was determined by measuring radioactivity of membranes at zero time of incubation and subtracting from the value of solutes associated with the membranes. The percent activity was calculated as follows:

\[
\text{percent activity} = \left(\frac{\text{cpm in pellet}_{\text{total}} - \text{cpm in pellet}_{\text{nsc}}}{\text{cpm in pellet}_{\text{total}}}\right) \times 100
\]

Each value was normalized by dividing the percent transport activity by the total amount of protein in each reaction, when comparing PYCs preparations of different strains.

Guanosine Diphosphatase Assay—GDPase was assayed as described (7) in solubilized P100 fractions prepared from PYCs from strains JPY25 6c (VRG4) or JPY26 3d (vrg4-2). Inorganic phosphate was determined by the method of Ames (21). One unit of GDPase is defined as the amount of GDP-[3H]mannose that nonspecifically bound to the outside of membrane was determined by measuring radioactivity of membranes at zero time of incubation and subtracting from the value of solutes associated with the membranes. The percent activity was calculated as follows:

\[
\text{percent activity} = \left(\frac{\text{cpm in pellet}_{\text{total}} - \text{cpm in pellet}_{\text{nsc}}}{\text{cpm in pellet}_{\text{total}}}\right) \times 100
\]

Each value was normalized by dividing the percent transport activity by the total amount of protein in each reaction, when comparing PYCs preparations of different strains.

RESULTS

The vrg4 Mutant Is Defective in Both N- and O-Linked Sugar Modifications—VRG4 is required for N-linked glycosylation (10–12). To assay effects on O-linked glycosylation, we examined the glycosylation state of chitinase. Chitinase is a secreted protein that contains carbohydrates that are exclusively O-linked. Therefore, any effect on O-linked glycosylation can be detected by an electrophoretic mobility shift (22). Whole cell extracts were prepared from isogenic wild type and vrg4-2 cells and assayed by immunoblotting, using anti-chitinase antiserum. As a control, chitinase mobility was also examined in cells containing an mnn10-2 mutation, which are defective only in N-linked glycosylation (23). A mobility shift was detected in chitinase from vrg4-2 when compared with wild type cells but not in mnn10-2 cells (Fig. 1). This result demonstrates that the vrg4 mutation affects O-linked glycosylation and therefore is required for the glycosylation of both classes of proteins.

The vrg4 Mutant Is Defective in Sphingolipid Mannosylation—vrg4-2 cells display an aberrant morphology of intracellular membranes when viewed by electron microscopy (12). In vrg4 mutants, membranes accumulate but stain poorly with potassium permanganate. This observation suggested that the VRG4 gene product may be required for maintaining the normal protein/lipid ratio of these Golgi membranes whose staining properties are altered by the vrg4 mutation. The synthesis of sphingolipids in yeast requires vesicular transport to the Golgi and suggests that their synthesis occurs in this compartment (24). Therefore, it was of interest to determine whether the vrg4 mutation affected sphingolipid biosynthesis. In S. cerevisiae, there are three major classes of sphingolipids. These include the IPCs and the MIPC, and M(IP)2C (see Ref. 5 for review). To test the idea that VRG4 is required for sphingolipid biosynthesis, we compared [3H]inositol-labeled lipids in isogenic vrg4-2 mutant and wild type strains. Cells were labeled for 10 min with [3H]inositol and chased for 20 or 40 min. Lipids were extracted and analyzed by thin layer chromatography. The most significant difference between wild type and vrg4-2 cells was the failure of the vrg4-2 strain to accumulate MIPC and M(IP)2C (Fig. 2). These results demonstrate that VRG4 is required for the biosynthesis of sphingolipids and suggest that the defect specifically affects the Mannosylated forms.

Development of an in Vitro GDP-mannose Transport System Based on Permeabilized Yeast Cells—The effect of the vrg4 mutation on glycoprotein and sphingolipid biosynthesis suggested that VRG4 is generally required for mannosylation in the Golgi. A simple model that could explain the pleiotropic phenotype of the vrg4 mutant is that VRG4 is required for the accumulation or transport of GDP-mannose into the lumen of the Golgi.

To test this model, GDP-mannose transport activity was compared in isogenic wild type and vrg4-2 mutant strains. Lumenal GDP-mannose transport in vitro in yeast has been characterized using crude Golgi-enriched vesicles (7). Using this system, we routinely observed a decrease in the activity of mutant membranes compared with wild type (data not shown). However, this method involves large scale cell preparations, where reactions typically require the addition of milligram quantities of protein. To allow the processing of more samples simultaneously for comparative purposes, we sought to develop another system to measure GDP-[3H]mannose transport at an analytical scale. For this purpose, PYCs were used. PYCs are highly competent for glycosylation in vitro when supplemented with GDP-mannose (20) and therefore must be capable of efficient lumenal GDP-mannose transport.

GDP-mannose transport was characterized in permeabilized yeast cells containing a dpm1 mutation that results in a 90–95% decrease of dolichol phosphate-mannose synthase (Dpm1p) activity in vitro (25). This mutant background was required to eliminate a competing reaction catalyzed by Dpm1p, in which GDP-mannose donates mannose to form dolichol phosphate-mannose (Dol-P-Man) that in turn acts as the mannose donor for glycosylation in the ER. This ER reaction, which is quite efficient in vitro, would otherwise obscure the Golgi transport of GDP-mannose (7). A comparison of the [3H]mannose uptake into sealed membranes of isogenic strains that were wild type (JPY25 6b) or that contained the dpm1 mutation (JPY25 6c)
demonstrated that mannose incorporation into Dol-P-Man accounted for greater than 60% of the observed $^3$H uptake (data not shown). Therefore, all of the experiments described below were conducted with isogenic strains harboring the dpm1 mutation, which did not otherwise affect the growth properties of these strains (data not shown).

To assay GDP-mannose uptake, after incubating PYCs in the presence of GDP-[H]mannose, the amount of [H]mannose associated with washed vesicles was compared with that which remained in the supernatant (S100). Vesicles were prepared by centrifugation at 100,000 g (P100) with extensive washes to remove bound radiosolutes. A time course of $^3$H uptake suggested that transport of GDP-mannose was quite efficient. Typically 20–30% of the $^3$H in the reaction was recovered in the P100 fraction after 6–8 min, corresponding to an uptake of about 25 pmol of GDP-mannose (Fig. 3A). The rate of transport was linear with time up to 6 min (Fig. 3A) and with protein concentration in a range from 0.4 to 1.2 mg/ml (Fig. 3B). Transport was temperature-dependent; optimal transport occurred at 30 °C, was slightly reduced at 25 and 42 °C, and inhibited at temperatures above 60 °C (data not shown).

GDP-mannose uptake was completely inhibited by the addition of detergent (0.1% Triton X-100) with transport reduced to less than 2%, demonstrating that the accumulation of GDP-mannose requires intact vesicles. Similarly, inclusion of 4 mM 4,4-diisothiocyanostilbene-2,2-disulfonic acid, a stilbene derivative that is known to inhibit transport of nucleotide sugars in both mammalian (26) and yeast (7) systems, completely inhibited activity. As demonstrated previously, (7), transport was not dependent on energy nor on divalent cations as the addition of ATP, Mg²⁺, or EDTA did not affect the efficiency of transport (data not shown). However, we infer that removal of Mg²⁺ or inclusion of EDTA did affect the activity of endogenous acceptor glycosyltransferases that utilized the labeled mannose, since the transport was stimulated about 2-fold in the presence of Mg²⁺ (data not shown).

The physical properties of lumenal radioactive material was

![Figure 2](image.png)  **Fig. 2. Analysis of sphingolipids in vrg4-2 and wild type cells.** Sphingolipids in RSY255 (VRG4) or NDY5 (vrg4-2) were pulse-labeled with [H]myoinositol and chased for 20 or 40 min with unlabeled inositol, extracted, and separated by thin layer chromatography as described under “Materials and Methods.” The assignment of phosphatidylinositol (PI), IPCs, MIPC, and M(IP)₂C, denoted by arrows, is based upon a comparison of their mobility on TLC with those reported in the literature and upon their relative abundance (6).

![Figure 3A](image.png)  **A**  **Fig. 3. GDP-mannose transport in permeabilized yeast cells as a function of time and protein concentration.** PYCs (prepared from strain JPY25 6c) were incubated in reaction buffer containing 3 µM GDP-mannose (GDP-man) and 50 nCi of GDP-[H]mannose (15 Ci/nmol) in a final volume of 25 µl, as described under “Materials and Methods.” A, shows the transport of GDP-[H]mannose as a function of time, in which reactions were carried out in a final protein concentration of 0.5 µg/µl. B, shows the protein dependence of the reactions, carried out for 6 min at 30 °C under conditions described above. 20 pmol of GDP-mannose transport corresponds to a luminal uptake of 27% of the GDP-mannose in the reaction. Typical values for the absolute cpm transported into vesicles prepared from a 25-µl reaction range from 6,000 to 12,000 cpm in over 10 separate experiments.  **Fig. 3B**
examinined by analyzing the transport reaction products after phase partitioning. This separates lipid-linked oligosaccharides, which partition into the organic phase from protein-water-soluble products as described by Waechter et al. (27). By this assay, most of the radioactive products (87%) that associated with the membranes were water-soluble (GDP-mannose) or chloroform/methanol-insoluble (protein). We conclude that GDP-mannose transport in PYCs appears to have all of the hallmarks previously described for this activity in crude Golgi membranes (7).

The VRG4 Gene Product Is Required for Lumenal Golgi GDP-mannose Translocation—To test the model that VRG4 is required for GDP-mannose transport, PYCs were prepared in parallel from wild type and vrg4 mutant cells, and their transport activity was compared. Transport activity in VRG4 (JPY25 6c) and vrg4-2 (JPY26 3d) strains was examined as a function of time. In contrast to wild type cells, where greater than 25% of the exogenous GDP-[3H]mannose was transported, vrg4-2 membranes displayed a severe defect in GDP-mannose uptake (<2% transport) (Fig. 4). This defect was partially complemented in the vrg4-2 mutant strain by a plasmid bearing the VRG4 gene (Fig. 4). This is consistent with the observation that this plasmid does not fully complement the vrg4-2 mutant glycosylation phenotype in vivo (data not shown).

To determine whether the effect of the vrg4-2 mutation was specific for GDP-mannose uptake, the activity of another Golgi protein was assayed in solubilized P100 fractions prepared from wild type and vrg4-2 PYCs. As shown in Table II, the level of GDPane activity in wild type or vrg4-2-derived P100 fractions was essentially indistinguishable. Vrg4p is therefore specifically required for GDP-mannose uptake.

VRG4 Is a Resident Golgi Protein—The VRG4 gene product is required for a number of different Golgi functions (12). If these effects are due to its role in nucleotide sugar uptake, Vrg4p would be predicted to reside in the Golgi complex. To determine the intracellular localization of Vrg4p, the VRG4 gene was tagged at the carboxyl terminus with three tandem copies of the HA epitope (see "Materials and Methods"). Even when tagged with three copies of the HA epitope, when expressed as a single copy, Vrg4-HA3p in whole cell extracts was barely detectable by immunoblot analyses (Fig. 5A). Although the HA-tagged form of VRG4 did not complement the slow growth phenotype of the vrg4-2 mutant to the same extent as the wild type VRG4 gene, it was able to complement the sensitivity to hygromycin B as well as the lethality of a VRG4 deletion (data not shown). This suggests that the carboxyl-terminal addition of the HA epitope does not significantly alter the normal function of the Vrg4 protein.

The intracellular location of Vrg4-HA3p was examined by indirect immunofluorescence, using antibody directed at the HA epitope. A punctate pattern of fluorescence, characteristic of the Golgi complex, was observed in cells expressing Vrg4-HA3p (Fig. 5B). This staining pattern was similar to another Golgi-localized protein, Och1p, an initiating α,1,6-mannosyltransferase (Fig. 5B). One difference in the staining pattern of these two proteins was that generally more punctate spots were observed in the Vrg4-HA3p-expressing cells. In most of the Vrg4-HA3p-expressing cells observed, the average number of HA-staining spots per cell observed by shifting the plane of focus was 20–25. This was confirmed by performing a Z-series in which the analysis of optical sections of 1-μm thickness through individual cells indicated an average number of 25 spots per cell (data not shown). Cells expressing Och1-HA3p contained between 7 and 10 spots/cell, and no qualitative differences were observed in cells overexpressing Och1p. From these results, we conclude that Vrg4p resides in the Golgi complex. Taken together with immunoelectron microscopy studies which suggest that the yeast Golgi is comprised of about 30 spot-like structures (28), it appears that unlike the more spatially restricted Och1p, the Vrg4 protein is broadly distributed throughout the Golgi complex.

Homology to VRG4 Predicts the Existence of Other Putative S. cerevisiae Nucleotide Sugar Transporters—VRG4 encodes a highly conserved protein. Thirteen different members have been identified including the Leishmania LPG2 and the Kluyveromyces lactis MNN2 gene products (12, 29, 30). In the case of Lpg2p and Mnn2p, both proteins have been implicated as nucleotide sugar transporters (29–31). A search of the S. cerevisiae genome data base identified several other yeast ORFs with sequence similarity to Vrg4, suggesting that these putative proteins may function in nucleotide sugar transport. These putative yeast proteins are listed by ORF name in Table III. One of these ORFs (Yer039p), which we have designated HVG1 (for Homologous to VRG4), encodes a predicted protein that is highly similar to Vrg4p (80% identical). Although the other proteins listed in Table III are more distantly related to Vrg4 and Hvg1p (about 25% identical and 45% similar along their length), each of these proteins are of a similar size (35–45
GDP-mannose Transport Requires VRG4

The Vrg4 protein sequence was used to search the S. cerevisiae genome data base using the BLAST algorithm (41), and the identified proteins were aligned using DNASTAR MegAlign program with the Clustal algorithm. The accession numbers for each are as follows: (hvg1 = Yem9/yer039p accession number, P40027); Yea4/yel004p accession number P40004; YMD8 accession number, Q03697; Yor306c, accession number, Q04835; Ym018C accession number, Z46659x21.

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DISCUSSION

The transport of GDP-mannose into the lumen of the Golgi is requisite for glycosylation of both proteins and lipids in S. cerevisiae. The work presented here shows that the VRG4 gene is required for this transport event and suggests that VRG4 encodes the nucleotide sugar transporter. Our major observations and conclusions are as follows. (i) vrg4 mutants accumulate under-mannosylated proteins and sphingolipids in vivo. (ii) Membranes from the vrg4 mutant are specifically impaired in the luminal uptake of GDP-[3H]mannose in vitro. Although these mutant membranes are drastically reduced in the ability to transport GDP-mannose, the activity of another Golgi enzyme, GDPase, is unaffected. (iii) VRG4 encodes a protein that is part of a large family of related proteins, some of which are known to function in nucleotide sugar transport. (iv) As predicted, Vrg4p is a resident of the Golgi complex. Although we cannot formally rule out the possibility that VRG4 encodes a regulator of the GDP-mannose transporter, the data demonstrate a pivotal role for Vrg4p in nucleotide sugar transport.

Several putative nucleotide sugar transporters exhibit a high degree of similarity to Vrg4p (12, 29). These include the Leishmanias Lpg2p which also regulates Golgi GDP-mannose transport (29) and the K. lactis Mnn2p which regulates UDP-GlcNAc transport (30, 31). Lpg2 is most similar to Vrg4, whereas Mnn2p is more related to another S. cerevisiae ORF, Yel004p (30). The similarity between Mnn2p and Vrg4p is somewhat surprising based upon their different substrate specificities. In K. lactis, glycoproteins are terminally modified by the addition of N-acetylglucosamine and require the luminal transport of UDP-GlcNAc in the Golgi. Glycoproteins in S. cerevisiae do not undergo this modification in the Golgi. Instead, they are modified entirely by mannose (see Ref. 2). Therefore, the homology between Mnn2p and Vrg4p must be...
clude domains that are not involved in substrate specificity. The vr4g mutation specifically affected MIPC and M(IP)2C biosynthesis. Coupled with the demonstration that VRG4 is a resident Golgi protein, these data are consistent with the hypothesis that mannosylation of IPC to form MIPC and M(IP)2C is catalyzed by a glycosyltransferase that resides in the Golgi and utilizes GDP-mannose (24). These results support the conclusions of Conzemmlen and co-workers (24), showing that the biosynthesis of MIPC and M(IP)2C is dependent on vesicular transport from the ER to Golgi, suggesting that these molecules are made in the Golgi.

VRG4 is an essential gene that is pleiotropically required for a number of different Golgi functions, including secretion and the maintenance of normal membrane morphology. These observations led to the proposal that Vrg4p plays an important role in establishing or maintaining the organization of the Golgi (12). The effect on sphingolipid biosynthesis may explain the pleiotropy associated with the vr4g phenotype. Sphingolipids are essential membrane components. Although their biological role in yeast is still unclear, there is evidence that sphingolipids modulate the activity of the plasma membrane ATPase (32) and are involved in phospholipid biosynthesis (33) and anchoring of cell-surface glycosylphosphatidylinositol-linked proteins (34). Furthermore, the enrichment of sphingolipids in organelles of the secretory pathway (35) supports the notion that the processes of protein secretion and the intracellular trafficking of sphingolipids are linked processes. VRG4 may indirectly affect these processes by inhibiting the synthesis of MIPC and M(IP)2C in the Golgi. The lack of these mature forms indirectly affect these processes by inhibiting the synthesis of MIPC and M(IP)2C, which in turn affects a range of biological functions, including secretion.

What is the essential role of VRG4? Both N- and O-linked glycosylation are essential, but it appears that only modifications that occur in the ER are vital. For instance, mutations that block the transfer of the preassembled lipid-linked oligosaccharide onto the asparagine residues of proteins are lethal (Ref. 36 and see Ref. 37 for review). Similarly, mutants that block the transfer of the preassembled lipid-linked oligosaccharide onto the asparagine residues of proteins are lethal. Mutations that completely lack the enzymes which catalyze the addition of the saccharide onto the asparagine residues of proteins are lethal. Mutations that block the transfer of the preassembled lipid-linked oligosaccharide onto the asparagine residues of proteins are lethal. For instance, mutations including secretion.

Biosynthesis of MIPC and M(IP)2C is dependent on vesicular transport from the ER to Golgi, suggesting that these molecules are made in the Golgi.