Molecular and Phenotypic Analysis of *CaVRG4*, Encoding an Essential Golgi Apparatus GDP-Mannose Transporter

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Cell surface mannan is implicated in almost every aspect of pathogenicity of *Candida albicans*. In *Saccharomyces cerevisiae*, the Vrg4 protein acts as a master regulator of mannan synthesis through its role in substrate provision. The substrate for mannosylation of proteins and lipids in the Golgi apparatus is GDP-mannose, whose lumenal transport is catalyzed by Vrg4p. This nucleotide sugar is synthesized in the cytoplasm by pathways that are highly conserved in all eukaryotes, but its lumenal transport (and hence Golgi apparatus-specific mannosylation) is a fungus-specific process. To begin to study the role of Golgi mannosylation in *C. albicans,* we isolated the *CaVRG4* gene and analyzed the effects of loss of its function. *CaVRG4* encodes a functional homologue of the *S. cerevisiae* GDP-mannose transporter. CaVrg4p localized to punctate spots within the cytoplasm of *C. albicans* in a pattern reminiscent of localization of Vrg4p in the Golgi apparatus in *S. cerevisiae*. Like partial loss of *ScVRG4* function, partial loss of *CaVRG4* function resulted in mannosylation defects, which in turn led to a number of cell wall-associated phenotypes. While heterozygotes displayed no growth phenotypes, a hemizygous strain, containing a single copy of *CaVRG4* under control of the methionine-repressible *MET3* promoter, did not grow in the presence of methionine and cysteine, demonstrating that *CaVRG4* is essential for viability. Mutant *Candida vrg4* strains were defective in hyphal formation but exhibited a constitutive polarized mode of pseudohyphal growth. Because the *VRG4* gene is essential for yeast viability but does not have a mammalian homologue, it is a particularly attractive target for development of antifungal therapies.

Candida albicans is the most important human fungal pathogen. It causes infections that range from superficial colonization of oral and vaginal tissues to life-threatening infections in severely immunocompromised hosts. An essential step for the colonization and infection of host tissues is adhesion, which is initiated by the outermost components of the fungal cell wall. Cell wall-associated mannosylated proteins (mannans) on the external layer of the cell wall have been implicated as key determinants that mediate these initial and critical interactions between the fungus and its host (for reviews, see references 8, 10, and 11). The mannose branches on these glycoproteins, attached by N- and O-glycosidic linkages, are the structures recognized during the immune response against the pathogen (for reviews, see references 3, 14, and 15). Therefore, the enzymes that regulate addition of the mannose molecules are the focus of intense research for understanding fungal biology and mechanisms of host defense and as potential antifungal drug targets.

Biogenesis of mannoproteins in *Saccharomyces cerevisiae* has been well characterized (for a review see reference 40). Following the initial glycosylation steps in the endoplasmic reticulum, yeast mannoproteins are elongated by addition of mannose or mannosylphosphate in the Golgi apparatus. Extension

and branching of N-linked oligosaccharides in the Golgi apparatus result in structures that contain up to 200 mannose units per site, while each O-linked chain is extended up to 5 mannose units (16). In addition to protein mannosylation, sphingolipids and glycophosphatidylinositol (GPI) anchors are also modified by addition of mannose in the Golgi apparatus (5, 47). The sugar donor for all of these Golgi apparatus-localized reactions is GDP-mannose, whose site of synthesis is the cytoplasm (for a recent review see reference 6). Utilization of GDP-mannose by the mannosyltransferases requires transport of this compound into the lumen of the Golgi apparatus by a specific nucleotide sugar transporter (1) that is encoded by the *VRG4* gene (19). Through its role in substrate provision, the activity of Vrg4p is the first and rate-limiting step in the Golgi apparatus-mediated mannosylation of cell surface glycoproteins in yeast cells. While none of the genes that encode various Golgi protein or lipid mannosyltransferases appear to be essential, deletion of the *VRG4* gene is lethal in *S. cerevisiae*. Therefore, while a defect in any one pathway can be tolerated in this yeast, a complete loss of Golgi mannosylation leads to death.

Much of the machinery that governs oligosaccharide biosynthesis in *C. albicans* and *S. cerevisiae* appears to be conserved. The structure of the outer chains of N-linked oligosaccharides on glycoproteins in *C. albicans* is similar, although not identical, to the structure of the outer chains of N-linked oligosaccharides on glycoproteins in *S. cerevisiae* (53). Genes related to *S. cerevisiae* mannosyltransferase genes have been identified as functional homologues (50, 54–56) and by their sequence sim-

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TABLE 1. *C. albicans* and *S. cerevisiae* strains used in this study

Strain	Parental strain	Genotype	Reference or source
C. albicans strains			
CA14	SC5314	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434*	22
JPC16J	CAI4	*ura3*Δ::λ*imm434 vrg4*Δ::*hisG-URA3-hisG/ura3*Δ::λ*imm434 VRG4*	This study
JPC16J-1	CAI4	*ura3*Δ::λ*imm434 vrg4*Δ::*hisG/ura3*Δ::λ*imm434 VRG4*	This study
BWP17	CAI4	*ura3*Δ::λ*imm434 his1*::*hisG arg4*::*hisG/ura3*Δ::λ*imm434 his1*::*hisG arg4*::*hisG*	58
ANC1	BWP17	*ura3*Δ::λ*imm434 his1*::*hisG arg4*::*hisG vrg4*Δ::*hisG-URA3-hisG/ura3*Δ::λ*imm434 his1*::*hisG arg4*::*hisG VRG4*	This study
ANC2	BWP17	*ura3*Δ::λ*imm434 his1*::*hisG arg4*::*hisG vrg4*Δ::*hisG/ura3*Δ::λ*imm434 his1*::*hisG arg4*::*hisG VRG4*	This study
ANC3	BWP17	*ura3*Δ::λ*imm434 his1*::*hisG arg4*::*hisG vrg4*Δ::*URA3-Met3p-/ura3*Δ::λ*imm434 his1*::*hisG arg4*::*hisG vrg4*Δ::*hisG*	This study
S. cerevisiae strains			
SEY6210		*MAT*α *ura3-52 his3*Δ*200 trp1*Δ*901 lys2-801 suc2-*Δ*9 leu2-3,112*	S. Emr
RSY255		*MAT*α *ura3-52 leu2-211*	R. Sheckman
NDY5	RSY255	*MAT*α *ura3-52 leu2-211 vrg4-2*	41
XGY14	W303-1A	*MAT*α *ura3-1 his3-11 trp1-1 ade2-1 leu2-3,112 can1-100 GAL1p-VRG4*::*HIS3*	25

ilarities. As a first step towards studying the global effects of mannan defects on pathogenesis, here we describe identification of the *C. albicans VRG4* (*CaVRG4*) gene and phenotypic characterization of *C. albicans vrg4* mutants. Conditional loss of *CaVRG4* function leads to lethality, reduction in the amount of cell wall mannan, and profound induction of morphological abnormalities.

MATERIALS AND METHODS

Strains, media, growth conditions, and transformation protocols. The *C. albicans* and *S. cerevisiae* strains used in this study and their genotypes are shown in Table 1. *S. cerevisiae* strains were routinely grown on YPAD (1% yeast extract, 2% Bacto Peptone [Difco], 50 mg of adenine sulfate per liter, 2% glucose). *C. albicans* strains were grown on YPAD supplemented with 50 μg of uridine (Uri) per ml [YAPD(+Uri)] (in medium designations a plus sign before a compound indicates that the compound is present in the medium, and a minus sign before a compound indicates that the compound is not present in the medium). *C. albicans* ANC3 (*MET3p-VRG4/vrg4*Δ) was maintained on SD(−Ura,−Met, −Cys) (0.67% yeast nitrogen base without amino acids supplemented with 2% glucose and amino acids but lacking methionine [Met], cysteine [Cys], and uracil [Ura]). To repress the *MET3* promoter, *C. albicans* strains were grown in SD or YPAD(+Uri) containing 5 mM methionine and 2 mM cysteine. To induce hyphal formation in solution, strains were inoculated at a concentration of 5×10^6 cells/ml into YPAD(+Uri) supplemented with 10% fetal calf serum and, as indicated below, with methionine and cysteine and were grown at 37°C. To examine morphological defects on solid media, strains were grown on Spider medium (1% nutrient broth, 1% mannitol, 2% potassium phosphate [pH 7.2]) or YPAD(+Uri) with 4% fetal calf serum, solidified with 2% agar. When necessary (see below), these media were supplemented with 2 mM methionine and 0.5 mM cysteine.

C. albicans was transformed by the method described by Gietz et al. (26), with the following modifications. For each transformation, 5 ml of cells grown in YPAD(+Uri) (optical density at 600 nm [OD$_{600}$], 2) was harvested, washed once with 100 mM lithium acetate, and incubated with 5 to 10 μg of transforming DNA and 50 μg of salmon sperm DNA in 40% polyethylene glycol–100 mM lithium acetate in a 360-μl (final volume) mixture. After overnight incubation at 30°C, the cells were spread on selective medium.

Plasmid construction. All DNA manipulations were carried out by using standard protocols (43). pCaHHB contains the genomic clone of the entire *CaVRG4* open reading frame (ORF), as well as 3.3 kb of upstream flanking sequences and 0.6 kb of downstream flanking sequences, cloned in the *Hin*dIII/*Bam*HI site of pRS316, a *URA3/CEN6* yeast vector (46). pSK⁻CaVRG4 R/X contains the *CaVRG4* ORF, flanked by an *Eco*RI site upstream of the initiating start codon and an *Xba*I site downstream of the TAG stop codon. This 1.1-kb fragment was cloned into the *Eco*RI/*Xba*I site of pTαO (25) to produce pTiM1, which placed the *CaVRG4* ORF under control of the constitutive triose phosphate isomerase (*TPI1*) promoter for expression in *S. cerevisiae*. Digestion with *Xho*I in the *URA3* gene in pTiM1 targeted integration of this gene into the *S. cerevisiae ura3-52* locus. To produce a hemagglutinin (HA)-tagged allele, the

CaVRG4 ORF was amplified by PCR so that it included an *Eco*RI site upstream of the initiating codon and a 3′ *Pst*I site that replaced the TAG stop codon. The *Eco*RI/*Pst*I fragment was cloned into pSK⁻P/X HA$_3$ (37), which resulted in in-frame fusion of *CaVRG4* to sequences encoding three copies of the HA epitope to produce pSK⁻M1-HA$_3$. An *Eco*RI/*Xba*I fragment from pSK⁻M1-HA$_3$ was subcloned into pTαO to produce the integration plasmid pTiM1-HA$_3$, which was digested with *Xho*I to target integration in *S. cerevisiae*. To produce green fluorescent protein (GFP)-tagged alleles, the *CaVRG4* ORF was amplified by PCR as a *Bgl*II/*Sac*I fragment and subcloned into pAW6 (a gift from A. Warenda, State University of New York, Stony Brook). pAW6 encodes the yeast optimized GFP yEGFP (13) at the *Mlu*I/*Bgl*II sites of YPB1-ADHp. The resulting plasmid, pCaV:GFP, contained the *CaVRG4* ORF, tagged at the 3′ end with sequences encoding GFP, under the control of the strong *Candida ADH* promoter in a *Candida URA3 ARS*-containing vector.

pTiM1-A316D contains the *Cavrg4-A316D* allele, which carries a single missense mutation. The GCA codon, encoding Ala^{316}, was changed to GAT (encoding Asp) by site-directed mutagenesis with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) used according to the manufacturer's instructions.

Disruption plasmid pSK⁻Cavrg4Δ::URA3 was constructed in several steps. First, 200 bp of the 5′ end (as an *Eco*RI/*Bam*HI fragment) and 200 bp of the 3′ end (as a *Bam*HI/*Xba*I fragment) flanking the *CaVRG4* ORF were amplified by PCR and cloned into pBluescript SK(−), which resulted in a unique *Bam*HI site between the 5′ and 3′ *CaVRG4* sequences. The 2.86-kb *Bgl*II/*Bam*HI *hisG-CaURA3-hisG* fragment from p5921 (22) was inserted into this *Bam*HI site, resulting in a plasmid (pSK⁻Cavrg4Δ::URA3) containing the *hisG-CaURA3-hisG* region flanked by exactly 200 bp of the 5′ and 3′ ends of *CaVRG4*. This fragment was released prior to transformation by digestion with *Sac*I and *Hin*dIII.

To generate a derivative of *CaVRG4* that was under the control of the *MET3* promoter, pDisCaVRG4 was constructed. About one-half of the 5′ portion of the *CaVRG4* ORF (673 bp), including the first ATG, was amplified by PCR and cloned in the *Bam*HI/*Pst*I site of pCaDIS (9). Linearization of this plasmid at a unique *Bst*EII site within the *CaVRG4* sequence targeted this fragment to integrate at the chromosomal locus of *CaVRG4*, simultaneously truncating the endogenous *VRG4* gene and inserting the *MET3p*-driven *CaVRG4* gene.

Isolation of chromosomal DNA and Southern hybridization. Overnight cultures (4 ml) were harvested, washed with water, and suspended in 0.4 ml of TENTS (2% Triton X-100, 1% sodium dodecyl sulfate [SDS], 100 mM NaCl, 10 mM Tris [pH 8.0], 1 mM EDTA). After addition of 0.4 ml of phenol-chloroform (1:1), cells were broken by vortexing them with glass beads. Nucleic acids in the aqueous phase were ethanol precipitated and digested with 10 μg of RNase per ml, and the remaining chromosomal DNA was ethanol precipitated and dissolved in 100 μl of Tris-EDTA. DNA probes were prepared by the random priming method with a Prime-It Fluor labeling kit (Stratagene). Southern transfer and hybridization were carried out as described previously (17). Detection was carried out with the Illuminator chemiluminescent detection system (Stratagene) used according to the manufacturer's instructions.

Cloning and sequence analysis of the *C. albicans VRG4* gene. A single 3.5-kb *Bam*HI/*Hin*dIII fragment, containing the 5′ end of the *C. albicans VRG4* gene

and flanking sequences, was detected by hybridization to the *S. cerevisiae VRG4* (*ScVRG4*) gene. To isolate this *Bam*HI/*Hin*dIII fragment, a mini-genomic library enriched for the DNA fragment was prepared. A 100-μg portion of genomic *C. albicans* CAI4 DNA was digested with *Bam*HI and *Hin*dIII, and fragments that were between 3.0 and 5.0 kb long were size fractionated and cloned into pBluescript SK(−) (Stratagene). The resulting library was used to transform *Escherichia coli* for use in a modified bacterial hybridization screening analysis, performed as described previously (17), in which increasingly smaller pools of transformants were screened by Southern blot analysis to identify individual positive colonies. In the initial hybridization procedures we employed the *ScVRG4* gene as a probe. A sequence from the *Candida* sequencing project that resembled the 3′ portion of the *ScVRG4* gene allowed the corresponding *C. albicans* fragment to be amplified by PCR. This fragment was subsequently used as a homologous probe. Positive clones were confirmed by Southern blot analysis. An additional 1.2-kb *Hin*dIII/*Hin*dIII fragment, containing the 3′ end of the *CaVRG4* ORF and flanking DNA, was isolated as described above to allow reconstruction of the entire *C. albicans VRG4* gene. Sequence analysis was performed by the dideoxy chain termination method using a Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech). DNA sequence analysis was performed with an automated LI-COR 4000L DNA sequencer.

Disruption of the *CaVRG4* locus. Gene disruption was performed in two steps. The first allele of *CaVRG4* was deleted by utilizing the *URA* blaster protocol and the *hisG-CaURA3-hisG* module (22). pSK⁻CaVRG4Δ::URA3 (see above) was linearized with *Hin*dIII and *Sac*I and transformed into *C. albicans* BWP17 (58). Uracil prototrophs were isolated on SD(−Ura) medium. Putative heterozygotes were screened by Southern blotting to confirm disruption of the *CaVRG4* locus (e.g., strain ANC1). Forced excision of the *URA3* gene by selection on 5′-fluoroorotic acid (5′-FOA) allowed recovery of uracil auxotrophs. A suitable strain (ANC2) was verified by Southern blot analysis and transformed with pDISCaVRG4 (see above) in order to simultaneously delete the remaining *VRG4* allele and introduce the *MET3p-VRG4* allele. The genotypes of the resulting hemizygous strains (e.g., ANC3) were verified by Southern blot analysis (Table 1).

Complementation analyses. The *CaVRG4* gene was introduced into *S. cerevisiae* NDY5, which contains the *vrg4-2* allele (41), or *S. cerevisiae* XGY14, which contains the glucose-repressible *GAL1p-VRG4* allele (25). *CaVRG4* and *ScVRG4* were introduced into each of these strains on integrative plasmids pTiM1 and pTαO-ScVRG4 (25), respectively. Complementation of the hygromycin B-sensitive phenotype of NDY5 was assayed by growth on YPAD agar supplemented with 30 μg of hygromycin B per ml (18). Complementation of the lethality associated with the glucose-dependent repression of *VRG4* expression in XGY14 was assayed by comparing growth on YPA containing galactose and growth on YPAD (25).

Alcian blue binding assay. Alcian blue dye binding assays were carried out as described previously (38), with the following modifications. *C. albicans* strains were grown to the stationary phase in YPAD(+Uri) with or without Met/Cys at 30°C. After washing and resuspension in H_2O, each of the cultures was divided into two aliquots. One aliquot was dried to determine the dry weight, while the other was subjected to Alcian blue dye binding. The amounts of dye bound by different *C. albicans* strains were determined spectrophotometrically (at 600 nm) by measuring the optical density of Alcian blue that remained in the supernatant after the binding reaction. For each sample, values (see Fig. 8B) were normalized to the cell dry weight and expressed as a percentage of the value obtained for parental strain BWP17 that had been grown in YPAD, as follows: DB = (OD_{600} of dye solution − OD_{600} of cell supernatant)/cell dry weight; and relative dye binding = (DB of strain/DB of BWP17 grown in YPAD) (100), where DB is dye binding.

Immunoprecipitation, Western blot, and immunofluorescence analyses. HA-tagged CaVrg4p was detected by immunoblot analysis of whole-cell lysates prepared from *S. cerevisiae* by trichloroacetic acid precipitation as described previously (12), using anti-HA mouse antibodies. Primary antibodies were bound to horseradish peroxidase (HRP)-coupled mouse immunoglobulin G and were detected by the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

For analysis of concanavalin A (ConA)-reactive proteins, proteins in whole-cell lysates were isolated and processed as described above, but the membranes were blocked with 0.2% Tween 20 in phosphate-buffered saline. Glycoproteins were probed with 0.2 μg of HRP-labeled ConA (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline and were detected by chemiluminescence.

HA-tagged proteins were immunoprecipitated by incubating a 0.5% Triton X-100 detergent extract with anti-HA mouse antibodies as described previously (24). Proteins in whole-cell lysates and in anti-HA immunoprecipitates were solubilized in Laemmli sample buffer and fractionated by SDS–12% polyacryl-

amide gel electrophoresis (PAGE). After transfer, proteins on membranes were blotted with anti-HA rabbit polyclonal antibodies (Santa Cruz Biotechnology), and secondary HRP-conjugated anti-rabbit antibodies were detected by chemiluminescence.

Strains harboring CaVRG4-GFP plasmids (pCaV:GFP and pAW6) were grown in SD(−Ura) medium to an OD_{600} of 2, spotted onto glass slides, and viewed immediately by fluorescence microscopy. Cell were stained with Calcofluor white as described previously (27). Indirect immunofluorescence analysis of yeast cells expressing ScVrg4-HAp or CaVrg4-HAp and data analyses were performed as described previously (19).

In situ acid phosphatase assay. Native gel electrophoresis and activity staining of acid phosphatase from *C. albicans* were carried out as described by Schweingruber et al. (45) for the homologous *Schizosaccharomyces pombe* enzyme, with the following modifications. Cells were grown in YPAD(+Uri) to the log phase (OD_{600}, 5), and acid phosphatase activity was induced by overnight cultivation at 30°C in 20 ml of phosphate-reduced YPAD(+Uri) depleted of phosphate as described previously (59). After the cells were washed once in 62.5 mM Tris-HCl (pH 6.8), they were resuspended in 200 μl of lysis buffer (59) and broken by vortexing with glass beads (three times, 20 s each). The lysate was centrifuged for 10 min, and the resulting supernatant was transferred and centrifuged for 10 min to remove insoluble precipitates. The supernatant was mixed with 0.5 volume of sample buffer (59), and 5 μl was applied to a gel and subjected to 5% native gel electrophoresis at 4°C as described previously (45). After incubation for 10 min in 100 mM sodium acetate (pH 4.0) (SA), the gel was incubated at 37°C in SA containing 0.5 mg of α-naphthyl acid phosphate (Sigma) per ml for 1 h. Color was developed by adding 50 ml of SA (prewarmed to 60°C) containing 0.7 mg of *o*-dianisidine (tetrazotized; Sigma) per ml and incubating the preparation at 60°C for 10 min. Endoglycosidase H (endo H) (New England Biolabs) was added, but samples were digested in the absence of heat or detergent denaturation. Endo H-digested samples were resolved by 8% polyacrylamide native gel electrophoresis but otherwise were treated as described above.

Nucleotide sequence accession number. The *CaVRG4* gene and protein sequences have been deposited in the GenBank database under accession number AF164627.

RESULTS

Isolation and analysis of the *C. albicans VRG4* gene. Using genomic Southern analysis, we identified and isolated a DNA fragment that cross-hybridized to the *S. cerevisiae VRG4* gene (see Materials and Methods). Subsequent cloning and sequence analysis demonstrated that this fragment exhibited sequence homology to the 5′ end of the *S. cerevisiae VRG4* ORF. The full-length *CaVRG4* gene was reconstructed by isolating a genomic fragment that contained the 3′ end of *CaVRG4* and downstream flanking sequences (see Materials and Methods). The sequence of the entire 7.7-kb *Hin*dIII fragment was determined. In addition to the *CaVRG4* gene, this fragment also contained in its 5′ portion a sequence that is very similar to the *S. cerevisiae ADE4* gene, suggesting that the *CaVRG4* gene is adjacent to and 3′ with respect to *CaADE4*.

The sequence of the predicted CaVrg4 protein (GenBank accession no. AF164627) and alignment of this sequence with the *S. cerevisiae* Vrg4p sequence are shown in Fig. 1. The *CaVRG4* ORF encodes a 371-amino-acid polypeptide with a predicted molecular mass of 41.2 kDa; the molecular mass of ScVrg4p is 36.8 kDa. The two proteins are related along their lengths (65% identical and 79% similar) except for an additional 33-amino-acid N-terminal region in CaVrg4p that is not present in ScVrg4p.

***CaVRG4* encodes a functional homologue of *S. cerevisiae* VRG4.** Although deletion of the *S. cerevisiae VRG4* gene is lethal, *vrg4-2* is a viable allele. Like growth of other *S. cerevisiae* glycosylation mutants, growth of a *vrg4-2* mutant is inhibited by the aminoglycoside hygromycin B (4, 18). As a test for functionality, the *CaVRG4* gene was examined to determine its

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CaVrg4p   1   MGVISFYLIGQLLYLIRKKYTTTYRQQQQYQYNMDSKHSTSSSSSGSLATRISNSGPISIAAYCLSSILMTVTNKYVLSGFSFNLNFFLL
ScVrg4p   1   ---------------------------------M-SELKTGHAGHNPWAS-VANSGPISILSYCGSSILMTVTNKFVVNLKDFNMNFVML

CaVrg4p  91   AVQSIVCIVTIGSLKSLNIITYRQFNKDEAKKWSPIAFLLVAMIYTSSKALQYLSIPVYTIFKNLTIILIAYGEVIWFGGKVTTMALSSF
ScVrg4p  55   FVQSLVCTITLIILRILGYAKFRSLNKTDAKNWFPISFLLVLMIYTSSKALQYLAVPIYTIFKNLTIILIAYGEVLFFGGSVTSMELSSF

CaVrg4p 181   LLMVLSSVIAYYGDNAAVKSHDDAFA---------LYLGYFWMLTNCFASAAFVLIMRKRIKLTNFKDFDTMYYNNLLSIPILLICSFIF
ScVrg4p 145   LLMVLSSVVATWGDQQAVAAKAASLAEGAAGAVASFNPGYFWMFTNCITSALFVLIMRKRIKLTNFKDFDTMFYNNVLALPILLLFSFCV

CaVrg4p 262   EDWSSANVSLNFPADNRVTTITAMILSGASSVGISYCSAWCVRVTSSTTYSMVGALNKLPIALSGLIFFEAAVNFWSVSSIFVGFGAGLV
ScVrg4p 235   EDWSSVNLTNNFSNDS----LTAMIISGVASVGISYCSGWCVRVTSSTTYSMVGALNKLPIALSGLIFFDAPRNFLSILSIFIGFLSGII

CaVrg4p 352   YAVAKQKQQKEQSQQLPTTK
ScVrg4p 321   YAVAKQKKQQAQ----PLRK
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FIG. 1. Alignment of the *S. cerevisiae* and *C. albicans* Vrg4 proteins. Identical residues are shaded. The GenBank accession number of *ScVRG4* is L33915.

ability to suppress the drug sensitivity of an *S. cerevisiae vrg4-2* mutant. Expression of the *CaVRG4* gene, driven by the constitutive *S. cerevisiae TPI1* promoter, fully restored growth of the *vrg4-2* mutant on medium supplemented with hygromycin B (Fig. 2A).

As a second test for functionality, complementation of the lethality associated with loss of *VRG4* function was examined. The *CaVRG4* gene was introduced into an *S. cerevisiae* strain containing *VRG4* under the control of the glucose-repressible *GAL1* promoter. This strain grows normally in the presence of galactose but does not grow when *VRG4* gene expression is repressed in the presence of glucose. Expression of the *CaVRG4* gene and expression of the *ScVRG4* gene in this strain resulted in comparable growth rates on glucose-containing media (Fig. 2B), further suggesting that the *CaVRG4* gene is a functional homologue of its *S. cerevisiae* counterpart.

ScVrg4p is specific for transport of GDP-mannose, and a region required for binding to GDP-mannose has been mapped to a cytoplasmically oriented region of the protein within the GALNK motif (25). The *vrg4-2* allele has a single missense mutation in the GALNK region (A286D) that impairs binding to GDP-mannose (25). As another, more stringent test for the functional redundancy of the *C. albicans* and *S. cerevisiae* Vrg4 proteins as GDP-mannose transporters, we examined whether this single amino acid, which is a critical component of the GDP-mannose binding domain, is functionally conserved in the CaVrg4 protein. Site-directed mutagenesis was used to replace the corresponding amino acid, Ala[316] of the CaVrg4 protein, with Asp (see Materials and Methods), and the *Cavrg4-A316D* allele was analyzed for functionality by complementation. When expressed in an *S. cerevisiae* strain containing the *vrg4-2* allele, the *Cavrg4-A316D* allele did not complement the growth defect on medium supplemented by hygromycin B (Fig. 2C). This mutant gene also did not complement growth of the *GAL1-VRG4* strain on glucose-containing media (data not shown). These results demonstrate that mutation of this single amino acid, which was previously shown to define the substrate specificity of GDP-mannose transport by Vrg4p, results in loss of CaVrg4p function and that the same

amino acid is important for the functions of both proteins. Together with data which suggest that CaVrg4p resides in the Golgi apparatus (see below), these results imply that the *CaVRG4* gene mediates lumenal transport of GDP-mannose into the Golgi apparatus.

CaVrg4 protein resides in the Golgi apparatus. To examine the properties of the CaVrg4 protein, an allele that encodes HA-tagged CaVrg4p was constructed and expressed in *S. cerevisiae*. The HA-tagged CaVrg4 protein was functional in *S. cerevisiae*, as the *CaVRG4-HA* allele could complement an *S. cerevisiae vrg4* mutant (data not shown). When examined by immunoblot analysis in whole-cell protein extracts, the level of CaVrg4-HAp that accumulated at steady state was much lower (about 10-fold lower) than the level of ScVrg4-HAp (Fig. 3A, lanes 1 and 2). Detection of CaVrg4p at levels that were comparable to ScVrg4p levels required enrichment by immunoprecipitation (lanes 3 and 4). The reduced level of CaVrg4p was probably not due to differences between the transcription rates of the *S. cerevisiae* and *Candida VRG4-HA* genes, since both genes were integrated at the same locus and expressed under the control of the *TPI* promoter. Since the level of *CaVRG4* gene expression fully complemented *S. cerevisiae vrg4* mutants, the presence of even very low levels of CaVrg4p at steady state is apparently sufficient for function in *S. cerevisiae*. CaVrg4p migrated more heterogeneously and at a higher molecular weight than ScVrg4p during SDS-PAGE (Fig. 3A, lanes 1 and 2). The heterogeneity with which CaVrg4p migrated indicates that it may undergo posttranslational modification, although this was not investigated further. The increased molecular weight of CaVrg4p is consistent with its predicted additional N-terminal domain that is not present in *S. cerevisiae* Vrg4p.

In *S. cerevisiae* the Vrg4 protein resides in the Golgi compartment, and this localization is critical for function. Mutations in the N-terminal Vrg4p domain that cause mislocalization result in lethality (24). Since CaVrg4p could functionally substitute for *S. cerevisiae* Vrg4p, we inferred that it was also localized in the Golgi apparatus. To confirm that this was the case, the intracellular locations of CaVrg4-HAp and ScVrg4-HAp in *S. cerevisiae* were examined by indirect immunofluo-
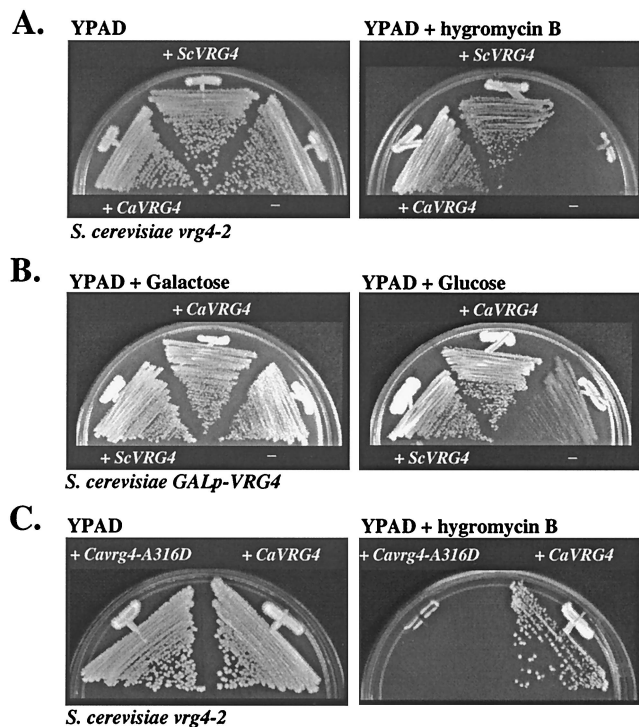
FIG. 2. Complementation of *S. cerevisiae vrg4* mutants by the *CaVRG4* gene. (A) Complementation of hygromycin B-sensitive growth of the *S. cerevisiae vrg4-2* mutant. Integrative plasmids expressing *CaVRG4* (pTiM1) or *ScVRG4* (pTαO-VRG4) (25) were introduced into NDY5 (*vrg4-2*), as described in Materials and Methods. Cells were plated on YPAD with or without 30 μg of hygromycin B per ml and incubated at 30°C for 3 days. (B) Complementation of the lethality associated with loss of *VRG4* function, as assayed in the *S. cerevisiae GAL1p-VRG4* strain. Integrative plasmids expressing *CaVRG4* (pTiM1) or *ScVRG4* (pTαO-VRG4) (25) were introduced into *S. cerevisiae GAL1p-VRG4* strain XGY14 as described in Materials and Methods. Strains were incubated for 3 days on YPA plates containing galactose or glucose. (C) Integrative plasmids containing wild-type *CaVRG4* (pTiM1) or the *Cavrg4-A316D* allele (pTiM1-A316D) introduced into *S. cerevisiae vrg4-2* strain NDY5 and assayed for complementation of the hygromycin B-sensitive growth phenotype as described above.

rescence analysis. CaVrg4-HAp displayed the same fluorescence pattern as ScVrg4-HA, albeit with a reduced intensity, residing in punctate spots throughout the cytoplasm that distinguished the Golgi apparatus in *S. cerevisiae* (Fig. 3B). The decreased intensity of CaVrg4-HAp fluorescence is in agreement with our finding that suggested that the level of expression of this protein in *S. cerevisiae* was reduced (Fig. 3A). This finding demonstrated that CaVrg4p contains the information for localization to the Golgi apparatus of *S. cerevisiae*.

Little is known about the structure of the *Candida* Golgi apparatus or its spatial distribution during morphogenetic switching. To examine CaVrg4p localization in live *Candida* cells, a fragment encoding the codon-optimized yEGFP (13) was fused in frame to the 3′ end of *CaVRG4*. The fusion construct was placed under control of the *ADH1* promoter in an *ARS/URA3*-containing plasmid. A plasmid that was identical except that it lacked the *CaVRG4* sequences was also constructed. Both plasmids were introduced into a wild-type strain
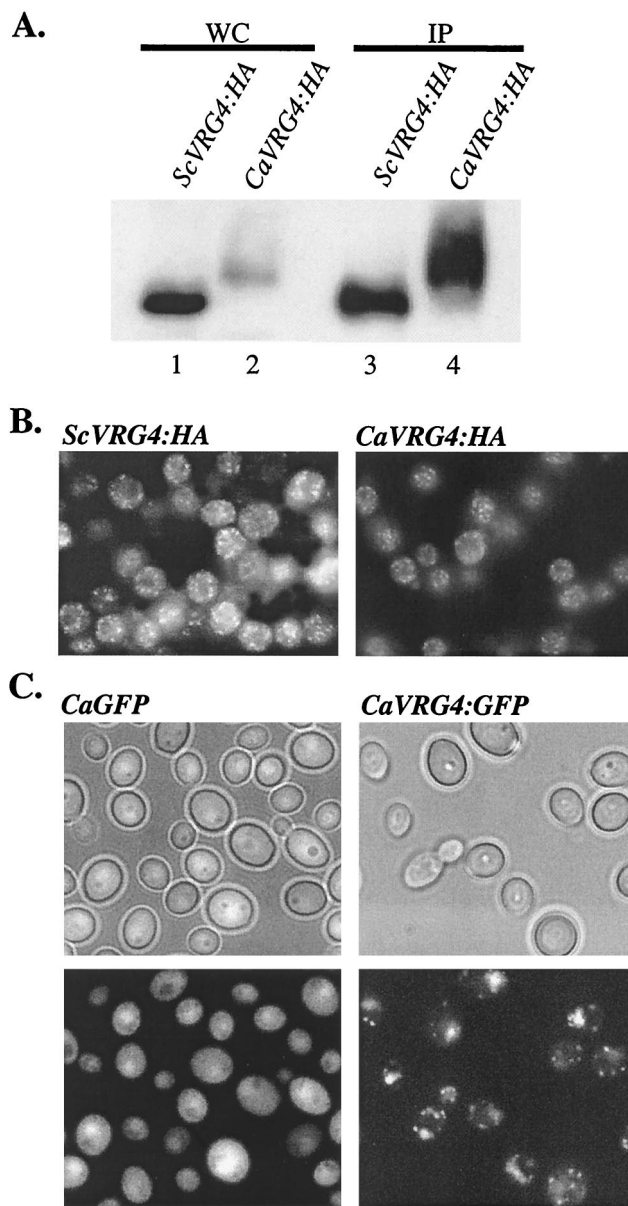

FIG. 3. Expression and intracellular localization of CaVrg4p. (A) Western blot analysis of CaVrg4-HA and ScVrg4-HA proteins. Whole-cell protein extracts were prepared from *S. cerevisiae* strains that were grown in YPAD to repress the endogenous *GAL1p-VRG4* gene (XGY14) (25) and harbored plasmids expressing either *ScVRG4-HA* (pTiVRG4-HA) (25) or *CaVRG4-HA* (pTiM1-HA). Equivalent amounts of proteins were directly applied to SDS-polyacrylamide gels (WC) (lanes 1 and 2) or were first immunoprecipitated with anti-HA mouse antibodies (IP) (lanes 3 and 4), and then the proteins were Western blotted with anti-HA rabbit antibodies and detected by chemiluminescence as described in Materials and Methods. (B) Indirect immunofluorescence of *S. cerevisiae* strains expressing *ScVRG4-HA* or *CaVRG4-HA*. *S. cerevisiae* strain XGY14 expressing *ScVRG4-HA* or *CaVRG4-HA* (as described above) was grown in YPAD to the log phase. Cells were fixed and treated with anti-HA antibodies, followed by fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G. (C) *CaVRG4-GFP* (pCaV:GFP) or *GFP* alone (pAW6) was expressed in *C. albicans* strain ANC2, and live cells were examined by fluorescence microscopy.

(BWP17) and a heterozygotic *vrg4 Candida* strain (ANC2), and GFP localization was monitored by fluorescence microscopy. No apparent difference between the morphology or phenotype of cells expressing ADH-driven CaVRG4-GFP and the morphology or phenotype of the control cells was observed. Cells expressing GFP alone exhibited an evenly distributed pattern of fluorescence throughout the cytoplasm, except for the vacuole. In contrast, the CaVrg4-GFP fusion protein localized in punctate spots (Fig. 3C). Its localization pattern was similar to, but in some ways distinct from, that seen in *S. cerevisiae*. In *Candida*, there were fewer spots, which tended to be more rod-like than the spots in *S. cerevisiae*. In agreement with these observations, previous studies in which the workers compared the morphologies of different yeasts by electron microscopy suggested that there was a difference in the appearance of structures hypothesized to be the Golgi apparatus in *C. albicans* and *S. cerevisiae* (42). Although preliminary, the different localization patterns of CaVrg4p in *S. cerevisiae* and *C. albicans* suggest that the structures or distributions of the Golgi apparatus in these two organisms may be different.

CaVRG4 is an essential gene. In order to determine the phenotypic consequences of loss of *CaVRG4* function, we attempted to construct a homozygous *vrg4Δ* strain. One allele of *CaVRG4* was deleted by using the *URA*-blaster technique (22). The disruption replaced the entire *VRG4* ORF with the *CaURA3* gene that was flanked by *Salmonella enterica* serovar Typhimurium *hisG* repeats (see Materials and Methods). Uracil prototrophs (e.g., strain ANC1) were plated on 5′-FOA-containing medium. Forced excision of the *URA3* gene and deletion of *CaVRG4* in 5-FOA-resistant uracil auxotrophs (e.g., strain ANC2) were confirmed by Southern blot analysis (Fig. 4B). To disrupt the second copy of *CaVRG4*, this procedure was repeated, but no homozygous null mutants were recovered. This procedure was performed with both *C. albicans* CAI4 and BWP17. Analysis of more than 50 transformants recovered after the second round of transformation indicated that the *Cavrg4Δ::hisG-URA3-hisG* disruption fragment had preferentially integrated into the previously disrupted allele (data not shown). This biased integration was due either to selective recovery of wild-type cells because of the lethality associated with loss of the *CaVRG4* function or to an allele-specific bias in homologous recombination. As *VRG4* is an essential gene in *S. cerevisiae*, the former possibility was the preferred model.

As an alternative strategy to test the null phenotype, we constructed a hemizygous strain that contained a single, conditional *VRG4* allele (Fig. 4A). The remaining copy of *VRG4* was truncated in a *VRG4/vrg4Δ::hisG* strain (ANC2), and a methionine-repressible *MET3p*-driven *VRG4* allele was simultaneously introduced (see Materials and Methods) (9). Uracil prototrophic transformants were selected under conditions that allowed *CaVRG4* expression (i.e., methionine and cysteine were not present in the growth medium), and the genotypes of the resulting strains (e.g., ANC3) were verified by Southern blot analysis (Fig. 4B). The strains grew on media lacking Met/Cys, although less well than the parental *VRG4/VRG4* or *VRG4/vrg4Δ* strains grew, suggesting that regulated expression by the *MET3* promoter did not induce *VRG4* at levels or in temporal patterns equivalent to those induced by the *VRG4* promoter. The strains did not grow on solid SD medium sup-
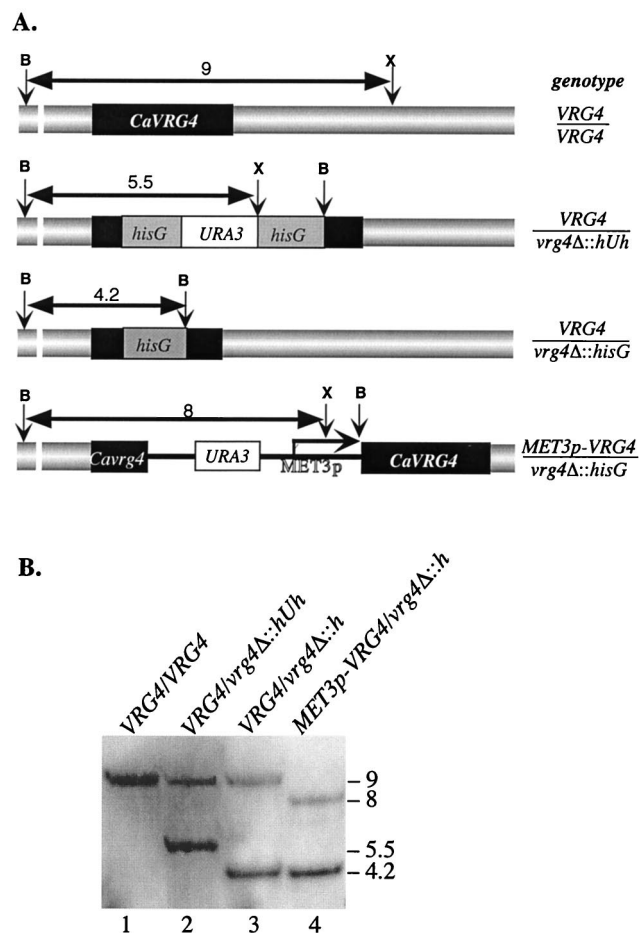

FIG. 4. Disruption of the *CaVRG4* gene. (A) Schematic restriction maps of *CaVRG4* and each of the disrupted alleles used to construct the heterozygous *VRG4/vrg4Δ* and hemizygous *MET3p-VRG4/vrg4Δ* strains. B and X indicate the positions of *Bam*HI and *Xba*I restriction sites, respectively, in the *CaVRG4* wild-type, *vrg4Δ::hisG-URA3-hisG*, *vrg4Δ::hisG*, and *MET3p-VRG4* alleles, and the predicted size (in kilobases) of each of the fragments is indicated. (B) Southern blot analysis of *C. albicans* genomic DNA digested with *Bam*HI and *Xba*I and isolated from wild-type *VRG4/VRG4* (BWP17), heterozygous *VRG4/vrg4Δ::hisG-URA3-hisG* (ANC1) and *VRG4/vrg4Δ::hisG* (ANC2), and hemizygous *MET3p-VRG4/vrg4Δ::hisG* (ANC3) strains. The 3.5-kb *Bam*HI/*Hind*III fragment that was used as a probe hybridized uniquely to each of the *Bam*HI/*Xba*I or *Bam*HI/*Bam*HI fragments that are indicated by the arrows in panel A.

plemented with 5 mM methionine and 1 mM cysteine (Fig. 5). This result demonstrated that *CaVRG4* is an essential gene.

In contrast to its behavior on solid SD(+Met,+Cys) medium, the hemizygous *MET3p-VRG4/vrg4Δ* strain continued to grow in liquid media (SD and YPAD) that were supplemented with Met/Cys, although its doubling rate was highly variable compared to that of the parental strain. One factor that contributed to this variability was the density of the inoculum. When cells were inoculated at a very low density (OD_{600}, <0.05), methionine-dependent *VRG4* repression was relatively efficient, while cultures inoculated at an OD_{600} greater than 0.5 reached an OD_{600} comparable to that of wild-type cell cultures after 12 h (data not shown). Presumably, the uptake or metabolism of methionine in dense cultures depleted the effective
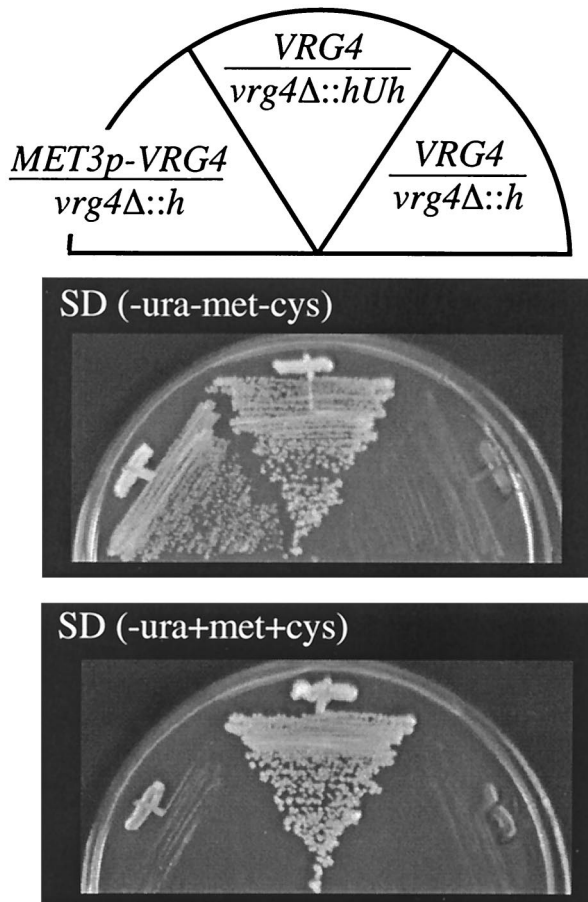
FIG. 5. *CaVRG4* is an essential gene. Heterozygous uracil prototrophic and auxotrophic strains ANC1 (*VRG4/vrg4Δ::hisG-URA3-hisG*) and ANC2 (*VRG4/vrg4Δ::hisG*) and the hemizygotic *MET3p-VRG4 vrg4Δ::hisG* strain ANC3 were plated on SD(−Ura) medium in the absence or in the presence of methionine and cysteine.

methionine and/or cysteine so that the concentration was below the concentration required for efficient repression of growth.

Although not reported previously, concentrations of methionine and cysteine greater than 10 and 5 mM, respectively, also affected the growth and morphology of the *VRG4/VRG4* parental strains (CAI4 and its derivative, BWP17). At these concentrations or higher concentrations, methionine and cysteine reduced the growth rates and accelerated hyphal formation, effects that were exacerbated by *URA3* prototrophy (data not shown). Therefore, for most of the analyses described below, the effects of *MET3p-VRG4* repression were examined in liquid media supplemented with 5 mM methionine and 2 mM cysteine, and overnight cultures grown in SD(−Met,−Cys, −Ura) that were diluted to an OD_{600} of about 0.2 were used. These conditions had no detectable effect on the growth of the parental strains and reduced, but did not eliminate, the growth of the *MET3p-VRG4* strain. Since growth was not eliminated, these growth conditions provided the opportunity to analyze the phenotypes associated with a partial loss of *VRG4* function.

Analysis of glycosylation and cell surface defects in *Candida vrg4* mutants. As an indirect measure of mannosylation de-

fects, the electrophoretic mobilities of glycoproteins in wild-type and mutant *Candida* cell extracts were compared. Protein extracts were prepared from cultures of the parental *VRG4/VRG4*, heterozygous *VRG4/vrg4Δ*, and hemizygous *MET3p-VRG4* strains grown in YPAD with or without additional Met/Cys. Proteins were fractionated by SDS-PAGE and examined by Western blotting with the mannose-specific lectin HRP-ConA in order to detect changes in the molecular weights of mannoproteins (Fig. 6A). Even when extracts were prepared from cells that had been grown in the absence of additional Met/Cys, the HRP-ConA-reactive proteins in the *MET3p-VRG4/vrg4Δ* strain (ANC3) had a lower average molecular weight than the HRP-ConA-reactive proteins in either the parental wild-type or heterozygous *VRG4/vrg4Δ* strain (Fig. 6A, lane 5). This result suggested that YPAD contains sufficient levels of endogenous methionine and/or cysteine to repress *MET3p-VRG4* expression. In extracts from mutant *Cavrg4* strains grown in the presence of additional Met/Cys, there was a marked decrease in higher-molecular-weight ConA-reactive proteins and a further increase in the lower-molecular-weight ConA-reactive proteins (Fig. 6A, lanes 5 and 6). These results suggest that mannosylation of proteins is severely affected by loss of *CaVRG4* function.

The increased level of lower-molecular-weight glycoproteins described above was consistent with a *vrg4*-dependent mannosylation defect. To confirm this, we wanted to analyze the effect of the *vrg4* mutation on carbohydrate modification of a single glycoprotein. Gel mobility shifts of secreted glycoprotein reporters, such as invertase, chitinase, and acid phosphatase, whose activities and secretion are not dependent on glycosylation, have been important tools for analyzing glycosylation mutants of *S. cerevisiae*. Therefore, we tried to use this approach to analyze the well-characterized *C. albicans* acid phosphatase. *C. albicans*-secreted acid phosphatase is a ∼130-kDa mannoprotein with a pH optimum of 3.6 to 4.5 (39).

To determine if acid phosphatase is undermannosylated in the *vrg4* mutant, native gel electrophoresis was used to analyze its mobility. Crude extracts were prepared under nondenaturing conditions from hemizygous *MET3p-VRG4* and wild-type strains grown in low-phosphate YPAD to induce acid phosphatase expression. Protein extracts were not treated or incubated with endo H to remove N-linked oligosaccharides. Acid phosphatase was visualized in the gel with a colormetric activity stain (Fig. 6B). Acid phosphatase migrated as a diffuse band whose average molecular weight was lower in *vrg4*-derived extracts (Fig. 6B, lanes 1 and 2). After removal of N-linked oligosaccharides with endo H, acid phosphatase from both *vrg4* mutant and wild-type strains migrated more rapidly, but it was still somewhat diffuse. Consistent with the idea that the remaining heterogeneity might be due to O-linked oligosaccharides whose additions are also affected by loss of *VRG4*, the mobility of acid phosphatase in *vrg4* extracts was slightly greater than the mobility in wild-type extracts (Fig. 6B, lanes 3 and 4). These results demonstrate that the *vrg4* mutation affects protein mannosylation.

Cell surface hydrophobicity is considered to be an important virulence determinant of *C. albicans*. The length of oligomannosides, linked to protein outer chain mannose via phosphodiester bonds (i.e., phosphomannan), has been correlated with cell surface charge, leading to hydrophilic or hydrophobic *C.*
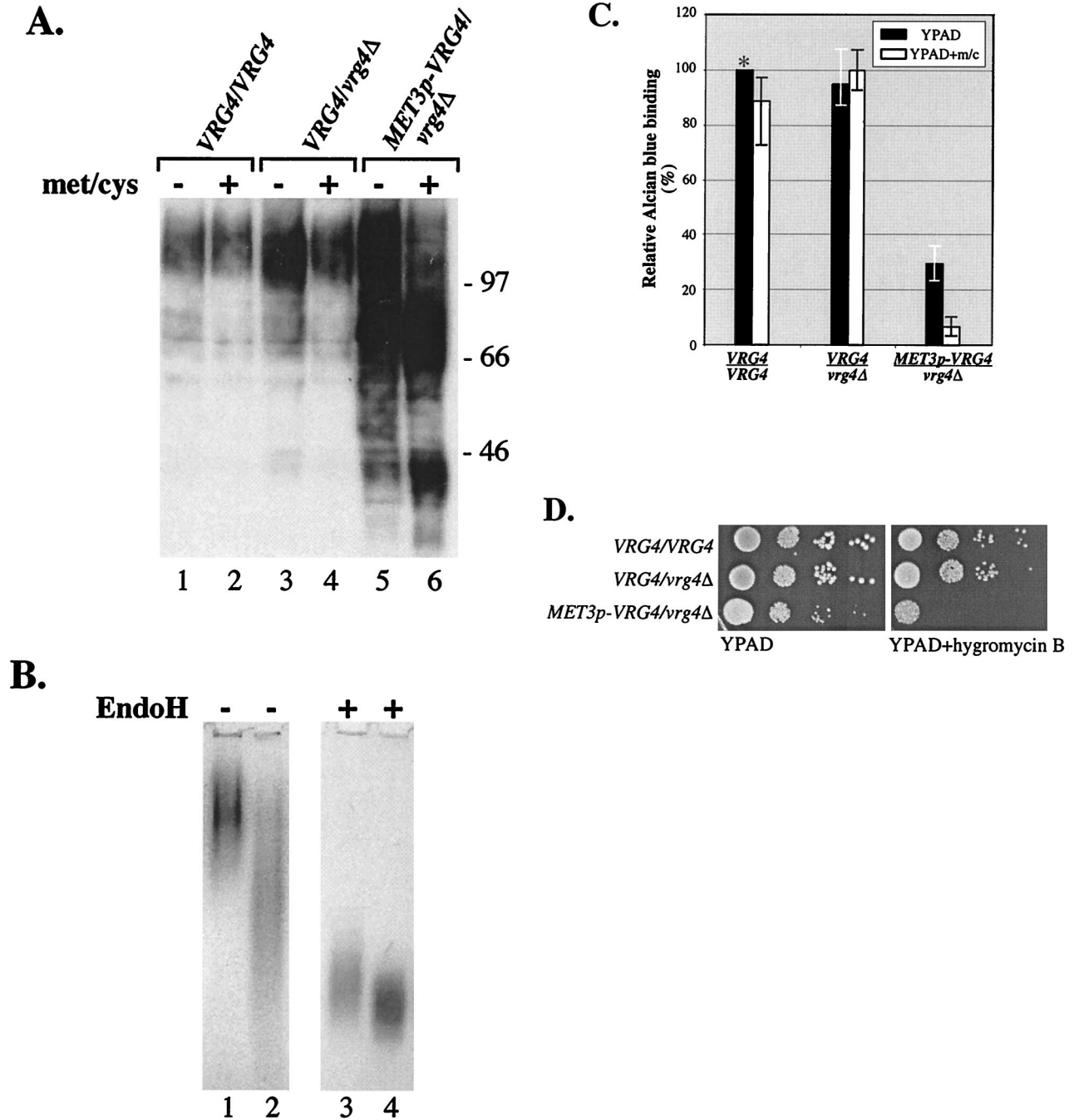
FIG. 6. Loss of *CaVRG4* function leads to mannosylation and cell wall-associated defects in *C. albicans*. (A) Proteins were isolated from wild-type strain BWP17, *VRG4/vrg4Δ::hisG* strain ANC2, and hemizygotic *MET3p-VRG4/vrg4Δ::hisG* strain ANC3 grown in YPAD with or without additional methionine and cysteine. After fractionation of equivalent amounts of protein by SDS–8% PAGE, mannoproteins were blotted with ConA-HRP and detected by chemiluminescence, as described in Materials and Methods. (B) Acid phosphatase in wild-type strain BWP17 (lanes 1 and 3) and *vrg4* mutant ANC3 (lanes 2 and 4) assayed by native gel electrophoresis and visualized colormetrically (see Materials and Methods). Extracts were not treated or incubated with endo H prior to electrophoresis, as indicated at the top. (C) The strains used in the experiment whose results are shown in panel A were incubated with Alcian blue and subjected to dye binding assays (see Materials and Methods). Relative dye binding activity was calculated by determining the percentage of dye bound compared to the amount bound by the parental strain (BWP17) that had been grown in YPAD (see Materials and Methods). Each bar represents an average based on the results of four experiments. m/c, methionine and cysteine. (D) The strains used in the experiment whose results are shown in panel A were serially diluted and plated on YPAD or YPAD that was supplemented with 150 μg of hygromycin B per ml.
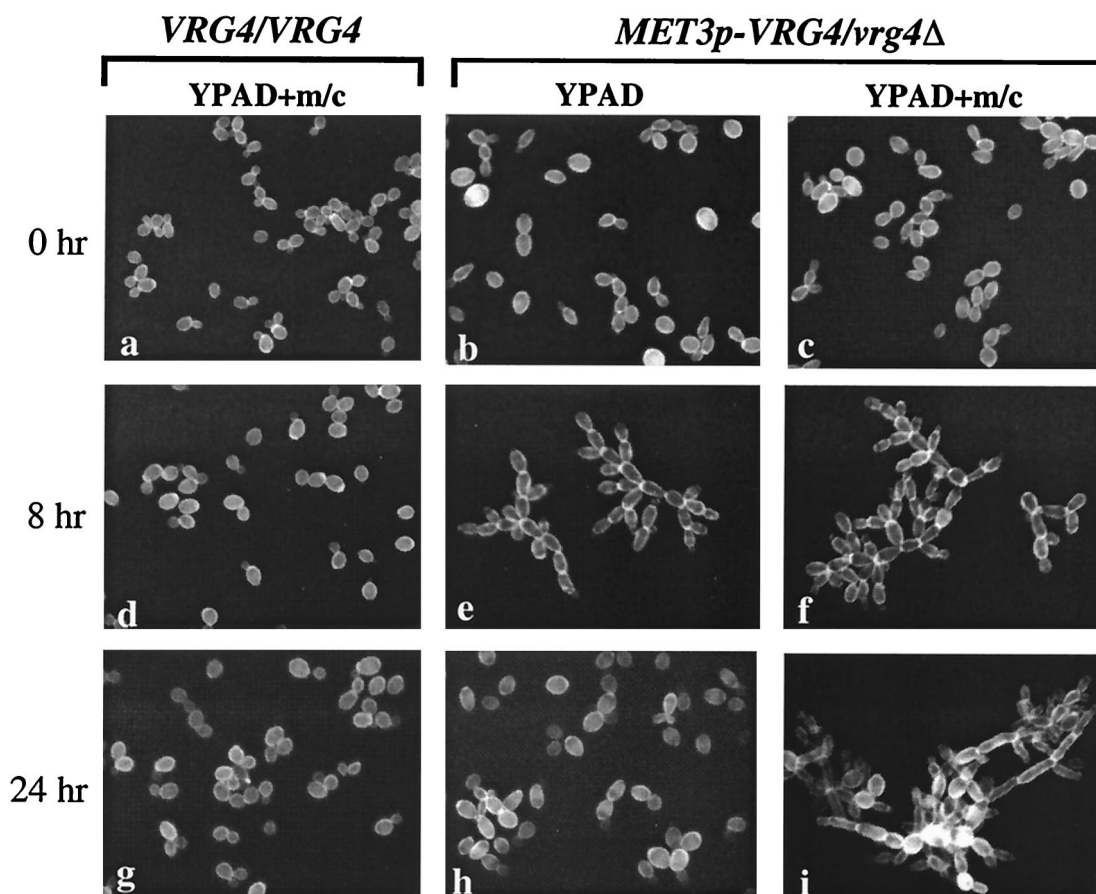
FIG. 7. Repression of Ca*VRG4* promotes pseudohyphal growth. Overnight precultures grown in SD(−Met/Cys) plus 10% YPAD were diluted into YPAD or YPAD containing additional methionine and cysteine (5 and 2.5 mM, respectively). At the times indicated, aliquots were removed, stained with Calcofluor white, and examined by fluorescence microscopy using a UV filter.

albicans strains (32, 33). In *S. cerevisiae*, mannosylphosphate gives a net negative charge to cell surface mannoproteins, which in turn allows binding to the dye Alcian blue under weakly acidic conditions (23). To test whether *CaVRG4* affects the level of cell surface phosphomannan, we measured the extents of Alcian blue binding to parental wild-type and *Cavrg4* mutant strains (see Materials and Methods). As shown in Fig. 6C, the wild-type and *vrg4* heterozygotic strains displayed equivalent dye binding affinities, whether they were grown in the presence or in the absence of additional Met/Cys. In contrast, the *MET3p-VRG4* strain was markedly defective in dye binding capacity. The *MET3p-VRG4* strain exhibited a threefold reduction in dye binding activity when it was grown in YPAD, and the amount of dye bound was less than 10% of the amount of dye bound by the parental wild-type strain when it was grown in YPAD containing Met/Cys. These results suggested that *CaVRG4* is required for phosphomannan addition and imply that, as in *S. cerevisiae*, phosphomannan addition in *C. albicans* requires a mannosylation step in the Golgi apparatus that utilizes GDP-mannose as a substrate.

S. cerevisiae mutants with defects in protein glycosylation or cell wall synthesis display a number of characteristic phenotypes when they are grown on media supplemented with sodium vanadate, hygromycin B, and Calcofluor white. To deter-

mine whether defects in mannan biosynthesis in *C. albicans* result in similar drug phenotypes, we examined the growth of *Cavrg4* strains on media supplemented with these compounds. The *MET3p-VRG4* hemizygous strain ANC3 was hypersensitive to hygromycin B and did not grow at all on YPAD plates containing 150 μg of hygromycin B per ml (Fig. 6D). Growth of the parental strains, CAI4 and BWP17, and growth of the *VRG4/vrg4*Δ heterozygotes were not affected, even at a hygromycin B concentration of more than 200 μg/ml (data not shown). In contrast to the effects of hygromycin B, a defect in *CaVRG4* had no effect on the sensitivity of growth to Calcofluor white or resistance to sodium vanadate (data not shown), both of which are phenotypes displayed by *Scvrg4* mutants. These results further underscore the differences that exist between *S. cerevisiae* and *C. albicans* with regard to cell wall composition and/or drug uptake and efflux mechanisms.

Effect of *CaVRG4* on cellular morphology. Sustained growth of a hemizygotic *MET3p-VRG4* strain in liquid media (either YPAD or SD, supplemented with Met/Cys) eventually led to cultures whose optical densities approached those of cultures of the parental strains. However, the increased optical densities did not wholly reflect increased numbers of cells. Rather, repression of *VRG4* expression resulted in a profound change in cell morphology (Fig. 7). Initially, the cells grew as short
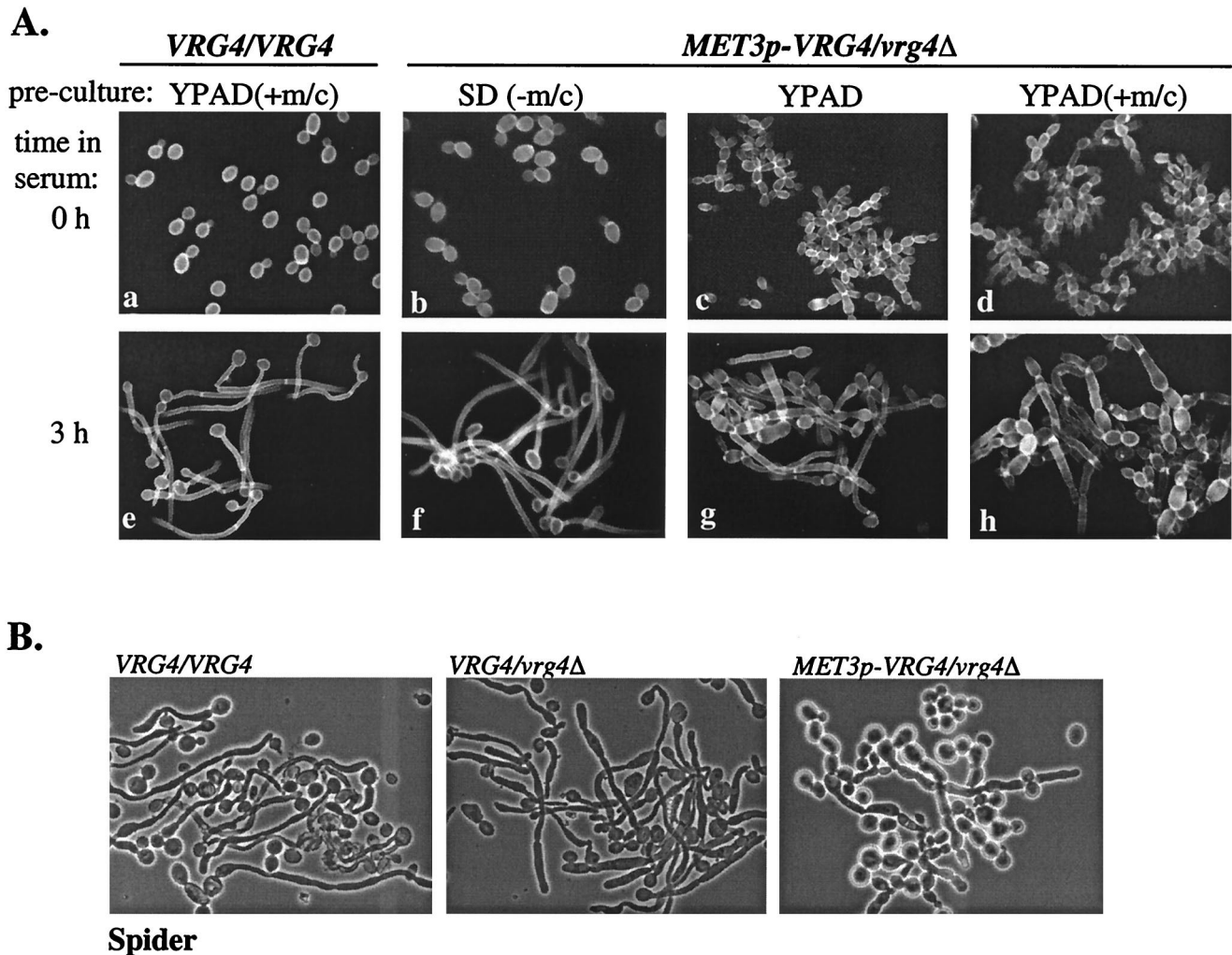
A.

B.

Spider

FIG. 8. Repression of *VRG4* prevents hyphal formation on liquid and solid media. (A) *C. albicans* parental wild-type strain BWP17 and *MET3p-VRG4* hemizygous strain ANC3 were precultured in SD(−Met/Cys), YPAD, or YPAD(+Met/Cys) for 12 h prior to induction of hyphal formation by growth at 37°C in YPAD containing 10% bovine calf serum. At zero time or 3 h after serum induction, aliquots were collected, stained with Calcofluor white, and examined by fluorescence microscopy. (B) *Candida* strains BWP17, ANC1 (*VRG4/vrg4*), and ANC3 were spotted on Spider medium plates and incubated for 3 days at 37°C. Individual cells from each colony were examined microscopically.

chains, which aggregated. After 4 to 8 h, long chains of cells attached to or branching from one another were observed, and by 24 h after inoculation most of the cells in each population had assumed a polarized, pseudohyphal mode of growth, and there were obvious constrictions at the sites of septation (Fig. 7). After 24 h in the presence of Met/Cys, many cells became vacuolated and unrefractive, as determined by light microscopy, and there was evidence of considerable cell lysis in the cultures (data not shown). Most of the cells also exhibited irregularities in surface staining by Calcofluor white, suggesting that the thickness of the cell wall was not uniform. The characteristic clumpiness and chain mode of growth occurred even in the absence of additional Met/Cys in YPAD. This was consistent with the results (Fig. 6), which demonstrated that cells grown in YPAD were defective in mannosylation and that the endogenous levels of Met/Cys in rich medium are sufficient for *MET3* promoter repression. After 24 h of growth in YPAD (not supplemented with additional Met/Cys) there was no ev-

idence of the elongated, attached cells (Fig. 7) or pseudohyphae that dominated the cultures after 8 h. This observation suggested that continued growth in liquid media might decrease the level of Met/Cys below the level required to repress the *MET3* promoter. Together, these results demonstrate that a defect in Golgi mannosylation induces the yeast cells to a transition to pseudohyphal growth and that this transition is reversible.

Effect of *CaVRG4* on hyphal formation. It is presumed that hyphal formation plays a key role in the pathogenesis of *C. albicans*. As hyphal formation requires substantial cell wall biosynthesis and/or remodeling, we expected that a defect in mannan biosynthesis would impair hyphal growth. To examine the effect of *CaVRG4* on hyphal formation, the morphologies of *Cavrg4* strains that were induced to form hyphae under various conditions were examined. Strains were precultured in various liquid media, as indicated in Fig. 8A, and were induced to form hyphae by dilution in YPAD containing 10% calf

serum at 37°C. No differences in the rate or appearance of hyphae formed by the *MET3p-VRG4* strains were observed when strains were compared to the parental strain and the organisms were precultured in SD(−Met,−Cys) (Fig. 8A, micrograph f). Both the parental strain and the *MET3p-VRG4* hemizygous strain initially formed germ tubes (data not shown) and then formed true hyphae that lacked constrictions between septa. This result was surprising since our previous results demonstrated that *MET3p-VRG4* expression was partially repressed in YPAD, as shown by the growth of the strain as chains of cells or pseudohyphal cells. No evidence of these growth forms was observed during hyphal induction in YPAD. Since hyphal induction in liquid occurs very rapidly (within 1 to 3 h), one possibility to explain the efficiency of hyphal formation in the *MET3p-VRG4* strain was that transcriptional repression of the *MET3* promoter phenotypically lagged or was recessive to induction of hyphae. To test this possibility, cells precultured in SD(−Met/Cys) were transferred and first grown in YPAD with or without additional Met/Cys for 12 h prior to hyphal induction. As shown in Fig. 8A, repression of *VRG4* expression prior to hyphal induction by growth in YPAD (micrograph g) or in YPAD that contained additional Met/Cys (micrograph h) resulted in cells that failed to form germ tubes (data not shown) or true hyphae. Instead, transfer of these cells into serum-containing media at 37°C resulted in rapid induction of pseudohyphae. Under these conditions, even after prolonged incubation in the presence of serum, no true hyphae were seen in *MET3p-VRG4* cell cultures. The mutant strains also did not form hyphae when they were grown at 37°C on mannitol-containing Spider medium plates (Fig. 8B) or on solid YPAD containing 4 or 10% serum (data not shown), whether the media were supplemented or not supplemented with additional Met/Cys. On solid media at 37°C, all strains displayed similar, rough colony morphologies after 2 days of incubation in the presence of Met/Cys or after 5 days of incubation in the absence of Met/Cys, demonstrating that methionine and/or cysteine dramatically accelerated filamentation (data not shown). Despite similar colony morphologies, microscopic examination of cells indicated that the parental and *VRG4* heterozygous strains formed true hyphae, while only pseudohyphae or elongated cells were observed for the *MET3p-VRG4* strains (Fig. 8B). These results demonstrate that *CaVRG4* is required for hyphal formation.

DISCUSSION

In this work we examined the role of mannosylation in *C. albicans* by analyzing the *VRG4* gene. *CaVRG4* was cloned on the basis of its structural similarity to the *ScVRG4* gene and was shown to be its functional homologue. Like *ScVRG4*, *CaVRG4* is an essential gene, implying that there is no other functionally redundant *Candida* gene that encodes GDP-mannose transport activity. Construction of a hemizygous *Candida* strain containing a conditional *MET3p-VRG4* allele enabled us to study partial loss of *VRG4* function and the requirement of this function for mannosylation. As in *S. cerevisiae*, the CaVrg4 protein is required for protein hypermannosylation for phosphomannan addition and appears to reside in the Golgi apparatus. All of the data suggest that CaVrg4p is the GDP-mannose transporter that regulates mannosylation in the Golgi

apparatus in the same manner that has been demonstrated for *S. cerevisiae*.

In *S. cerevisiae*, proper wall construction is essential for maintaining and defining cell shape. Because of the degree of cell wall expansion required to form a hypha, a defect in the synthesis of any wall component is predicted to have severe effects on filamentation in *Candida* cultures. Compatible with this idea, we found that even a partial loss of *CaVRG4* function completely blocked hyphal formation under all conditions tested. Thus, *CaVRG4* can be added to the growing list of *Candida* genes involved in cell wall synthesis that, when mutated, are uniformly impaired to some degree in hyphal formation. These genes include the genes required for synthesis or assembly of glucan (e.g., *CaKRE9*, *PHR1*, and *PHR2*) (21), chitin (e.g., *CHS1*) (35, 52), and mannan (e.g., *MNN9*, *MNT1*, *SRB1*, *PMT1*, and *PMT6*) (7, 50, 55–57). While filamentation defects may be a secondary effect due to inactivation of a hyphal regulator in some mutants, the phenotypic similarity of the mutants indicates that the cell wall has an essential role in hyphal formation.

Marked induction of polarized growth was an unexpected phenotype displayed by *C. albicans vrg4* mutants. Pseudohyphae or elongated chains of cells that remained attached to one another also suggested that there was polar budding. The elongated cell phenotype was not expected because it is precisely the opposite of what is seen in *Scvrg4* mutants and in most *S. cerevisiae* cell wall mutants, which typically arrest as large, unbudded cells that are defective in polarized growth (34). The relationship between pseudohyphal growth and hyphal growth in *C. albicans* is unclear. One model to explain induction of these elongated morphological forms in *Cavrg4* mutants assumes that pseudohyphae are intermediate forms of hyphae or that the two cell types are induced in response to similar signals. Accordingly, pseudohyphal growth may simply represent a general response to stress. Cells become elongated because mitosis is delayed and the cells continue to grow apically rather than isotropically. Certain *Candida* cell wall mutants (for instance, *mnn9* and *chs1* mutants) produce similarly elongated morphological forms (35, 50, 52). On the other hand, other *Candida* cell wall mutants do not produce an elongated cell phenotype but accumulate as large budded cells that are reminiscent of the cells of *S. cerevisiae* cell wall mutants (e.g., the *gas1*-related *phr1* mutants [44]). Therefore, the transition to pseudohyphal growth due to loss of *CaVRG4* function is probably not a general stress response to cell wall weakening representing a cell wall integrity pathway. In *S. cerevisiae*, compensatory changes in the composition, assembly, and depolarized localization of cell wall components are activated to ensure the integrity of the cell wall (48), and there is evidence that some aspects of this pathway are similarly activated in *C. albicans* (30). Further phenotypic analysis of *vrg4* and other *Candida* cell wall mutants is required to determine the basis for the morphological abnormalities that we observed in *Cavrg4* mutants, how they relate to a defect in mannosylation, and whether this phenotype is indicative of some type of cell wall-weakening response.

Although *CaVRG4* is clearly a functional and structural homologue of the *S. cerevisiae VRG4* gene, one difference between these two genes that may be related to differences in their mutant phenotypes is notable. In stark contrast to

CaVRG4, the Sc*VRG4* promoter region contains several consensus recognition elements for the G_1 cell cycle transcription factors Swi4/Swi6 (T/CACGAAAA) and MluI (ACGCGT) at positions −125, −245, −267, and −307 relative to the translational start site. As a result, Sc*VRG4* transcription is strongly cell cycle dependent, and maximal levels of both mRNA and protein occur late in G_1, before bud emergence (28; Dean, unpublished results). The cell cycle transcriptional machinery appears to be conserved in *S. cerevisiae* and *C. albicans* (2, 31). Therefore, the absence of these conserved *cis*-acting elements in the *CaVRG4* promoter region suggests that regulation of *VRG4* in *C. albicans* may be different from regulation of the gene in *S. cerevisiae* or that additional, uncharacterized *cis* and *trans* elements mediate induction of *CaVRG4* transcription late in G_1. The transcription of many *S. cerevisiae* genes whose products regulate cell wall biosynthesis is similarly cell cycle dependent, presumably to meet the high demand for cell wall expansion by an emerging bud (28, 51). It will be interesting to determine if *Candida* accommodates the need for de novo mannan synthesis during bud emergence or hyphal growth by upregulating CaVrg4p levels or by some other mechanism.

Hypermannosylated cell surface proteins are characteristic of most, if not all, fungal species, although the degrees to which these proteins may be subsequently decorated in the Golgi apparatus by additional types of sugars vary. Unlike *S. cerevisiae* oligosaccharides, N-linked oligosaccharides of *C. albicans* contain acid-labile phosphodiester-linked β-1,2-oligomannoside branches, whose lengths have been proposed to contribute to cell surface charge and pathogenicity (33). Although the evidence is indirect, reduced binding to Alcian blue has been strongly correlated with the absence of surface phosphomannan in *S. cerevisiae*, as shown by studies of *MNN6*, which encodes the *S. cerevisiae* Golgi mannosylphosphate transferase (38). As expected, we observed a dramatic reduction in binding to Alcian blue in *Cavrg4* mutants, suggesting that there was a reduction in surface phosphomannan and hence in β-1,2-oligomannoside branches. Another factor that has been proposed to contribute to surface hydrophobicity is the presence of sialylated galactosyl glycoconjugates on the surface of *C. albicans* (49). A search of the *Candida* genome sequence failed to identify any genes related to the genes that encode CMP-sialic transporters, whose activity is predicted to be required for synthesis of the glycoconjugates. The only gene related to the genes encoding nucleotide sugar transporters that could be identified in the *Candida* genome was *HUT1*, whose sequence is most similar to the sequences of the subgroup of genes which encode nucleotide sugar transporters that transport UDP-galactose (29, 36). Although deletion of *HUT1* results in little or no detectable change in the phenotype of *S. cerevisiae* (36), it will be interesting to determine whether the same is true for *C. albicans.*

We used native gel electrophoresis and in situ activity staining of secreted *C. albicans* acid phosphatase to directly assess the role of *VRG4* in protein mannosylation. Although widely used for analysis of mutants with mutations affecting secretion or glycosylation *of S. cerevisiae* proteins, these reporter assays have not been utilized for *C. albicans* glycoprotein analyses. Two predicted acid phosphatase-encoding genes, *PHO5* and *PHO12*, can be identified in the *C. albicans* genome based on sequence homology. Pho12p has a predicted molecular mass of

53.3 kDa, contains eight recognition sites for addition of N-linked oligosaccharides, and contains clusters of Ser/Thr residues typical of regions modified by sugar chains that are O linked. In contrast, *PHO5* encodes a predicted 42.4-kDa protein that contains only one potential site for addition of N-linked chains. While migration on native polyacrylamide gels does not provide an accurate measurement of protein molecular weight, based on the mobility of Pho12p and its susceptibility to endo H, this protein is the best candidate for the low-phosphate induced protein that we detected by the in situ acid phosphatase assay described here. Moreover, multiple protein bands, representing the two different predicted phosphatases, were not readily detected with this assay, suggesting that *PHO12* encodes the major inducible secreted acid phosphatase in *C. albicans*.

CaVRG4 mechanistically affects several parameters that are critical for pathogenesis, including cell growth, viability, hyphal formation, and cell surface mannan, suggesting that *VRG4* is required for virulence. Consistent with this assumption, other *Candida* mutants that are defective in Golgi mannosylation in steps downstream of *CaVRG4* (e.g., *MNT1* mutants) are avirulent (7). Unfortunately, the morphological phenotype and severe aggregation induced by this mutation posed technical problems that prevented accurate estimation of adhesion to mammalian epithelial cells by *Cavrg4* mutants. Studies to directly examine the role of *CaVRG4* during pathogenesis in animal models are under way.

Numerous studies have established that cell surface mannoproteins are important for host cell adhesion by fungi, although the relative roles played by the proteins and the carbohydrate portions of cell surface mannoproteins are still not well understood. The *VRG4* gene encodes the only GDP-mannose transporter in *S. cerevisiae* and, as inferred by its essentiality, in *C. albicans*. Highly related *VRG4* homologues can also be identified in *S. pombe*, *Candida glabrata*, *Aspergillus*, and even nonfungal species that contain mannosylated cell surface glyconjugates, such as plants and protozoans. In addition to viability of *S. cerevisiae* and *C. albicans*, *VRG4* is required for viability of *C. glabrata* (Nishikawa and Dean, unpublished data) and for virulence of *Leishmania* (20). Because *VRG4* is required for growth and survival of *Candida*, is conserved in fungi, and is not present in humans, it is likely to be an excellent broad-spectrum target for development of an antifungal compound.

In contrast to our understanding of *S. cerevisiae*, our understanding of the *Candida* cell wall is limited. While conservation in *S. cerevisiae* and *C. albicans* justifies the validity of using *S. cerevisiae* as a paradigm to direct studies of the *Candida* cell wall, the work presented here underscores the differences between these two organisms and the challenges that remain in this field.

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