

The Yeast *VRG4* Gene Is Required for Normal Golgi Functions and Defines a New Family of Related Genes*

(Received for publication, July 12, 1995, and in revised form, October 17, 1995)

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Sodium vanadate is an effective agent for the enrichment of yeast mutants with defects in glycosylation steps that occur in the Golgi complex (Ballou, L., Hitzeman, R. A., Lewis, M. S., and Ballou, C. E. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 3209–3212). We isolated and screened vanadate-resistant glycosylation mutants in the budding yeast, *Saccharomyces cerevisiae*, to identify any that may be defective in the secretory pathway, since changes in normal glycosylation may reflect defects within the secretory pathway. We identified one such mutant, allelic to *vrg4/van2*, that is defective in processes that occur specifically in the Golgi complex. Protein secreted from *vrg4* mutants lacks the outer chain glycosylation that is normally extended during passage through the Golgi. This mutant fails to retrieve soluble endoplasmic reticulum proteins from the Golgi and accumulates the Golgi-specific biosynthetic intermediate of the vacuolar protein, carboxypeptidase Y. Analyses of intracellular membranes by staining with the fluorescent lipophilic dye, DiOC₆, and by electron microscopy reveals a dramatic alteration in the membrane morphology of *vrg4* mutant cells. The *VRG4* gene encodes a 36.9-kDa membrane protein that is essential for cell viability. A sequence homology search has identified five related genes, establishing that *VRG4* is a founding member of a family of structurally similar genes. Taken together, these results suggest that the *VRG4* gene plays an important role in regulating Golgi functions and in maintaining the normal organization of intracellular membranes.

The Golgi complex is involved in the post-translational modification of glycoproteins and in the sorting of these proteins to their correct destination. Each of the individual Golgi cisternae is biochemically and functionally distinct, differing in both protein and lipid composition. In the case of the glycosyltransferases that mediate glycoprotein modifications, immunocytochemical and biochemical studies have clearly demonstrated that these enzymes are compartmentalized within particular cisternae. Successful glycan synthesis is dependent upon the compartmentalization and regulation of the glycosyltransferases that participate in these stepwise reactions.

Following the initial glycosylation steps in the endoplasmic

reticulum (ER),¹ yeast glycans are elongated by Golgi-localized mannosyltransferases to form glycoproteins with extended outer chains of 50 or more mannose units (for review, see Refs. 2 and 3). The outer chain, consisting of an α 1,6-linked mannose backbone, is normally highly branched with α 1,2- and α 1,3-linked mannoses (2–4). Several groups of yeast mutants with defects in glycosylation have been isolated, most notably the *sec* (secretion), *alg* (asparagine-linked glycosylation), and *mnn* (mannan) mutants. The *sec* mutants are conditional mutants with defects in transport steps through out the secretory pathway (5). The *alg* mutants are affected primarily in the synthesis of the core oligosaccharide that is added in the ER (6). The *mnn* mutants are blocked at various stages of outer-chain carbohydrate elongation that occur in the Golgi complex (1, 4, 7). Many of the *mnn* mutants do not appear to contain lesions in genes that encode glycosyltransferases (1, 4, 7–9). Rather, they likely affect other cellular functions associated with the secretory pathway that affect glycosylation in the Golgi (9).

As a first step toward identifying factors that participate in the correct localization of resident Golgi proteins, and therefore contribute to normal Golgi biogenesis, our efforts have been directed toward the characterization of mutants that have defects in Golgi-specific functions, specifically in glycosylation. The underlying prediction was that these glycosylation mutants would fall into two general classes: those containing defects in genes encoding the glycosyltransferases themselves, and those encoding proteins that regulate the activity or localization of these enzymes. In this report, we describe one glycosylation mutant that falls into the latter class. This mutant is allelic to *vrg4* (1) (also known as *van2*; see Refs. 10 and 11), a previously identified vanadate-resistant glycosylation mutant. Here, we present a phenotypic analysis of the *vrg4* mutant and a molecular analysis of the *VRG4* gene.

MATERIALS AND METHODS

Media and Yeast Strains—Yeast strains were grown in either YPAD (1% yeast extract, 2% peptone, 2% dextrose, 50 mg/liter adenine sulfate), YP (1% yeast extract, 2% peptone) to which 0.05% glucose was added (in experiments requiring invertase induction), or synthetic medium that contained 0.67% yeast nitrogen base and 2% glucose, supplemented with the appropriate auxotrophic requirements (12). YPAD was supplemented with 0.5 M KCl for the growth of all vanadate-resistant mutants, which are osmotically sensitive.

All yeast strains used in this study are listed in Table I. Yeast transformations were performed using the lithium acetate protocol, as described previously (13).

Isolation of Vanadate-resistant Mutants—Spontaneous mutants, resistant to 7 and 10 mM orthovanadate were isolated essentially as described, using MCY1093, MCY1094, or RSY255 as the starting strains (1). Resistant colonies arose at a frequency of approximately 10^{-5} after 3–5 days of incubation at 30 °C. Vanadate-resistant colonies were confirmed as such by restreaking onto YPAD plates containing

* This work was supported in part by Grant GM-48467 from the National Institutes of Health and a grant from the Council for Tobacco Research (to N. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L33915.

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¹ The abbreviations used are: ER, endoplasmic reticulum; kb, kilobase pair(s); BiP, binding protein; CPY, carboxypeptidase Y; PAGE, polyacrylamide gel electrophoresis.

TABLE I
Strains used in this study

Strain	Genotype
MCY1093	<i>MATα his4-539 lys2-801 ura3-52</i>
MCY1094	<i>MATα ade2-101 ura3-52</i>
RSY255	<i>MATα ura3-52 leu2-2111</i>
W3031a	<i>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100</i>
W3031b	<i>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100</i>
W3032n	<i>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100</i>
NDY22	<i>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 VRG4</i>
NDY17.4	<i>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 vrg4Δ:LEU2</i>
NDY1.4	<i>MATα ade2-101 ura3-52 ost4-2</i>
NDY7.4	<i>MATα ade2-101 ura3-52 ost4-3</i>
NDY10	<i>MATα ade2-101 ura3-52 vrg7-2</i>
NDY13.4	<i>MATα ura3-52 leu2-211 vrg9-1</i>
NDY4	<i>MATα ade2-101 ura3-52 alg11-1</i>
NDY5	<i>MATα ura3-52 leu2-211 mnn9-3</i>
2102p1.2	<i>MATα ura3-52 leu2-211 vrg4-2</i>
	<i>MATα ura3-52 his4-519 suc2-Δ9 leu2-3112 Δerd1</i>

7–10 mM sodium vanadate. For routine growth and storage, mutants were maintained on YPAD plates or YPAD liquid media supplemented with 0.5 M KCl.

Cloning and Sequencing the Wild Type VRG4 Gene—Strain NDY5 (*ura3-52 leu2-211 vrg4-2*) was transformed with a yeast genomic *CEN*-based library, carrying the *LEU2* selectable marker (from Phil Heiter). Prototrophic transformants were selected on medium lacking leucine and replica-plated onto media containing 50 μ g/ml hygromycin B. Plasmid DNA from hygromycin B-resistant colonies was isolated, amplified in *Escherichia coli*, and retransformed into the *vrg4* mutant to confirm complementing activity.

A 2.1-kb *EcoRI/HindIII* fragment capable of complementing the hygromycin B sensitivity of *vrg4* was sequenced by the dideoxy method (14) generating a nested deletion series using the ExoIII/ExoVII method (15). Both DNA strands were sequenced. DNA and predicted protein sequence comparisons against data bases were made using the BLAST algorithm (16) and analyzed using the GCG programs.

Plasmid Constructions—All DNA manipulations were carried out according to standard protocols (17). The 2.1-kb *EcoRI/HindIII* fragment containing the entire *VRG4* gene and regulatory sequences was subcloned into the vector pRS316 (18) to generate the *CEN* based plasmid, pRHL, containing the selectable marker, *URA3*. This plasmid was labeled with [³²P]dCTP (Amersham Corp.) using the random priming method and used to probe a nitrocellulose filter (kindly provided by J. Engebrecht), which contained separated yeast chromosomes (19).

The disruption plasmid pG5::LEU was constructed by inserting a *SmaI/SaI* fragment (blunt-ended with Klenow) containing the *LEU2* gene into the unique *HpaI* site that lies within the *VRG4* gene.

The integrative plasmid pG5i was constructed by cloning the *HindIII/EcoRI* fragment containing the entire *VRG4* gene into the *URA3*-containing pRS306. The plasmid was linearized at a unique *HpaI* site in the *VRG4* gene and transformed into strain NDY5.

Western Immunoblotting and Immunoprecipitations—Overnight cultures from mutant or wild type cells were diluted to 10⁷ cells/ml and grown for 3–4 h prior to protein extraction. Protein extracts were prepared as follows. Sodium hydroxide (to 0.25 M) and 2-mercaptoethanol (to 1%) were added directly to cell cultures (0.8 ml, approximately 2 \times 10⁷ cells). After 10 min on ice, trichloroacetic acid was added to 6%. After an additional 10 min on ice, the mixture was centrifuged in a microcentrifuge for 3 min at room temperature. The precipitate was washed vigorously in 1 ml of ice-cold acetone and centrifuged for 3 min. The pellet was suspended in 75 μ l of Laemmli's sample buffer (20) and heated at 95 °C for 3 min. Samples were centrifuged briefly to remove debris prior to electrophoresis on SDS-polyacrylamide gels (20). Analysis of carboxypeptidase Y and intracellular and secreted BiP (Kar2p) was performed by Western immunoblotting essentially as described (21), except that the secondary anti-rabbit antibody was conjugated to horseradish peroxidase and the immune complex detected by enhanced chemiluminescence (Amersham ECL). When comparing the levels of BiP that were secreted or accumulating intracellularly, equal numbers of cells were grown in YPAD media. Cells were then spun down and the cell pellet and supernatant separated. Intracellular proteins were extracted by the NaOH/ β -mercaptoethanol method as described above. Proteins in the culture supernatant were precipitated by the addition of 10 volumes of ice-cold acetone, followed by centrifugation at 10,000 \times g. In both cases, protein pellets were resuspended in equal volumes of

sample buffer prior to SDS-PAGE and Western blot analysis. Therefore, in comparing the levels of intracellular and extracellular BiP, equivalent amounts of proteins were examined.

Analysis of CPY by immunoprecipitation was carried out as described (21), except that cells were labeled with 200 μ Ci of [³⁵S]methionine and cysteine (Expre³⁵S³⁵S, DuPont NEN), and chased with the addition of a 10-fold chase solution of 50 mM methionine and 10 mM cysteine to a final concentration of 5 mM methionine and 1 mM cysteine.

In Situ Invertase Gel Assay—Native gel electrophoresis and activity staining of invertase was performed as described (4), with some modifications. Approximately 10⁸ cells (2–3 ml of an overnight culture, normalized for cell number) were washed with water and diluted into YP + 0.5 M KCl, containing 0.05% glucose to induce invertase expression. After 2 h, cells were centrifuged, washed twice with TP buffer (10 mM Tris-HCl (7.0); 1 mM phenylmethylsulfonyl fluoride), and resuspended in 20 μ l of TP buffer. Cells were lysed by vortexing with glass beads. 50 μ l of TP buffer containing 15% glycerol and 0.01% bromophenol blue was then added and samples were spun down briefly to remove debris. Lysates (5–20 μ l) were electrophoresed on a 5% acrylamide (30:0.8 acrylamide:bisacrylamide), 80 mM Tris-HCl (pH 7.3) native gel as described (4).

DiOC₆ Staining of Yeast Cells—Cells to be stained were freshly grown on YPAD plates overnight. Approximately 10⁷ cells were suspended in YPAD containing 0.5 M KCl. A stock solution of DiOC₆ (Eastman Kodak Co.) (1.0 mg/ml in ethanol) was titrated to obtain maximal staining of the nuclear envelope, ER, and associated membranous networks, which was achieved at a concentration of 10 μ g/ml. Cells were stained and observed in either Sylgard (Dow-Corning Products, Inc.) growth chambers, prepared as described (22) or on glass slides. Observations were made on a Bio-Rad MRC600 confocal microscope, using excitation and barrier filters appropriate for use with fluorescein.

Electron Microscopy—100 ml of yeast cells were grown to 10⁷ cells/ml in YPAD and rapidly fixed by the addition of 2 ml of 50% glutaraldehyde. Staining with permanganate was performed as described (23). Briefly, after washing in water, cells were incubated 3 h in 5 ml of 4% KMnO₄. Cells were then washed with distilled water and suspended in 2% uranyl acetate for 18 h at 4 °C. Fixed cells were dehydrated by washing with a graded series of ethanol and infiltrated in a 1:1 mixture of ethanol:Spurr's resin. Embedding was in Spurr's resin.

RESULTS

Isolation of the *vrg4* Mutant—We wished to isolate yeast mutants that affect Golgi-specific functions. Our approach was to select mutants defective in glycosylation. These were subsequently screened for other Golgi-specific defects. The rationale behind this approach is that secretion and glycosylation are closely coupled processes. Therefore, changes in normal glycosylation may reflect defects within the secretory pathway. Among the glycosylation mutants that we isolated, one mutant, which we later found to be allelic to *vrg4* (1), was identified that was defective in several processes specific to the Golgi. For clarity, we have retained the original designation of this mutant, *vrg4* (for vanadate-resistant glycosylation).

To select mutants with defects in glycosylation, yeast were

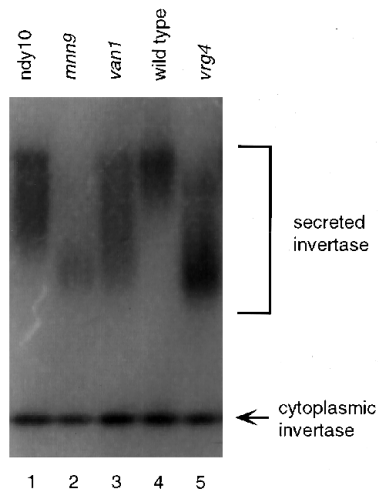


FIG. 1. Analysis of invertase glycosylation in vanadate-resistant mutants. Protein was isolated from wild type (lane 4) or vanadate-resistant isolates (lanes 1–3 and 5) and electrophoresed on 5% non-reducing polyacrylamide gels to detect native invertase using an activity stain as described under “Materials and Methods.” The position of glycosylated secreted invertase is indicated by brackets. The arrow denotes the position of nonglycosylated, cytoplasmic invertase. Lane 1 contains extracts from strain NDY10, which contains an uncharacterized glycosylation defect. Strain NDY4 (lane 2) contains the *mnn9* mutation, strain NDY7.4 contains the *van1* mutation (lane 3), and strain NDY5 contains the *vrg4* mutation (lane 5).

grown on medium supplemented with 10 mM sodium vanadate. Ballou *et al.* have found that resistance to sodium vanadate enriches for mutants defective in Golgi-specific glycosylation (1). They identified five complementation groups, all of which are defective in glycoprotein modifications that occur in the Golgi complex. Three of these are allelic to *mnn8*, *mnn9*, and *mnn10*, which have well characterized defects in glycoprotein outer chain structure (4, 8).

Spontaneous mutants that were resistant to 10 mM sodium vanadate arose at a frequency of approximately 10^{-5} . As expected from previous studies (1, 24), all exhibited varying degrees of sensitivity to the aminoglycoside, hygromycin B. Mutants were isolated from both *MATa* and *MAT α* strains. To assay for dominance of the vanadate resistance/hygromycin B sensitivity phenotype, the mutants were crossed to the parental strain of the appropriate mating type. By this criterion, all of the mutants we isolated were recessive. Mutants were also tested for temperature-sensitive growth and none were found to be temperature-sensitive.

Complementation analysis was performed by crossing mutants of opposite mating types and testing for vanadate resistance and hygromycin B sensitivity. This analysis indicated that the 28 isolates we obtained represent a minimum of eight different genes. Complementation analyses with a partial set of previously identified vanadate-resistant glycosylation mutants were performed. Among the eight vanadate-resistant mutants we isolated, we found mutants allelic to *vrg4* (1), *vrg7* (5) (also known as *van1*; see Ref. 10), and *mnn9* (7, 25). *mnn9* and *van1* are well characterized mutants, known to be defective in outer chain glycoprotein modifications that occur in the Golgi complex (1, 4). Little else is known about *vrg4*. Since we have not exhausted our search, it is unclear if other previously identified vanadate-resistant mutants (10, 26) are represented in the collection of mutants we isolated.

The glycosylation pattern of the secreted, periplasmic form of invertase was examined in the mutants to determine if all had defects in glycosylation (Fig. 1). Invertase exists in two states, a cytoplasmic, non-glycosylated form, and a secreted form. The secreted form is a highly glycosylated protein whose rate of

migration on native gels reflects the size and number of *N*-linked oligosaccharide chains it contains. Using an *in situ* gel assay (4), the electrophoretic mobility of invertase was visualized by an activity stain that monitors sucrose hydrolysis by invertase. A glycosylation defect can be detected by comparing the average rate of migration of invertase in extracts prepared from mutant and wild type cells.

All of the mutants expressed the periplasmic form of invertase that migrated with an increased electrophoretic mobility compared to wild type. A subset of these are shown in Fig. 1. All of the mutants also expressed the cytoplasmic, nonglycosylated form of invertase that comigrated with wild type cytoplasmic invertase (see arrow in Fig. 1). Since both forms of invertase are derived from the same gene, we infer that the change in mobility of the secreted invertase in each of these mutants is due to a decrease in carbohydrate modifications rather than differences in protein structure. The electrophoretic resolution of this procedure is insufficient to detect differences in individual carbohydrate modifications. Despite this, several general classes of defects were apparent. The most severe glycosylation phenotype is exemplified by the mutation in isolate NDY5, which carries the *vrg4* mutation (Fig. 1, lane 5). Invertase in this mutant migrates with a mobility similar to that of the *mnn9* mutant (Fig. 1, lane 2), that lacks an outer chain and contains an oligosaccharide consisting of 10–14 mannoses (4). The mobility of invertase was somewhat variable, and in some experiments we observed a small fraction of invertase in the *vrg4*–2 mutant that co-migrated with the fully glycosylated form (for example see Fig. 6B, lane 3). The source of this variability is unknown. However, in all cases, the bulk of invertase that is secreted in this mutant migrates with an increased mobility. This result suggests that, like *mnn9*, *vrg4* severely impairs elongation of outer chain carbohydrates.

The *vrg4* Mutant Accumulates the Golgi Intermediate of Carboxypeptidase Y—We reasoned that if any of the mutants are defective in the secretory pathway within the Golgi complex, rather than in a glycosyltransferase, other phenotypic abnormalities that affect Golgi functions might be apparent. As one measure of a defect in transport through the Golgi, each mutant was assayed for the relative ratios of the different biosynthetic intermediates of the vacuolar protein, CPY. The biosynthetic pathway of CPY is well understood (27). As CPY transits the secretory pathway, it undergoes a series of modifications resulting in discrete ER, Golgi, and vacuolar forms, which can be distinguished electrophoretically. CPY is synthesized as a precursor (“p1”) that is core-glycosylated in the ER (67 kDa). After transport through the Golgi, CPY is further modified by addition of sugars (“p2”), increasing the molecular mass (69 kDa). Proteolytic processing in the vacuole results in the mature form (“m”) of CPY (61 kDa). At steady state, CPY is predominantly in the mature, vacuolar form. A delay or block in transport through the secretory pathway can be detected by the accumulation of the ER or Golgi intermediates.

Protein extracts from mutant and parental cells were subjected to immunoblot analyses using antiserum raised against CPY. Only one mutant, *vrg4*, showed an accumulation of the ER and Golgi form of CPY (Fig. 2A, lane 6). Most of the other mutants accumulated mature CPY that was indistinguishable from that of the parental, wild type cells. The two exceptions were mutants that accumulated forms of CPY that migrated even faster than mature CPY (Fig. 2A, compare lanes 1 and 2 with lane 7). The increased mobility in these two glycosylation mutants was due to a reduction in the number or size of core oligosaccharides that are added in the ER.² This increased

² N. Dean, unpublished data.

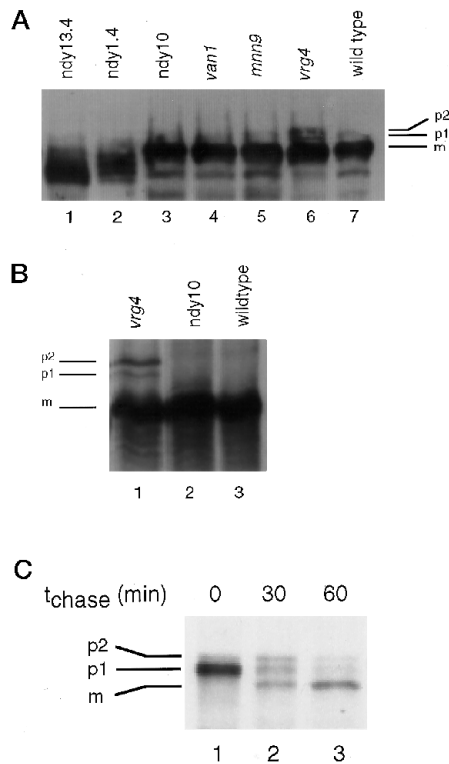


FIG. 2. The *vrg4* mutant accumulates the ER and Golgi intermediates of carboxypeptidase Y. In panels A–C, the ER, Golgi and mature forms of CPY are denoted by p1, p2, and m, respectively. **Panel A,** Western blot analysis of CPY in vanadate-resistant mutants (lanes 1–6) and wild type cells (lane 7). Protein was prepared and analyzed as described under “Materials and Methods.” Strain NDY13.4 (lane 1) contains an uncharacterized *alg*-like mutation; strain NDY1.4 (lane 2) contains a mutation in the *OST4* gene; see legend in Fig. 1 for strain description of other mutants in lanes 3–6. Note that among these mutants, only *vrg4* (lane 6) accumulates the Golgi and ER form. All the other mutants except NDY13.4 (lane 1) and NDY1.4 (lane 2) are indistinguishable from wild type (lane 7). **Panel B,** immunoprecipitation of CPY from mutant and wild type cells. Proteins in *vrg4* mutant (lane 1), NDY10 (lane 2), and wild type cells (lane 3) were labeled with [³⁵S]methionine for 10 min and chased for 15 min. CPY was immunoprecipitated and subjected to SDS-PAGE, as described under “Materials and Methods.” **Panel C,** kinetic analysis of CPY in *vrg4* mutant cells. Proteins in *vrg4* mutant were labeled with [³⁵S]methionine for 20 min and chased for 0 min (lane 1), 30 min (lane 2), and 60 min (lane 3) with cold methionine/cysteine. CPY was immunoprecipitated and subjected to SDS-PAGE, as described under “Materials and Methods.”

electrophoretic mobility of CPY is diagnostic of early glycosylation mutants with lesions in the ER (28). This increased mobility is not observed in *vrg4*, suggesting that the glycosylation defect caused by the *vrg4* mutation is not due to an ER glycosylation defect. The accumulation of the ER and Golgi forms of CPY in the *vrg4* mutant was not a consequence of its glycosylation defect, since none of the other glycosylation mutants accumulated these forms of CPY (Fig. 2A, lanes 1–5).

To further confirm the presence of the ER and Golgi forms of CPY in the *vrg4* mutant, immunoprecipitation studies using anti-CPY antibodies were performed on metabolically labeled cells. CPY was immunoprecipitated from cells that were pulse-labeled with [³⁵S]methionine and chased for variable lengths of time. In the *vrg4* mutant there was an accumulation of both the ER and Golgi form after a 15-min chase, with a detectable increase in the Golgi form (Fig. 2B, lane 1). In the parental strain, after 15 min, all of the CPY had chased into the mature form (Fig. 2B, lane 3). In the *vrg4* mutant, most of the ER and Golgi CPY intermediates eventually chased into the mature form, but only after 60 min (Fig. 2C, lane 3). Some experimental variability in the kinetics of CPY transport to the vacuole in

the *vrg4*–2 mutant was observed in pulse-chase experiments. This variability may be a result of differences in the growth stage of mutant cells, which grow poorly in the synthetic medium used to deplete the intracellular pools of methionine. However, when analyzed at steady state, the accumulation of the p1 and p2 forms of CPY was always seen in the *vrg4* mutant. No secreted CPY was ever observed in the medium (data not shown), suggesting that the effect of this mutation is not due to a general perturbation of the secretory pathway. Since most of the CPY was correctly delivered to the vacuole, these results suggest that transport through primarily the Golgi is delayed, but not blocked in this mutant.

The VRG4 Gene Is Required for the Retention of BiP in the ER—As a test for sorting defects within the Golgi, we investigated whether any of the different mutant strains secreted soluble ER proteins into the culture media. The sorting of soluble resident ER proteins in yeast occurs via a receptor-mediated recycling mechanism (21, 29). Soluble HDEL-bearing ER proteins are free to leave the ER, along with other nascent secreted proteins. Upon reaching the Golgi complex, they are recognized by a Golgi-localized receptor. The receptor-ligand complex returns to the ER in a retrograde transport step (29). Since the decision to sort HDEL-bearing ER proteins occurs in the Golgi, failure to retain ER proteins reflects a Golgi sorting defect.

Proteins in culture supernatants from mutant and wild type cells were precipitated and assayed for the presence of the resident ER protein, BiP, by Western immunoblot analyses. Wild type cells do not secrete significant levels of BiP into the culture supernatant, as BiP is efficiently retained in the ER (Fig. 3, lane 5). By this assay, we found that among the different vanadate-resistant mutants, only *vrg4* had an ER retention defect, and secreted BiP at levels comparable to the ER retention mutant, *erd1* (ER retention defect) (30, 31) (Fig. 3, compare lanes 4 and 6). The intracellular level of BiP is induced as part of the unfolded protein response. To examine if the secretion of BiP in *vrg4* was an indirect result of increased BiP synthesis, which in turn saturates HDEL retention, we compared the intracellular level of BiP in the *vrg4* mutant to that of wild type cells by immunoblot analysis of protein extracts. An equal number of mutant and wild type cells were washed and intracellular proteins were extracted, precipitated, and assayed for the presence of the resident ER protein, BiP, by Western immunoblot analyses. The result of this experiment demonstrated that the level of intracellular BiP in *vrg4* and wild type cells was indistinguishable (Fig. 3B, compare lanes 1 and 2). None of the other vanadate-resistant glycosylation mutants secreted more BiP than did wild type cells. However, several mutants did contain markedly increased intracellular levels of BiP, presumably due to increased levels of misfolded proteins as a result of glycosylation defects (data not shown). These results demonstrate that the secretion of BiP in *vrg4* was not an indirect effect of elevated levels of misfolded proteins due to inappropriate glycosylation. Rather, these results suggest that the *vrg4* mutation affects the receptor-mediated retrieval of BiP from the early Golgi.

The VRG4 Gene Is Required for the Maintenance of Normal Membrane Morphology—The multiple defects observed in the *vrg4* mutant were consistent with the idea that the *vrg4* lesion causes a direct or indirect malfunctioning of the Golgi complex. To examine the overall morphology of membranes in the *vrg4* mutant, we examined the pattern of membrane staining in wild type and *vrg4* mutant cells treated with a lipophilic vital stain, DiOC₆ (3,3'-dihexyloxycarbocyanine iodide). As in higher eukaryotes, at high dye concentrations, this fluorescent dye stains membranous components of the secretory pathway in

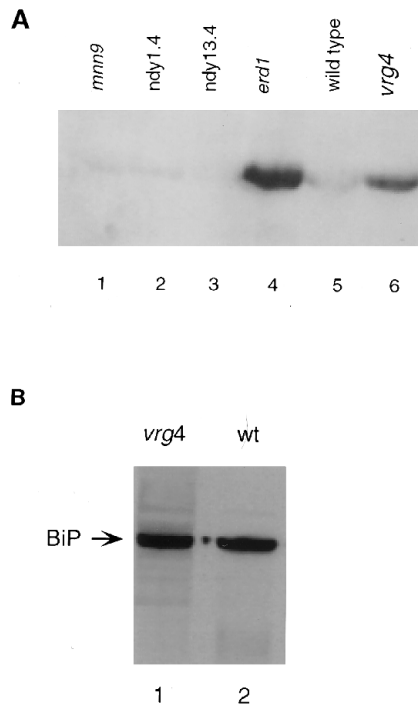


FIG. 3. VRG4 is required for the retention of BiP in the ER. Panel A, Western immunoblot of BiP in culture supernatants from mutant and wild type cells. Equivalent amounts of protein in media from four different vanadate-resistant mutants were analyzed as described under "Materials and Methods" using antibodies against the C-terminal HDEL peptide. These are compared with supernatants derived from *erd1* (lane 4) and parental strains (lane 5). Note that of the vanadate-resistant mutants, only *vrg4* (lane 6) fails to retain BiP intracellularly. Panel B, Western immunoblot of intracellular BiP in wild type and *vrg4* mutant cells. Cultures ($\sim 2 A_{600}$ units) were spun down to separate cells from culture supernatants and total intracellular protein extracted from washed, cell pellets as described under "Materials and Methods." Equivalent amounts of protein from each sample were subjected to SDS-PAGE and immunoblotted with anti-HDEL antibodies.

yeast, in particular the ER, Golgi, vesicles, and associated structures (22, 32).

A comparison of the staining pattern revealed striking differences in the endomembrane system of wild type and *vrg4* mutant cells. Wild type cells showed a characteristic network of ER membranes just below the plasma membranes and peripheral to the nucleus (Fig. 4, panels B and D). This was in contrast to the highly vesiculated appearance of *vrg4*. While the mutant also stained membranes below the plasma membrane and peripheral to the nucleus, the most evident difference in mutant cells was a fragmented, vesiculated appearance of stained membranes (Fig. 4, panels A and C).

A feature of *vrg4* that was usefully highlighted by DiOC₆ staining was the heterogeneity of mutant cells. (Fig. 4, panel C). Wild type cells are of a uniform size and shape, while *vrg4* cells are variable and formed large, clumped aggregates. The tendency to form aggregates was a general feature of the vanadate-resistant mutants we isolated and is a phenotype described previously (1). The vesiculation of intracellular membrane in *vrg4* was unlikely to be a consequence of this aggregation phenotype. While other glycosylation mutants also exhibited this phenotype, the staining patterns were very distinct from *vrg4*. This is exemplified by the staining pattern of *mnn10*, whose membranes had a more punctate-like appearance (Fig. 4, panel E).

We observed other phenotypes that were common to these vanadate-resistant mutants including osmotic sensitivity, reduced sporulation frequency, poor spore viability, reduced spheroplasting frequency, and 2–8-fold decreased growth rate

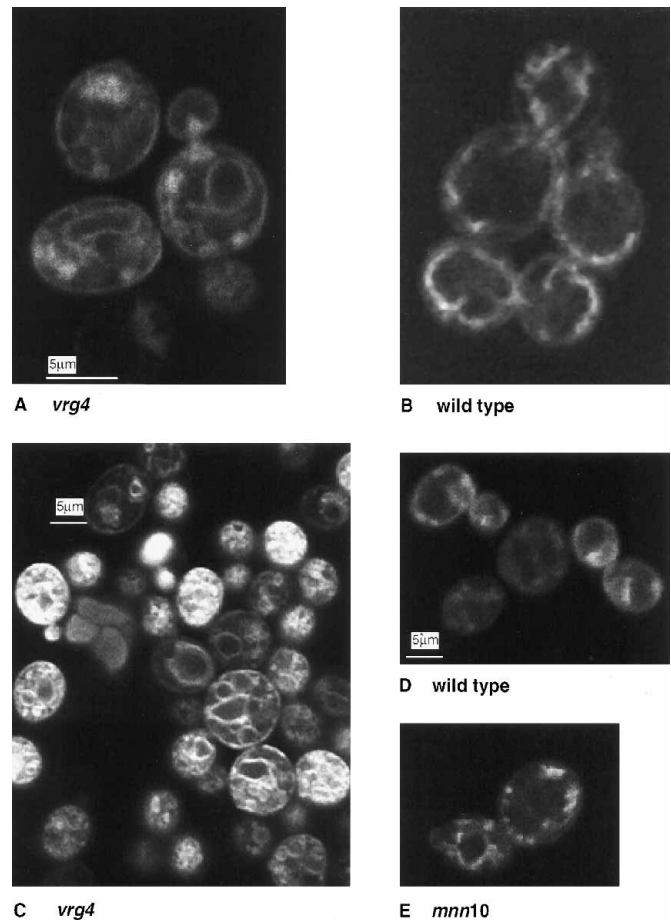


FIG. 4. DiOC₆ staining of wild type, *vrg4*, and *mnn10* cells. Mutant and wild type cells ($\sim 10^7$ cells) were treated with 10 $\mu\text{g/ml}$ of DiOC₆ (as described under "Materials and Methods") and visualized by confocal microscopy. Shown are *vrg4* (panels A and C), wild type (panels B and D), and *mnn10* (panel E) cells. All cells were viewed with a 60 \times oil immersion lens, but in panels A and B, an additional 2.5-fold magnification was provided through the zoom function in the software. Bar represents 5 μm .

(data not shown). Presumably, these were a consequence of cell wall defects due to defects in glycosylation. Because the entry or accumulation of DiOC₆ in mutant cells may be influenced by these defects, the concentration of DiOC₆ for both wild type and *vrg4* cells was carefully titrated and staining patterns were examined at different dye concentrations. Since the *vrg4* mutant grows with a decreased growth rate, we also examined the staining patterns at different growth stages. In all cases, we observed the same increased, fragmented membrane morphology as well as heterogeneity in *vrg4* cells, which was never observed in wild type cells (data not shown).

The morphology of intracellular membrane structures in *vrg4* was examined at higher resolution by thin-section electron microscopy. Cells were rapidly fixed in glutaraldehyde and membranes stained with potassium permanganate (Fig. 5). Again, a marked difference between *vrg4* and isogenic wild type parental cells was observed. Wild type cells exhibit highly contrasted, intensely staining membranous structures. Mutant membranes lack this contrast and appeared to be devoid of membrane-bound organelles entirely (Fig. 5, compare panels A and B), although a faintly stained nucleus was always observed. This phenotype was seemingly different from that of DiOC₆ staining, where the mutant membranes appear to be fragmented and highly vesiculated, but relatively abundant. Based upon these differences, it seems likely that, unlike wild type membranes, the mutant membranes were somehow al-

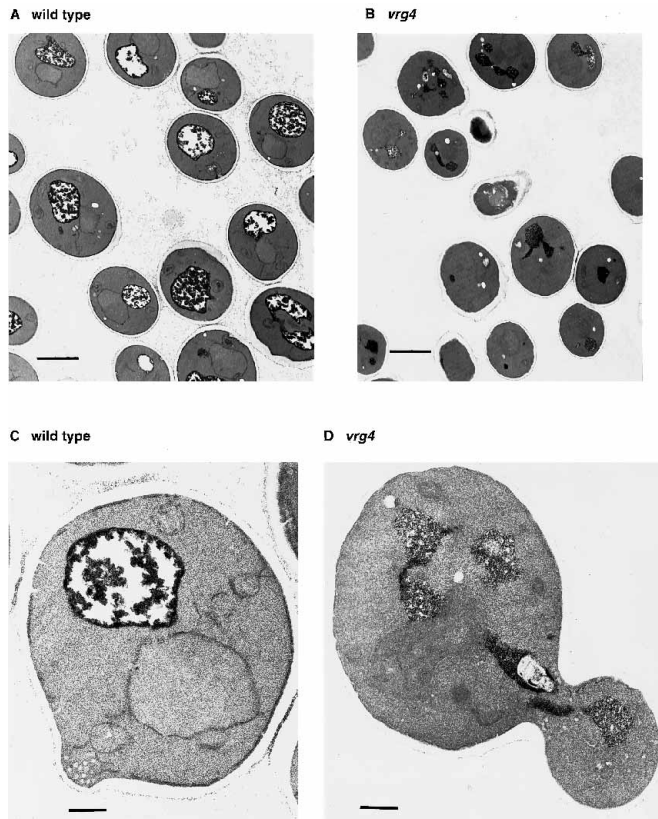


FIG. 5. Thin-section electron micrographs of *vrg4* and wild type yeast cells. *vrg4* mutant cells (panels B and D) or wild type (panels A and C) cells were stained with potassium permanganate and prepared as described under "Materials and Methods." Bars represent 2 μ m (panels A and B) and 500 nm (panels C and D).

tered in their permanganate staining properties. At higher magnifications, membranes and vesicles become more apparent, but clearly were distinct in their staining properties (Fig. 5, compare panels C and D). Unlike wild type cells, which typically contain a single vacuole, the *vrg4* mutant had small fragmented vacuolar-like structures that contain a higher amount of electron dense material. From these analyses we conclude that the *VRG4* gene is required for a normal endomembrane system.

Cloning and Analysis of the VRG4 Gene—Vanadate-resistant mutants fail to grow on media containing hygromycin B at concentrations where wild type cells grow normally (1). We exploited this drug sensitivity as a means to clone the wild type *VRG4* gene. Mutants were transformed with a *CEN*-based yeast genomic library, containing the *LEU2* gene as a selectable marker. Leucine prototrophs were selected and replica-plated onto media supplemented with 50 μ g/ml hygromycin B. Six hygromycin-resistant colonies were isolated. Plasmids isolated from each of these colonies were distinct, but contained overlapping restriction fragments. All six plasmids conferred hygromycin B resistance when retransformed into the *vrg4* mutant. Further subcloning isolated the complementing activity to a 2.1-kb *EcoRI/HindIII* fragment (Fig. 9A). Hybridization of the 32 P-labeled *EcoRI/HindIII* fragment to separated yeast chromosomes mapped this gene on chromosome XV (data not shown). Expression of the cloned fragment containing the putative *VRG4* gene in the *vrg4* mutant restores the ability of these cells to retain ER proteins (Fig. 6A, compare lanes 3 and 4) and rescues the invertase glycosylation defect (Fig. 6B). A slight amount of invertase that was underglycosylated could still be detected in *vrg4* mutant cells that harbored the cloned gene (Fig. 6B, lane 2). Cell growth during invertase induction

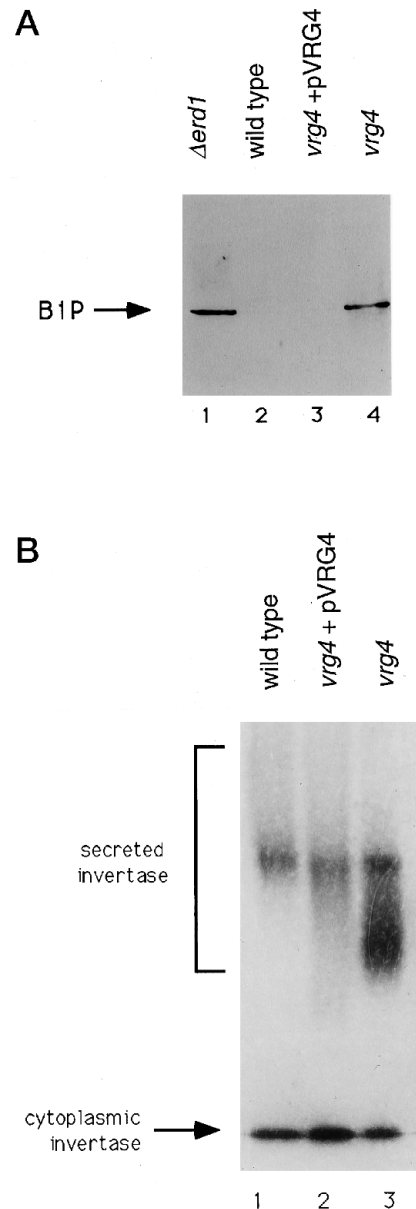


FIG. 6. The cloned *VRG4* gene rescues both the glycosylation and ER retention defects of *vrg4*. Panel A, Western immunoblot of proteins in culture supernatants from wild type (lane 2), *erd1* (lane 1), *vrg4* mutant (lane 4), and *vrg4* mutant harboring a plasmid bearing the *VRG4* gene (lane 3). The mutant *vrg4* harboring a plasmid bearing the cloned gene no longer secretes BiP into the culture media. Panel B, the *vrg4* mutant harboring a plasmid bearing the wild type *VRG4* gene recovers the ability to glycosylate invertase normally. Shown is a native *in situ* invertase assay (performed as in Fig. 1) comparing the glycosylation state of invertase from wild type (lane 1), *vrg4* mutant (lane 3), and mutant harboring the complementing clone (lane 2).

was not carried out under conditions that favor plasmid selection. Therefore, this apparent leakiness may have been due to plasmid loss in some of the cells assayed.

To confirm that the cloned fragment contained the *VRG4* locus, the *EcoRI/HindIII* fragment was cloned in an integrative plasmid (pRS306) that contains the selectable marker, *URA3*. The plasmid was linearized at a unique site within the *VRG4* portion to allow homologous recombination at the *vrg4* locus and used to transform *vrg4 ura3* cells. *Ura*⁺ transformants were then crossed to a *VRG4 ura3* strain. The resulting diploid was sporulated and tetrads dissected. This analysis demonstrated a 2:2 segregation pattern for *Ura*⁺/*Ura*⁻ and a 4:0 pattern for hygromycin resistance/hygromycin sensitivity, in-

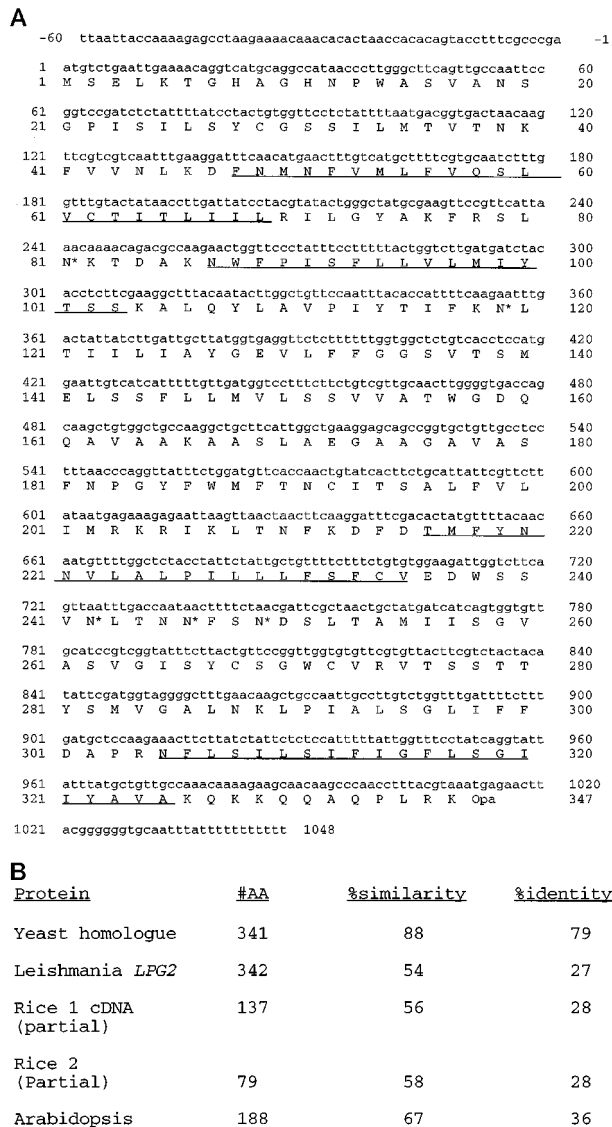


FIG. 7. The nucleotide and predicted amino acid sequence of the VRG4 gene and its relation to other genes. Panel A, the five potential glycosylation sites, at amino acid positions 81, 119, 242, 246, and 249, are denoted by asterisks. Four potential membrane-spanning domains, comprised of at least 20 uncharged residues and flanked by charged residues are underlined (and correspond to the black bars in Fig. 8). Recently, this sequence was found to be the same as that of the VAN2 gene (GenBank™ accession no. U15599 (11)). Panel B, summary of percent amino acid identities and similarities shared between the yeast Vrg4 protein and other members of this family (gap alignment program used the Needleman and Wunsch algorithm in the GCG program). The accession numbers for those sequences in the data base are as follows: yeast homologue, U18796 (gene *YER039c*); Leishmania, U26175; rice 1, D24450; rice 2, D24744; Arabidopsis, T45513.

dicating that the cloned fragment is tightly linked to *VRG4* and most likely does contain the *VRG4* locus (data not shown).

DNA sequence analysis of the 2.1-kb fragment revealed the presence of two open reading frames (Fig. 9A). Further analyses mapped the complementing activity to the larger open reading frame, within a 1.6-kb *HindIII/EcoRV* fragment. The nucleotide and predicted amino acid sequence of this region is shown in Fig. 7.

The *VRG4* DNA sequence encodes a predicted protein of 36.9 kDa. There are five potential recognition sites for *N*-linked glycosylation (indicated by asterisks in Fig. 7A). Hydrophobicity analysis (33) (Fig. 8) suggests that the protein is hydrophobic, containing multiple membrane-spanning domains.

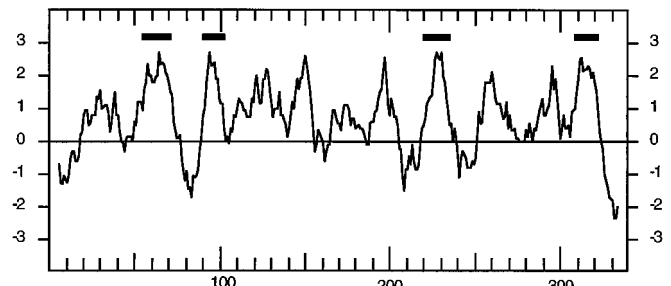


FIG. 8. Hydropathy plot of the predicted Vrg4p. Hydrophobicity was calculated according to the method of Kyte and Doolittle (19), using a window of 11 amino acids. The black bar represents the potential transmembrane domains, underlined in Fig. 7.

VRG4 Encodes a Protein Required for Viability—We analyzed the effect of replacing the wild type version of the *VRG4* gene with a null allele. A standard one-step gene disruption was performed in order to replace the chromosomal copy of the *VRG4* gene with a disrupted allele of *vrg4*, containing an insertion of the *LEU2* gene (34). A linear DNA fragment containing the disrupted version was used to transform a wild type diploid homozygous for the *leu2-3,112* mutation. The disruption of one allele was confirmed by Southern blot analysis of genomic DNA (data not shown).

Diploids were sporulated and dissected tetrads were analyzed for cell viability (Fig. 9). The resulting tetrad analysis demonstrated that the *VRG4* gene encodes a protein that is essential for cell viability. Only those *Leu*⁺ segregants carrying the wild type copy of *VRG4* were viable. Haploid segregants carrying the null allele were inviable. Spores carrying the *vrg4* disruption were able to germinate since microcolonies containing about 20 cells were formed. The conclusion from this experiment is that this gene product is required for the vegetative growth of these cells.

VRG4 Defines a Related Family of Proteins—A sequence homology search identified five genes with significant homology to *VRG4* (Fig. 7B). The first is an uncharacterized open reading frame on yeast chromosome V encoding a putative protein that is 79% identical and 88% similar to Vrg4p. Though remarkably similar, this protein does not perform a function redundant to that of Vrg4p as the disruption of *VRG4* leads to loss of viability (see below). Furthermore, this gene cannot complement a *vrg4* mutant even when overexpressed.²

The second *VRG4*-related gene is the Leishmania homologue, *LPG2*. The Vrg4p and Lpg2p proteins are 28% identical and 54% similar. Like *vrg4*, the Leishmania *lpg2* mutant is defective in mediating modifications that are specific to the Golgi (35). The *VRG4* gene does not rescue a Leishmania *lpg2* mutant (35), and likewise the *LPG2* gene fails to complement the *vrg4* mutant.³ It is unknown whether or not this lack of complementation reflects the evolutionary divergence between these two organisms, or whether yet another *VRG4*-like gene exists that is more functionally related to *LPG2*.

A search of the data base of expressed sequence tags (DBEST) has identified an additional set of cDNAs that encode putative proteins with a high degree of homology to *VRG4*, though no functions have been ascribed to these. These include one Arabidopsis (*Arabidopsis thaliana*) and two rice (*Oryza sativa*) genes. In the case of these three genes, sequence alignments suggest that these three cDNAs represent partial sequences. A summary of the similarity between *VRG4* and these related genes is shown in Fig. 7B. The identification of these related genes suggests that the *VRG4* gene product serves a

³ J. B. Poster and N. Dean, unpublished observations.

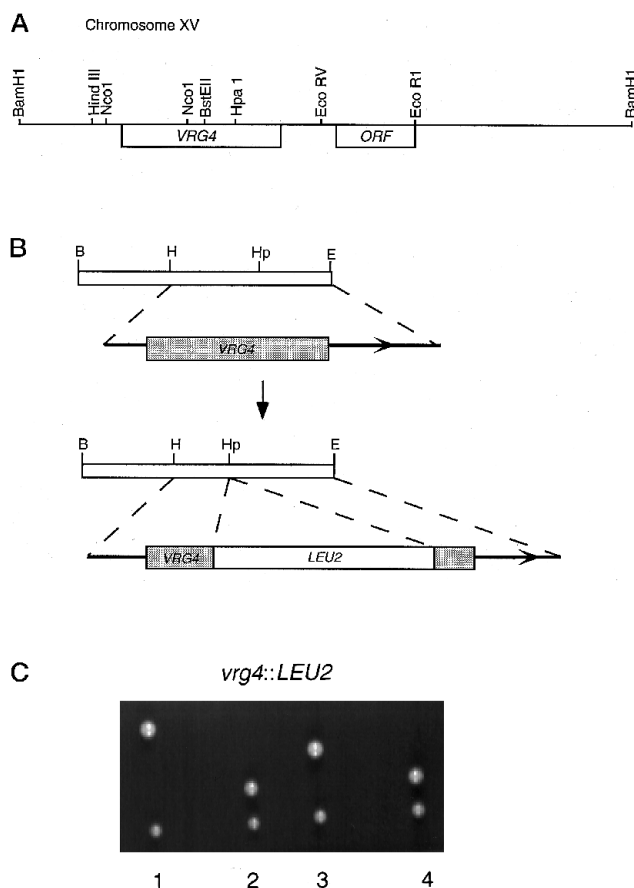


FIG. 9. **VRG4 encodes a protein required for viability.** Panel A is a schematic representation of the restriction map of the region surrounding the *VRG4* gene, on chromosome XV. Panel B is a schematic diagram of the strategy used to create the *vrg4::LEU2* disruption plasmid that was used to replace one copy of the chromosomal wild type *VRG4* allele in a diploid. B, H, Hp, and E refer to *Bam*HI, *Hind*III, *Hpa*I, and *Eco*RI restriction sites. Panel C, tetrad analysis of diploid strains heterozygous for the *VRG4* disrupted allele. Tetrads obtained from the sporulation were dissected on YPD plates, with four spores on one column. These were incubated for 3 days at 30 °C. Each column is labeled numerically.

highly conserved function, both in *Saccharomyces cerevisiae* and in other distantly related eukaryotes.

DISCUSSION

The analysis of oligosaccharide modification and the extent of glycosylation has been a major tool for the analysis of glycoprotein localization within the secretory pathway. Extending this idea, we have screened yeast mutants with Golgi-specific glycosylation defects to identify any that affect protein transport in the Golgi. We have identified one mutant in this screen, *vrg4*, that has a severe glycosylation defect and in addition, affects transport processes that occur specifically in the Golgi complex. Recently, Kanik-Ennulat *et al.* (11) have identified the *VRG4* gene as *VAN2*, a gene that can mutate to confer vanadate resistance. The *van2-93* mutant was isolated as a vanadate-resistant isolate and, similarly, was shown to be required for viability and to secrete underglycosylated invertase. The *vrg4* mutant is defective in the retrieval of HDEL-bearing ER proteins, the transport of CPY through the Golgi and, morphologically, exhibits an aberrant endomembrane system. Together, these phenotypes suggest that this protein has an important role in regulating normal Golgi function.

Like *vrg4*, several other mutants, including *pmr1* and *erd1*, have been identified that affect the secretory pathway and are

also defective in glycosylation. Both *pmr1* and *erd1* are resistant to vanadate, although not to the same degree as *vrg4*.² The *PMR1* (plasma membrane ATPase-related) gene encodes a Golgi-localized Ca^{2+} ATPase and leads to the same underglycosylation of invertase as seen in *mnn9* (36, 37). While the mechanism for the effect of *pmr1* on the secretory pathway is not understood, it is likely to be the result of its effect on Ca^{2+} flux into or out of the Golgi (36, 37). The *erd1* mutation affects the retention of HDEL-bearing ER proteins and also leads to the underglycosylation of invertase (30, 31). Like *PMR1*, *ERD1* may play a role in maintaining some aspect of Golgi structure or ionic environment that, when perturbed, results in pleiotropic effects on both glycosylation and secretion (30). The deleterious effect of the *vrg4* mutation on Golgi functions argues that Vrg4p may perform a similar function. The Leishmania *VRG4* homologue, *LPG2*, immunolocalizes to the Golgi apparatus (35), suggesting that Vrg4p is resident in that compartment and may play a direct role in mediating Golgi functions. Given its effect on the sorting of ER proteins and on the outer chain glycosylation of invertase, processes that are thought to occur in an early yeast Golgi compartment (21), it is likely that Vrg4p affects an early Golgi compartment, although it may, in addition, influence later compartments as well.

What is the essential function of Vrg4p? Although necessary for normal glycosylation, the protein is probably not a glycosyltransferase. Thus far only four yeast genes that encode Golgi-specific glycosyltransferases have been isolated. These include *MNN1* (25, 38), *MNT1* (also known as *KRE2*) (39, 40), *OCH1* (41), and *MNN10* (42). Like Golgi-localized glycosyltransferases in higher eukaryotes, these gene products share certain structural features. All are Type II membrane proteins, with single transmembrane domains and large C-terminal luminal domains. The predicted Vrg4 protein sequence does not match this consensus. Furthermore, yeast can survive, albeit poorly, in the absence of outer chain glycosylation. Since the *VRG4* gene is required for viability, it is unlikely that its essential function is carbohydrate modification.

The aberrant morphology of mutant membranes, most clearly seen by thin-section electron microscopy, suggests at least one important function in which the *VRG4* gene does play a role. The mutants lack the high staining contrast of wild type permanganate-stained membranes. The mechanism by which permanganate stains membranes is not well understood. It is likely due to the deposition of MnO_2 at the polar ends of lipids in the membrane (43). The clarity of permanganate-stained membrane is also thought to be due, in part, to the loss of protein components in the membrane as a result of oxidative cleavage of proteins by KMnO_4 (43). In either case, the altered staining characteristics of mutant membranes reflects an alteration in their lipid or protein composition, suggesting that the essential role of *VRG4* is to establish or maintain the normal lipid/protein ratio of these membranes.

The combined effects of *VRG4* on a number of Golgi-specific functions, coupled to its effect on membrane morphology, suggest that Vrg4p plays an important role in establishing or maintaining the organization of this organelle. Analyses of this protein, as well as those defective in other vanadate-resistant mutants that indirectly affect the Golgi complex, will further our understanding of the factors that regulate the structure and function of this organelle.

Acknowledgments—We thank Clint Ballou for yeast strains and Ron Hitzeman for valuable discussions and for performing the complementation analysis with *vrg4*. We are especially grateful to Jackie Partin for technical assistance with electron microscopy, Bill Therkauf for help with the confocal microscope, and Debbie Brown and Nancy Hollingsworth for critical reading of the manuscript.

Note Added in Proof—We have recently analyzed the localization of epitope-tagged Vrg4 protein by indirect immunofluorescence. Like the *Leishmania* homologue, yeast Vrg4p is localized in the Golgi complex, supporting our conclusion that its affect on Golgi functions is direct.

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