Distinct Protein Domains of the Yeast Golgi GDP-mannose Transporter Mediate Oligomer Assembly and Export from the Endoplasmic Reticulum*

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The substrates for glycan synthesis in the lumen of the Golgi are nucleotide sugars that must be transported from the cytosol by specific membrane-bound transporters. The principal nucleotide sugar used for glycosylation in the Golgi of the yeast Saccharomyces cerevisiae is GDP-mannose, whose lumenal transport is mediated by the VRG4 gene product. As the sole provider of lumenal mannose, the Vrg4 protein functions as a key regulator of glycosylation in the yeast Golgi. We have undertaken a functional analysis of Vrg4p as a model for understanding nucleotide sugar transport in the Golgi. Here, we analyzed epitope-tagged alleles of VRG4. Gel filtration chromatography and co-immunoprecipitation experiments demonstrate that the Vrg4 protein forms homodimers with specificity and high affinity. Deletion analyses identified two regions essential for Vrg4p function. Mutant Vrg4B proteins lacking the predicted C-terminal membrane-spanning domain fail to assemble into oligomers (Abe, M., Hashimoto, H., and Yoda, K. (1999) FEBS Lett. 458, 309–312) and are unstable, while proteins lacking the N-terminal cytosolic tail are stable and multimerize efficiently, but are mislocalized to the endoplasmic reticulum (ER). Fusion of the N terminus of Vrg4B to related ER membrane proteins promote their transport to the Golgi, suggesting that sequences in the N terminus supply information for ER export. The dominant negative phenotype resulting from overexpression of truncated Vrg4-DΔN proteins provides strong genetic evidence for homodimer formation in vivo. These studies are consistent with a model in which Vrg4p oligomerizes in the ER and is subsequently transported to the Golgi via a mechanism that involves positive sorting rather than passive default.

The Golgi complex serves as the intracellular site for the terminal carbohydrate modifications of proteins and lipids. These modifications are essential for life and play a variety of important biological roles, from protein folding to the regulation of cell surface properties. The substrates for the carbohydrate modification of both glycoproteins and glycolipids in the Golgi are nucleotide sugars, whose site of synthesis is the cytosol. These molecules must be transported into the Golgi lumen by membrane-bound nucleotide sugar transporters (NSTs) to be utilized by the glycosyltransferases. The current model for the transport of nucleotide sugars by the NSTs involves a one-for-one exchange reaction, in which the lumenal transport of a nucleotide sugar from the cytoplasm is coupled to the equimolar exit of the corresponding nucleotide monophosphate (2–4). The nucleotide monophosphate is generated through the action of the glycosyltransferases and nucleoside diphosphatases in the lumen of the Golgi. As a consequence of their role in substrate provision, the NSTs play an indispensable role in glycoconjugate synthesis, best evidenced by the severe phenotype of mutants with defects in Golgi transport of nucleotide sugars (for review, see Refs. 5 and 6).

Many NST activities have been reported, which differ from one another in their substrate specificity. The diversity of glycosylation reactions in the mammalian Golgi requires the transport of many different nucleotide sugars and a correspondingly large number of NSTs. In contrast, the vast majority of carbohydrate modifications in the yeast Golgi are restricted to mannose additions that utilize GDP-mannose as the nucleotide sugar substrate (7). In the yeast, Saccharomyces cerevisiae, lumenal GDP-mannose transport requires the VRG4 gene product (8). Mutations in this gene lead to a loss of nucleotide sugar transport in vitro and the underglycosylation of glycoproteins and glycosphingolipids in vivo. A deletion of the VRG4 gene is lethal, demonstrating that mannosylation in the Golgi is essential (9). Its strong homology to the Leishmania GDP-mannose transporter as well as to a large number of other NSTs (8, 10) argues that the Vrg4 protein functions as a transporter per se, rather than as a regulator of transport. It is the concerted action of both the glycosyltransferases and the NSTs that ultimately dictate glycoconjugate synthesis. Although much is known about the glycosyltransferases, relatively little is known about the biochemical and molecular basis by which the NSTs transport nucleotide sugars from the cytosol into the Golgi. We have undertaken an analysis of the Vrg4 protein to gain a better understanding of NST function. Here data are presented that suggest this protein functions as a homodimer. In addition, deletion analysis of mutant proteins has enabled us to identify a region of the protein that is essential for its exit from the ER and proper Golgi localization but that, when mutated, does not interfere with protein stability or oligomerization. This N-terminal region is distinct from a C-terminal domain required for oligomerization (1) and protein

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The abbreviations used are: NST(s), nucleotide sugar transporter(s); TEV, tobacco etch virus; PCR, polymerase chain reaction; ORF, open reading frame; HA, hemagglutinin; PMSF, phenylmethylsulfonyl fluoride; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; UTR, untranslated region; TMD(s), transmembrane domain(s).

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stability. Like Vrg4 proteins lacking the cytosolic N-terminal domain, these Vrg4C proteins are also retained in the ER (1) but probably because they are misfolded and incompletely assembled. When present on a protein that resides in the ER, a sequence homologous to the N terminus of Vrg4p targets the heterologous protein to the Golgi, suggesting that sequences in the N terminus act as a positive ER export signal.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media—**Standard yeast media and genetic techniques were used (11). Hygromycin B sensitivity was tested on yeast extract/petrole/adenine sulfate/dextrose plates (YPAD) supplemented with 50 μg/ml hygromycin B (Roche Molecular Biochemicals) as described previously (12). SEY6210 (MATa ade2–1 ura3–52 leu2–3, 112 his3–11 trp1–1 leu2–3, 112 can1–100) was used as the parental strain for the construction of XGY10 and XGY12, in which the chromosomal VRG4 or GDA1, respectively, are replaced with those that contain a C-terminal tobacco etch virus (TEV) protease cleavage site fused to a protein A tag (see below). XGY11 was constructed from W303α (MATα ade2–1 ura3–1 his3–11 trp1–1 leu2–3, 112 can1–100) and contains a replacement of the normal Vrg4C locus with an allele in which Vrg4p is tagged at the N terminus with a triple HA-TEV tag and is under the control of the GAL1 promoter. NDY5 (MATα ura3–52 leu2–211 vrg4–2) (9) was used for complementation analysis of the Vrg4C protein. XGY13 is a derivative of RSY255 (MATa leu2–3, 112 can1–100) and contains an altered version of the vrg4–2 allele that encodes additional methionines in the N-terminal region of Vrg4p. XGY14 is a derivative of RSY255 and contains an altered version of the vrg4–2 allele that encodes additional methionines in the N-terminal region of Vrg4p.

**Plasmids—**Plasmids used in this study are listed in Table I. Standard molecular biology techniques were used for all plasmid constructions (19). To construct pRHL-myc3, the VRG4 gene was cloned in-frame to sequences encoding three tandem copies of the myc epitope (EQKLQISEEDL). A HindIII/SalI fragment containing the VRG4 ORF lacking the stop codon was isolated by PCR and cloned into pSK–P/MyC, a derivative of Bluescript SK (Stratagene). pSK–P/MyC carries a 172-base pair fragment encoding three tandem copies of the myc epitope, cloned between the PstI and XbaI sites of pBluescript SK. This fragment was placed under the control of the TDH3 promoter at the C terminus. SK–P/MyC was used to generate pRHL-myc3, which contains VRG4-myc3 under the control of its own promoter, on an EcoRI/HindIII fragment in the CEN6/URA3 vector, pRS316 (20). This EcoRI/HindIII fragment from pRHL-myc3 was cloned into YEplac181 (21) to generate YEplac181-RHL-myc3, to allow expression in a 2μ/LEU2 vector and into YeEp352 (22) to generate YeEp352-RHL-myc3, to allow expression in a 2μ/URA3 vector. Identical constructs, but containing the triple HA tag at the C terminus are pRHL-HA3, YEplac181-RHL-HA3, and YeEp352 RHL-HA3.

A series of plasmids containing vrg4 alleles with 5′ deletions were constructed by PCR amplification using pSK–P/MyC as the template plasmid (8). Each of the deletion constructs shares the same 3′ end, including sequences encoding the C-terminal triple HA-tag. KpnISmaI and NruISmaI fragments, amplified by PCR, containing deleted portions of the vrg4 gene were ligated into the KpnISmaI sites of pYEplac181 (20) to place them under the control of the glyceroldehyde-3-phosphate dehydrogenase (TDH3) promoter in a 2μ/LEU2 yeast expression plasmid. An ATG sequence in YEplac181 encodes the initiating methionine in each of these vrg4Lαv alleles. 5′ primers for these PCR reactions were designed to amplify from the coding sequence in the 16th, 45th, or 79th amino acid of Vrg4p, respectively, to generate the series pYEplac181-5′-NαH3, pYEplac181-5′-SαH3, and pYEplac181-5′-NαH3. Similarly, an EcoRI/StmaI fragment containing the entire wild type VRG4-1 gene was amplified by PCR and ligated into the EcoRI/PvuII sites of YeEp352GAP vector to create pYEplac181VRG4-1. An analogous series of plasmids, encoding Vrg4LαV proteins that were myc-tagged, was derived from the YeEpGAPαN plasmids described above by replacing the 3′-half of each
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VG4 gene with a HpaI/UcoI fragment from pRHL-nyc3. Each of the BamHI/SacI fragments were inserted into YEplac181. This series of plasmids encode Vgr4p with N-terminal deletions (Δ15, Δ44, and Δ78) and a C-terminal myc epitope, whose expression is under the control of the TDI3 promoter in a 2μL/2C plasmid.

To encode the N-terminal VG4 deletion mutants, we first made a vector (YEpGAN-myc3) containing a fragment encoding three copies of the myc epitope, flanked by an EcoRI/KpnI site and containing an initiating ATG, in YEpGAP. KpnI/UcoI DNA fragments containing deleted 3’ terminal of VG4 were amplified and ligated into the KpnI/UcoI sites of YEpGAP, in-frame and 3’ to the myc3 epitope. The first 669 codons of Vgr4p, which are the transmembrane region, were designed to be immunogenic using rules that last 60 amino acids of Vgr4p to generate YEpGAP36C-myc3, YEpGAP13C-myc3, and YEpGAP34C-myc3. These plasmids encodeVgr4p C-terminal deletions with an N-terminal myc epitope, whose expression is under the control of the TD3 promoter in a 2μ/L/2C plasmid.

An HA-tagged allele of the HVG1 gene was created in several steps. First, a fragment containing the HVG1 ORF, lacking the stop codon and flanked by a 5’ HindIII and a 3’ NsiI site was amplified by PCR from yeast genomic DNA and cloned into pSK P2X/HA3 (24) to generate pSK HVG1-HA3. This plasmid encodes Hvg1p with the HA3 epitope at the C terminus. A HindIII/NsiI fragment from SK-HVG1-HA3 containing HA-tagged HVG1 was cloned into pRS316TPI (9) to generate pRS TPI-HVG1-HA3. This places HA-tagged Hvg1p under the control of the TPI promoter in a CEN6/URA3 yeast expression plasmid. Yeptp1-HVG1-HA3 contains the SalI/SacI fragment from pRS TPI-HVG1-HA3, in pYEp352.

To construct a plasmid that encodes Hvg1p with a N-terminal extension homologous to that of Vgr4p, site-directed mutagenesis was used to change the stop codon located 60 base pairs upstream the first ATG of the HVG1 ORF to an arginine codon normally found at amino acid 78 of the Vgr4 protein. A HindIII/NsiI fragment containing the entire HVG1 ORF as well as 275 base pairs of 5’-flanking sequences was isolated by PCR and cloned into pSK P2X/HA3 to place the HA3 epitope at the 3’-end. This plasmid was used as template DNA for site-directed mutagenesis of HVG1, using the QuikChangeTM Site-Directed Mutagenesis kit (Stratagene). The mutagenic primers change the 7 of the stop codon (TGA) to a C to produce an arginine codon (CGA). This generated a 98-amino acid extension in the N terminus of HVG1 ORF to create pSK mHVG1-HA3. A 1.1-kilobase pair HindIII/NsiI fragment from pSK mHVG1-HA3 was cloned into pRS316TPI to make pRSTPI-mhVG1-HA3. This places the mutagenized HVG1 (mHVG1) under the control of the TPI promoter in a CEN6/URA3 yeast expression plasmid. Similar to Yeptp1-HVG1-HA3, Yeptp1-mHVG1-HA3, was also generated by using the SalI/SacI fragment from pRS TPI-HVG1-HA3, in pYEp352.

A fragment containing the entire YPL244C ORF was isolated by PCR from yeast genomic DNA and cloned into pSK P2X/HA3 to place the HA3 epitope at the 3’-end of Ypl244p. YeptGAPyl244-HA3 and YeptGAPpy2244344-HA3 were constructed by cloning a KpnI/UcoI fragment (amplified by PCR) into YEpGAP, YEpGAP-Cem3, Ypl244-41-HA3, and YEpGAP-Cem3-41-HA3, by fusing a fragment encoding the N-terminal 47 amino acids of Vgr4p in-frame into the EcoRI/KpnI site of YEpGAPpy2244344-HA3.

Preparation of Cell-free Lysates—Exponentially growing yeast cells (Ao20 = 1–3) were harvested and converted to spheroplasts with lyticase, as described previously (25). Spheroplasts from 3–4 OD units of cells were resuspended in 400 μl of ice-cold lysis buffer (150 mM NaCl, 10 mM HEPES-KOH (pH 7.5), 5 mM MgCl2, 1 mM PMSF) containing either 1% digitonin or 1% Triton X-100. 200 μl of extract were fractionated over a gel filtration column (Superose 6 HR 10/30, Amersham Pharmacia Biotech) equilibrated with 150 mM NaCl, 5 mM HEPES-KOH (pH 7.5), 5 mM MgCl2, 1 mM PMSF, containing either 1% Triton X-100 (for Triton extracts) or 1% digitonin (for digitonin extracts), using the FPLC system (Amersham Pharmacia Biotech). FPLC was performed at a flow rate of 0.2 ml/min, and 1-ml fractions were collected. Fractions were analyzed for the presence of Vgr4-HAp by immunoblotting with Rabbit anti-mouse IgGs for the detection of the protein A epitope or 12CA5 for the detection of the HA epitope, as described above.

Indirect immunofluorescence of yeast cells was performed as described previously (8). Samples were observed with a Zeiss Axioscop and photographed with a Sony DFC-9000 cooled CCD camera. Images were captured using NIH Image software, and all processing was done with Canvars (v.5) (Deneba).

RESULTS

Vgr4p Fractionates as a Homodimer during Chromatography—Using co-immunoprecipitation assays of detergent-solubilized, epitope-tagged Vgr4 proteins, coincident with other studies (1) we found that Vgr4p exits as a multimer. To investigate the oligomeric properties, the molecular weight of the Vgr4p-containing complex was examined by gel filtration chromatography. Detergent extracts prepared from yeast expressing an HA-tagged VGR4 allele were fractionated by FPLC over a Superose 6 column. This tagged allele can complement the hygromycin B sensitivity of a vrg4 mutant, indicating that this

Co-immunoprecipitation, Western Immunoblotting, and Immunofluorescence—The HA-tagged proteins were immunoprecipitated by incubating 400 μl of the detergent extract (described above) with 200 μl of a hybridoma cell culture supernatants containing the 12CA5 monoclonal anti-HA antibody and 25 μl of protein A-Sepharose (Amersham Pharmacia Biotech) at room temperature for 2 h. Immunoprecipitation of myc-tagged proteins was done identically, except we used culture supernatants containing the monoclonal anti-myc antibody and the incubations were carried out at 4°C overnight. The protein A-Sepharose beads and associated proteins were centrifuged and washed three times with the same lysis buffer (1% digitonin or 1% Triton X-100, 150 mM NaCl, 50 mM HEPES-KOH (pH 7.5), 5 mM MgCl2, 1 mM PMSF). After resuspending in Laemmli’s sample buffer and solubilizing at 4°C for 1 h, the proteins were fractionated by 10% SDS-PAGE, transferred to Immobilon-polyvinylidene difluoride membranes (Millipore) and immunoblotted with anti-HA (Y-11) or anti-myc A-14 rabbit polyclonal antibodies (Santa Cruz Biotechnology). Secondary anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham Pharmacia Biotech) were used at a 1:3000 dilution and detected by chemiluminescence (ECL, Amersham Pharmacia Biotech).

Whole cell protein extracts were prepared by trichloroacetic acid precipitation, as described previously (25). Proteins were separated by SDS-PAGE and detected by Western immunoblotting using anti-HA or anti-myc antibodies, as described previously (9). Culture supernatants, containing the monoclonal anti-HA antibody, 12CA5, or the monoclonal anti-myc antibody, 9E10, were used at a 1:10 dilution. Rabbit polyclonal IgG from the HA epitope (A-14) and mouse IgGs for the detection of the protein A tag, were used at a 1:5000 dilution. Secondary anti-rabbit or anti-mouse antibodies (Amersham Pharmacia Biotech), conjugated to horseradish peroxidase, were used at a 1:3000 dilution and were detected by chemiluminescence (ECL, Amersham Pharmacia Biotech).

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epitope does not alter the normal function of Vrg4p (8). Protein extracts were prepared under conditions that favored stable Vrg4p-containing oligomeric complexes, assayed by co-immunoprecipitation of HA- and myc-tagged Vrg4 proteins (e.g. see Fig. 4 and “Experimental Procedures”). Either 1% Triton X-100 or 1% digitonin was used to solubilize proteins, and extracts were fractionated in the same lysis buffer, to maintain stable complexes throughout the procedure. Fractions were analyzed for the presence of Vrg4-HAp by SDS-PAGE, followed by immunoblotting with HA-specific antibodies.

A comparison of the distribution of Vrg4-HAp and molecular weight standards showed that the peak position of Vrg4-HAp elution corresponds to a molecular mass of about 67 kDa (Fig. 1), which is about twice the predicted molecular mass of monomeric Vrg4p. The peak of Vrg4p elution was the same whether the fractionation was performed with buffer containing 1% Triton X-100 or 1% digitonin (Fig. 1). This result suggested that the size of Vrg4p-containing complex is similar in the presence of both 1% Triton and 1% digitonin. The majority of Vrg4p-HAp prepared from digitonin extracts was recovered in a sharp, symmetrical peak. No material of a lower molecular weight could be detected, suggesting that most of the Vrg4p extracted from cells by digitonin exists as a homogenous higher molecular weight species. In contrast, when prepared from Triton extracts, Vrg4p was recovered in a broader peak, with a trailing edge of fractions containing monomeric forms of Vrg4 that co-eluted with lower molecular weight standards. This observation is consistent with our results that demonstrated a reduced stability of the Vrg4p-containing complex in Triton X-100 compared with digitonin during co-immunoprecipitation (data not shown).

The predicted molecular mass of the Vrg4p monomer is 36 kDa, and under denaturing gel electrophoresis, it migrates with an apparent molecular mass of about 32 kDa. It is known that the interaction of detergents with some membrane proteins may alter their apparent molecular weight during gel filtration. Therefore we anticipated that the migration profile of Vrg4p-containing complexes would be an indication of the upper limit of the true molecular weight of the complex. Vrg4p elutes with an apparent molecular mass that is about twice that of the monomer. Taken together with co-immunoprecipitation results, these data suggest that Vrg4p exists as a homodimer, although its association with other low molecular weight molecules cannot be ruled out.

**Fig. 1.** Vrg4p exists in a ~65-kDa complex during gel filtration chromatography. Detergent extracts were prepared from a yeast strain (SEY6210) expressing Vrg4-HAp (pRHL-HA) and fractionated by FPLC over a Superose 6 column (see “Experimental Procedures”). Aliquots of fractions were subjected to SDS-PAGE and Western blotted with anti-HA antibodies. Only peak fractions are indicated.

**Fig. 2.** Amino acids at the N and C terminus of Vrg4p are required for viability. A, shown is a schematic diagram depicting the HA- and myc-tagged wild type and mutant Vrg4 proteins containing deletions at their termini. Hatched boxes represent the position of predicted transmembrane domains (see Fig. 9 for model). B, strain XGY11, containing Vrg4 under control of the glucose repressible GAL1 promoter was transformed with plasmids containing VRG4-HA or a vrg4Δ alleles (as indicated) and streaked onto YPA medium supplemented with galactose or glucose.

**Phenotypic Characterization of vrg4 Mutants Encoding Proteins with N- and C-terminal Deletions—**To identify regions of the Vrg4 protein important for function, a series of vrg4 mutant alleles were constructed that encode proteins with deleted termini. Each of these proteins were also epitope tagged at the nondeleted end to facilitate further analyses (see “Experimental Procedures”). Schematic diagrams of these truncated proteins are shown in Fig. 2A. Each vrg4Δ allele was first assayed for functionality by monitoring complementation of the hygromycin B growth sensitivity of the viable vrg4--2 mutant. While deletion of either the first 15 amino acids or the last 13 amino acids had little effect on protein function, deletion of the N-terminal 44 amino acids (vrg4-Δ44N, which lacks the N-terminal cytosolic tail) or the C-terminal 34 amino acids (vrg4-Δ34C, which lacks the last predicted TMD) impairs protein function. Mutant alleles encoding these truncated proteins completely failed to complement both the drug sensitive phenotype of the vrg4--2 mutant, as well as its glycosylation defect (data not shown).

To determine whether vrg4-Δ44N and vrg4-Δ34C are null alleles, complementation of the lethality associated with loss of VRG4 function was examined. Each of these mutant alleles was introduced into a yeast strain containing VRG4 under the control of the glucose-repressible GAL1 promoter. This strain grows normally in the presence of galactose, but fails to grow when VRG4 gene expression is repressed in the presence of glucose. As expected from our examination of glycosylation phenotypes (data not shown), deletion of the first 15 amino acids of Vrg4p had minor effects, since expression of vrg4-Δ15N supported the growth of the GAL1-VRG4 strain in the presence of glucose (Fig. 2B). Similarly, vrg4-Δ34C and vrg4-Δ13C also encode proteins that support viability. In contrast, neither vrg4-Δ44N, vrg4-Δ78N, nor vrg4-Δ34C supported the growth of the GAL1-VRG4 strain in the presence of glucose (Fig. 2B), demonstrating that these alleles encode nonfunctional GDP-mannose transporters.
The C Terminus of Vrg4p Is Required for Protein Stability—To study the role of these mutations on Vrg4 function, we examined their effect on protein stability by Western blot analysis. The steady state level of the mutant Vrg4ΔC proteins that were myc-tagged at the N terminus was quantitatively compared with that of the wild type Vrg4-myc protein, with anti-myc antibody (Fig. 3). While a deletion of 6 or 13 amino acids had no affect on protein stability, a deletion of an additional 21 amino acids (Vrg4Δ34Cp) that removes the predicted C-terminal TMD (see Fig. 9) destabilized the Vrg4 protein and resulted in levels 5–10-fold lower than wild type (Fig. 3). As described by Abe et al. (1), we also found that Vrg4 proteins lacking these C-terminal 35 amino acids failed to oligomerize and accumulated in the ER (data not shown). These results demonstrate that sequences predicted to comprise the last TMD in the C terminus of Vrg4p are required for protein stability and suggest that their retention in the ER is simply a result of their instability and failure to assemble.

Mutant Vrg4 Proteins Containing N-terminal Deletions Are Unstable and Maintain the Ability to homo-oligomerize. Detergent lysates were prepared from yeast (SY6210) harboring plasmids that encode myc-tagged Vrg4p protein (YEpGAPVRG4-N-myc3), Vrg4Δ6Cp (YEpGAPΔ6C-myc3), Vrg4Δ13Cp (YEpGAPΔ13C-myc3) or Vrg4Δ34Cp (YEpGAPΔ34C-myc3). Equivalent amounts of protein in each sample was separated by SDS-PAGE and immunobotted with anti-myc antibody as described under “Experimental Procedures.”

The experiments described above provide strong biochemical evidence that Vrg4p exists as a homodimer, and immunoblotted with anti-myc rabbit antiserum (Fig. 4, upper panel). By this assay, we found that the levels of the wild type and mutant Vrg4ΔN-myc proteins were virtually indistinguishable (Fig. 4, upper panel). This was also true of HA-tagged proteins or when these alleles were expressed from a low copy, CEN vector (data not shown). These results demonstrate that Vrg4 proteins containing a deletion of 15, 44, or 78 amino acids are as stable as their wild type counterparts.

To determine whether the N-terminal deleted proteins were affected in their oligomer assembly properties, a co-immunoprecipitation assay was used. The same detergent extracts used in the Western blot assay described above were incubated with the anti-HA monoclonal antibody. To measure the relative amount of myc-tagged mutant protein that associated with the HA-tagged mutant or wild type Vrg4 protein, proteins precipitated with anti-HA antibody were fractionated by SDS-PAGE and immunoblotted with anti-myc antibody as described under “Experimental Procedures.”

The C Terminus of Vrg4p is required for protein stability. The steady state level of the mutant Vrg4p was compared with that of the wild type Vrg4-myc protein, with anti-myc antibody (Fig. 3). While a deletion of 6 or 13 amino acids had no affect on protein stability, a deletion of an additional 21 amino acids (Vrg4Δ34Cp) that removes the predicted C-terminal TMD (see Fig. 9) destabilized the Vrg4 protein and resulted in levels 5–10-fold lower than wild type (Fig. 3). As described by Abe et al. (1), we also found that Vrg4 proteins lacking these C-terminal 35 amino acids failed to oligomerize and accumulated in the ER (data not shown). These results demonstrate that sequences predicted to comprise the last TMD in the C terminus of Vrg4p are required for protein stability and suggest that their retention in the ER is simply a result of their instability and failure to assemble.

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The experiments described above provide strong biochemical evidence that Vrg4p exists as a homodimer, but do not address the question of whether dimer formation is important for its in vivo function. To test this idea, we took advantage of the vrg4Δ44N allele, since it encodes a nonfunctional protein that maintains the ability to multimerize. If homodimerization is important for Vrg4p function in vivo, then
overexpression of the vrg4Δ44N allele should confer a dominant negative growth phenotype, via its ability to bind to and form inactive dimers with the endogenous wild type Vrg4p. High copy plasmids that overexpress either VRG4, vrg4Δ44N, or vrg4Δ78C allele were introduced into yeast. Each of these strains was streaked onto selective media supplemented with hygromycin B. Overproduction of Vrg4-Δ34Cp, which fails to multimerize (Ref. 1 and data not shown) or the wild type Vrg4p, had no effect on growth phenotype (Fig. 5). In contrast, overexpression of the vrg4Δ44N allele impaired growth on this medium; this strain grew as poorly as the vrg4Δ2 mutant. (Fig. 5). These genetic data agree well with the co-immunoprecipitation experiments described above (Fig. 4) that demonstrate N-terminal Vrg4 deletion proteins multimerize efficiently and also suggest that dimer formation is essential for GDP-mannose transport to occur in vivo.

Deletion of the N-terminal Domain of Vrg4p Results in Its Mislocalization—Since the vrg4Δ44N and vrg4Δ78N alleles encode proteins that are stable and that can assemble into oligomers, this raised the question of what is the biochemical basis for the loss of protein activity. GDP-mannose transport activity is associated with Golgi membranes (7), and the Vrg4-HA protein is localized to the yeast Golgi (8). One possible explanation for its inactivity is that the N terminus of Vrg4p is required for proper Golgi localization. To test whether these mutant Vrg4 proteins are mislocalized, we compared their intracellular location to that of the normal Vrg4 protein. Cells co-expressing VRG4-myc and vrg4Δ15N-HA, vrg4Δ44N-HA or vrg4Δ78N-HA were fixed with formaldehyde, and each protein was detected by indirect immunofluorescence using antibodies directed against the myc or HA epitope. As expected, deletion of the first 15 amino acids had no effect on protein localization; Vrg4Δ15N-HAp displayed the same punctate pattern characteristic of the yeast Golgi that was also observed for the wild type Vrg4 protein. In contrast, a deletion of 44 or 78 amino acids resulted in the mislocalization of the mutant proteins to the ER (Fig. 4), a proportion of Golgi-localized complexes must be hetero-oligomeric, containing both mutant and wild type subunits. This implies that normal homo-oligomers or mixed hetero-oligomers are competent for ER exit while mutant homo-oligomers are not. These data suggest that amino acids between 16 and 44 are required for export from the ER.

The N Terminus of VRG4 Can Direct an ER Protein to the Golgi—The accumulation of misfolded, unstable proteins or unassembled oligomeric proteins in the ER via quality control mechanisms is well documented. This idea can certainly explain the accumulation of Vrg4Δ4C proteins in the ER (1) as these mutant proteins appears to be both unstable (Fig. 3) and monomeric (Ref. 1 and data not shown). However, this idea does not satisfactorily account for the mislocalization of Vrg4ΔN44p and Vrg4ΔN78p in the ER, since both the mutant proteins are stable and fully competent to multimerize (Fig. 4).

To examine whether this N-terminal region of the protein may act as an ER export signal, we asked if it could target a resident ER protein to the Golgi. To approach this problem, we took advantage of a highly related Vrg4p homologue, encoded by the HVG1 gene. Although Hvglp is 88% identical along its length to Vrg4p, it is not functionally redundant and fails to complement a vrg4 mutant even when overexpressed (Exp. 8 and see Fig. 7). A significant difference between Vrg4p and Hvglp is that Hvgl is a natural truncated variant of Vrg4p,
lacking the N-terminal 98 amino acids that are present in Vrg4p (see alignment in Fig. 7). Although the function of Hvg1p is unknown, analysis of its intracellular localization by indirect immunofluorescence demonstrated that, unlike Vrg4p, Hvg1 resides in the ER and not in the Golgi (Fig. 8). Remarkably, the nucleotide sequences of the 5′-untranslated region (UTR) of HVG1 that correspond to the 5′-translated region of VRG4 have not significantly diverged, except for the presence of an in-frame stop codon (denoted by asterisk in Fig. 7) upstream the initiating methionine in HVG1 (see text). This stop codon was mutated to an arginine codon in mHVG1 (see text). Western blot analysis of the Hvg1-HA and mHvg1-HA proteins. Whole cell lysates from 10 OD units of yeast cells (SEY6210) expressing vector alone (lane 1), HA-tagged mHVG1-HA on a 2μ (lane 2) or CEN plasmid (lane 4) or HVG1-HA on a 2μ (lane 3) or CEN plasmid (lane 5) were immunoprecipitated with anti-HA monoclonal antibodies. The entire immunoprecipitate was subjected to SDS-PAGE, Western immunoblotted with anti-HA rabbit antibodies, and detected by chemiluminescence. C, strain NDY5, containing the vrg4–2 mutation, was transformed with 2μ or CEN-containing plasmids encoding mHvg1-HAp or Hvg1 and plated on medium containing 50 μg/ml hygromycin B. Also shown as a control is the growth phenotype of NDY5 (vrg4–2) and its isogenic wild type parental strain, RSY255 (VRG4). The intracellular localization of Hvg1-HA and mHvg1-HA was examined by indirect immunofluorescence, using anti-HA antibody. While Hvg1 is found primarily in the ER, mHvg1p, containing the additional N-terminal domain is found exclusively in the Golgi (Fig. 8A). This difference in localization cannot be attributed to differences in expression levels, since both proteins accumulated to comparable levels (Fig. 7B). Similarly, retention of Hvg1 in the ER is not due to its inability to multimerize, since both Hvg1 and mHvg1 can oligomerize (data not shown). Thus, the additional N-terminal domain is sufficient to cause the export of this protein from its normal location in the ER to the Golgi. We also analyzed the effect of fusing the Vrg4p N terminus onto a less related membrane protein. Ypl244c is a yeast ORF of unknown function that displays homology to the S. pombe UDP-galactose transporter. We found that this protein is pri-
marily localized in the yeast Golgi, although when overexpressed it can also be seen in the ER (Fig. 8B, Ypl244c-HAp). Removal of the N-terminal 41 amino acids of Ypl244cp causes its mislocalization to the ER (Fig. 8B, Ypl244c Δ41-HAp). However, when the N-terminal 47 amino acids of Vrg4p are fused to this truncated Ypl244c protein, this protein is again found primarily in the Golgi complex, although some ER staining is also observed (Fig. 8B, VN47-Ypl244c Δ41-HAp). These results suggest that sequences in the N terminus of Vrg4p can supply the information that promotes the ER export of this protein, which is normally found within its N terminus.

The N and C Termini of Vrg4p Face the Cytosol—The use of secondary structure algorithms and hydropathy analyses predict that Vrg4p contains between six and eight TMDs. To obtain more information about the function of the N and C termini and as a first step toward examining the membrane topology of the Vrg4 protein, it was of interest to examine whether these regions of the protein face the lumen of the Golgi or the cytosol. To do this we used a protease protection assay of epitope tags placed at the C or N terminus of Vrg4p. A 7-amino acid recognition site for cleavage by the TEV protease (26) was inserted at the junction of either an N-terminal triple HA tag or a C-terminal protein A tag (Fig. 9A). This TEV recognition sequence is not found in any predicted yeast ORF, so cleavage of these fusion proteins by TEV protease is highly specific.

Yeast strains were constructed in which the chromosomal allele of VRG4 was replaced by the N- or C-terminally tagged alleles. These strains, whose sole source of Vrg4p is from the tagged VRG4 alleles, display no growth or glycosylation defect (data not shown), suggesting that neither the N- nor the C-terminal tag affects normal Vrg4 protein folding.

To determine whether these epitopes were accessible to digestion by the TEV protease, membrane fractions were prepared from yeast strains expressing these tagged alleles and subjected to digestion with the TEV protease. After digestion, proteins were separated by SDS-PAGE and immunoblotted with antibodies against the HA or protein A tag. Both the N and C termini were digested in the presence of TEV protease (Fig. 9B). Since a C-terminal tag of a control protein, Gda1p, that resides in the lumen (27, 28) was protected from TEV cleavage, the digestion of the N and C termini of Vrg4p was not merely the result of leaky or inverted vesicles (Fig. 9B). Both Vrg4p and Gda1p were digested by TEV protease in the presence of Triton X-100, further demonstrating that the proteolytic protection of Gda1p was specifically due to its luminal orientation. Neither Gda1p nor Vrg4p was digested when an inhibitor of the TEV protease, ZnSO4, was included in the incubation, demonstrating the specificity of TEV cleavage.

**FIG. 8.** Addition of the related Vrg4p N terminus on Hvg1p or on Ypl244cΔ41-HAp causes their export from the ER to the Golgi. Indirect immunofluorescence of SEY6210 expressing mHVG1-HA or HVG1-HA (A) or Ypl244cΔ41-HAp, Ypl244cΔ41-HAp, or Vrg4 N47-Ypl244c Δ41-HAp (B). Fixed cells were treated with anti-HA antibodies, followed by fluorescein isothiocyanate-conjugated anti-mouse.

**FIG. 9.** The N and C termini of Vrg4p face the cytosol. A shows a schematic diagram of the Vrg4 and Gda1 epitope-tagged proteins containing TEV protease cleavage sites (depicted by an arrow) at the N or C terminus that were used for the protease protection assay shown in B, where the hatched boxes represent predicted membrane-spanning domains. B, microsomes were prepared from yeast strains containing Vrg4p with an N-terminal TEV tag (XY711), Vrg4p with a C-terminal TEV tag (XY710), or Gda1p with a C-terminal TEV tag (XY712), as described under “Experimental Procedures,” and treated with 20 units of TEV protease in the absence (−) or presence (+) of Triton X-100 or zinc sulfate. Proteins were separated by SDS-PAGE and analyzed by Western immunoblotting followed by chemiluminescence, using anti-HA antibodies or rabbit IgG for the detection of the protein A epitope. C depicts a model for the membrane topology of Vrg4 that combines the results from the protease protection assay, which experimentally place the C and N termini in the cytosol and hydropathy analysis, which predicts six to eight membrane-spanning segments.
These results demonstrate that both the N and C termini of Vrg4p face the cytoplasm and that this protein contains an even number of membrane spanning domains (see model in Fig. 9C).

**DISCUSSION**

Although nucleotide sugar transport activity was first described over twenty years ago, little is known about the molecular mechanisms that drive these reactions. We examined epitope-tagged alleles of VRG4 to study the properties of Vrg4p that relate to its ability to catalyze GDP-mannose transport into the Golgi. The experiments described here establish that this transporter normally functions as a multimer, probably as a homodimer. In addition, through our analyses of mutant alleles, we identified an essential domain that is required for ER export and Golgi localization but that is distinct from another domain required for protein stability and oligomer assembly.

An important result presented in this work is that the Vrg4 protein can interact with itself with high affinity and specificity. Moreover, the predicted molecular weight of the Vrg4 protein, in both Triton X-100 and digitonin, is twice the molecular weight of the monomer, suggesting that Vrg4p functions independently as a homodimer. We looked for evidence of its interaction with other proteins and found none. Vrg4p does not associate with either Gda1p or Ynd1p (data not shown), although an interaction with either of these proteins would not have been surprising, since they are the Golgi GDPases that generate the GMP that perpetuates the nucleotide sugar transport cycle (29, 30). These results are in agreement with radiation inactivation studies that suggest Gda1p is homodimeric (27). It therefore appears that the luminal transport of GDP-mannose, like the hydrolysis of GDP to GMP, is catalyzed by a homodimer that does not require an association with other membrane proteins for function.

With the exception of the Leishmania GDP-mannose transporter, which exists as a hexameric complex (31), recent reports indicate that other nucleotide sugar transporters including the human GDP-fucose (32) and UDP-GalNAc transporter (33) exist as homodimers. The murine CMP-sialic acid transporter, the Kluyveromyces lactis UDP-N-acetylglucosamine transporter, and the human UDP-galactose transporter contain leucine zipper motifs that have been postulated to function in oligomerization though the physical properties of these proteins have not yet been investigated (29, 34, 35). Therefore, dimerization or some higher order structure may be a general feature of nucleotide sugar transporters. Dimer formation of the yeast GDP-mannose transporter is clearly essential for its function in vivo, since inhibition of functional Vrg4p dimer formation in vivo, which we induced experimentally through the overexpression of the vrg4Δ44N allele, leads to growth defects.

How might dimer formation contribute to the transport of nucleotide sugars? Some clues may come from studies of the major facilitator superfamily of transporters, whose members are characterized by two structural units of six or seven TMDs, connected by a loop (see Ref. 36 for review). The current view is that this 2-fold symmetry enables the formation of a transmembrane channel through which solutes may pass. The presence of shared sequence motifs in the C- and N-terminal halves of these 12 TMD-containing proteins has led to the hypothesis that these transporters originally arose by a tandem intragenic duplication from primordial six TMD-containing protein (37). A computer analysis of all members of the major facilitator superfamily encoded by the S. cerevisiae genome identified 149 proteins, which include Vrg4p (38, 39). This is despite the fact that Vrg4p is predicted to contain only six to eight TMDs. The idea that Vrg4p exists as a dimer reconciles its anomalous inclusion in this family of transporters that are otherwise characterized by their conserved topology. Protein dimerization may facilitate the formation of a transmembrane channel that normally requires the packing of 12-14 TMDs.

In the case of the yeast GDP-mannose transporter, Abe et al. (1) recently demonstrated that the C terminus of Vrg4p is required for oligomerization. Mutant vrg4ΔC alleles encoding proteins lacking the last 12, 35, and 62 amino acids failed to complement the glycosylation defect of a vig4 (vrg4) mutant, and residues between the last 12 and 62 amino acids (predicted to encode the last TMD) are required for oligomer formation and are mislocalized in the ER (1). On the basis of these data, they suggest that these sequences may be involved in trafficking Vrg4p from the ER to the Golgi. We obtained similar results, but with some important differences. First, deletions of up to 13 amino acids at the C terminus had little effect on Vrg4p function. Second, and perhaps most importantly, while amino acids predicted to comprise the C-terminal TMD are required for oligomer formation, we found that these amino acids are also essential for protein stability. Proteins lacking this C-terminal TMD are nonfunctional, unstable, and accumulate in the ER, consistent with the idea that quality control mechanisms restrict the exit of these unassembled Vrg4 monomers from the ER and result in their degradation.

We found that the N-terminal cytosolic tail is required for ER export. Unlike the C terminus, deletion of this N-terminal region of the protein, between amino acids 15 and 44, does not affect protein stability or oligomer assembly. The demonstration that Vrg4Δ44N proteins efficiently homo-oligomerize with themselves suggests that their retention in the ER is not due to their failure to oligomerize in the ER. Three additional pieces of indirect evidence suggest that these sequences facilitate ER export. First, the Vrg4-related Hvg1 protein lacks this N-terminal sequence and normally resides in the ER. Second, the addition of a highly related N-terminal sequence onto Hvg1p results in its transport to the Golgi. Third, fusion of the Vrg4p N terminus to a truncated UDP-galactose homologue, Ypl244c, restores its localization to the Golgi.

Several possible mechanisms by which the N-terminal tail influences ER export can be envisioned. The first is that it may act as a cytoplasmic cargo recognition signal for the inclusion of Vrg4p into transport vesicles. Alternatively, the N terminus may assist a particular folding conformation that effects the kinetics of export from the ER. The transport of cargo between the ER and the Golgi is mediated by COPII-coated vesicles and requires the sorting and concentration of cargo into these vesicles (see Ref. 40 for review). Cargo selection may involve specialized accessory factors dedicated to a subset of cargo. Several yeast proteins, including Shr3p (41, 42), Gal2p (43), Erv14p (44), and Let1p (45), have been described recently that are candidates for these ancillary proteins that facilitate ER export of specific proteins in yeast. With the exception of Let1p, a relative of the COPII protein, Sec24p (45), whether these other proteins are directly involved in cargo selection is not known. They may simply influence some aspect of protein maturation that is required for the efficient trafficking. Since overexpression of VRG4 does not lead to any obvious secretion defects (data not shown), it seems unlikely that the N terminus interacts directly with the general COPII machinery. Further experiments will be required to distinguish whether this N-terminal tail plays a direct role in sorting via its interaction with some adapter or an indirect role via its influence on protein folding. In conclusion, our results have defined two structural domains of the yeast Golgi GDP-mannose transporter that are
critical for its function. The essentiality of this protein underscores the significance of glycosylation in the Golgi.

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