Roles of Candida albicans Dfg5p and Dcw1p Cell Surface Proteins in Growth and Hypha Formation

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The Candida albicans cell wall participates in both growth and morphological transitions between yeast and hyphae. Our studies here focus on Dfg5p and Dcw1p, two similar proteins with features of glycosylphosphatidylinositol-linked cell surface proteins. Mutants lacking Dfg5p are defective in alkaline pH-induced hypha formation; mutants lacking Dcw1p have no detected hypha formation defect. Both homozygote-triplication tests and conditional expression strategies indicate that dfg5 and dcw1 mutations are synthetically lethal. Therefore, Dfg5p and Dcw1p share a function required for growth. Epitope-tagged Dfg5p, created through an insertional mutagenesis strategy, is found in cell membrane and cell wall extract fractions, and endoglycosidase H digestion shows that Dfg5p undergoes N-linked mannosylation. Surprisingly, Dfg5p is required for expression of the hypha-specific gene HWP1 in alkaline media. Because Dfg5p is a cell surface protein, it is poised to generate or transmit an external signal required for the program of hypha-specific gene expression.

Candida albicans is an opportunistic fungal pathogen. It inhabits the gastrointestinal and genital tracts in most healthy individuals as a benign commensal organism. However, it can cause diverse infections when host or environmental factors permit tissue invasion or overgrowth. C. albicans cells are surrounded by a cell wall composed of β-glucan, chitin, and mannanprotein (4, 15). The cell wall is of interest for several reasons. First, it has a role in cell morphogenesis. C. albicans produces several morphologically distinct types of cells, such as yeast and hyphal cells (20), that differ in cell wall architecture and composition (4, 11, 36). Second, it has a role in virulence. The cell wall is the surface of contact between pathogen and host, and several cell wall proteins contribute to adherence, a major virulence trait (27, 36). Third, as an essential pathogen-specific structure, it comprises many targets for drug or vaccine therapy (9, 37).

Several strategies have permitted isolation of C. albicans cell wall protein genes. These strategies include purification and sequencing, expression cloning of surface antigens, functional cloning in Saccharomyces cerevisiae, and identification of C. albicans sequence homologs of characterized S. cerevisiae cell wall protein genes (4, 27). In addition, a recent report described a gene fusion library for cloning of C. albicans gene segments that direct secretion or surface localization in S. cerevisiae (24). Many well-characterized surface proteins have features of glycosylphosphatidylinositol (GPI)-linked surface proteins, including an N-terminal signal sequence and a C-terminal GPI anchor addition signal (15, 36). GPI anchors provide a mechanism for membrane association in many eukaryotes, but in fungi, the GPI moiety can also be used to provide a covalent linkage to cell wall β-glucan (15, 19, 36). Other classes of fungal cell wall proteins lack a GPI anchor (4, 15, 36). A major challenge is to establish the functional relationships between each C. albicans cell wall protein and morphogenesis and virulence and to assess each protein’s potential as a target for therapeutic strategies.

We have been interested in the mechanisms by which C. albicans recognizes and responds to its external environment. The response to external pH is of particular interest, because it includes a change in cell morphology: C. albicans grows as yeast cells at pH 4 and as hyphae at pH 8. In addition, many mutants defective in pH-responsive growth or morphogenesis in vitro are also defective in virulence in animal models (1, 6, 27). Many genes that govern C. albicans alkaline pH responses are conserved in S. cerevisiae and act in the Rim101p signal transduction pathway (7, 28, 29). In S. cerevisiae, mutations in these genes cause defects in the ability to invade agar, growth in alkaline media, and other aspects of growth and cell differentiation (16–18).

This report describes our characterization of two putative C. albicans cell wall protein genes, DFG5 and DCW1. Our interest in DFG5 was based on the fact that S. cerevisiae dfg5 mutants are defective in agar invasion (25). More recently, S. cerevisiae DFG5 has been found to promote growth at alkaline pH as well (12). Thus, it has seemed possible that C. albicans DFG5 might govern alkaline pH responses. Our interest in DCW1 was based on the fact that it is similar to DFG5. In the course of our studies, a published report described the characterization of S. cerevisiae DFG5 and DCW1 (14). We report here that in C. albicans, as in S. cerevisiae, DFG5 and DCW1 share a function that is required for growth. In addition, we have used scanning mutagenesis to epitope tag C. albicans Dfg5p and demonstrate...
its surface localization. The role of \textit{S. cerevisiae} Dfg5p in invasive growth is not understood. We have found unexpectedly that \textit{C. albicans} Dfg5p is required for expression of a hyphaspecific gene, thus arguing that Dfg5p or its dependent biological process has a regulatory role in hypha development.

### TABLE 1. Genotypes of the \textit{C. albicans} strains used in this study

| Strain | Genotype
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>DAY210</td>
<td>\textit{dfg5::hisG _ARG4}</td>
</tr>
<tr>
<td>DAY229</td>
<td>\textit{dcw1::hisG _ARG4}</td>
</tr>
<tr>
<td>DAY254</td>
<td>\textit{dfg5::dpl200 _ARG4}</td>
</tr>
<tr>
<td>DAY280</td>
<td>\textit{dfg5::dpl200 _UAU1 _DCW1}</td>
</tr>
<tr>
<td>ES1</td>
<td>\textit{dfg5::dpl200 _UAU1 _PHIS1 _DCW1}</td>
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<tr>
<td>ES5</td>
<td>\textit{dfg5::dpl200 _UAU1 _PHIS1::DFG5 _UAU1 _DCW1}</td>
</tr>
<tr>
<td>ES1</td>
<td>\textit{dfg5::dpl200 _UAU1 _PHIS1::DFG5 _UAU1 _DCW1}</td>
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<tr>
<td>ES5</td>
<td>\textit{dfg5::dpl200 _PHIS1::DFG5 _UAU1 _DCW1}</td>
</tr>
<tr>
<td>ES193</td>
<td>\textit{dfg5::dpl200 _UAU1 _PHIS1::DFG5 _UAU1 _DCW1}</td>
</tr>
<tr>
<td>ES193</td>
<td>\textit{dfg5::dpl200 _UAU1 _PHIS1::DFG5-1001 _UAU1 _DCW1}</td>
</tr>
<tr>
<td>ES193</td>
<td>\textit{dfg5::dpl200 _UAU1 _PHIS1::MET3::DFG5 _UAU1 _DCW1}</td>
</tr>
<tr>
<td>ES193</td>
<td>\textit{dfg5::dpl200 _UAU1 _PHIS1::MET3-DFG5 _UAU1 _DCW1}</td>
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* All strains have the genotype \textit{ura3::\_his4 \_his3 \_his1::hisG \_his1::hisG \_arg4::hisG \_arg4::hisG} along with the mutations or alterations indicated (underlined) and are derived from strain BWP17. Plasmid integration was targeted to the \textit{his1::hisG} locus by digestion with \textit{NruI}.
leaving behind a 15-bp insertion. Each plasmid insert was released with Pml and moved by in vivo recombination into vector pPDB78. The resulting plasmids were designated pDTCx, with x representing the DFG5 ORF nucleotide number immediately 5′ to each insertion. Plasmid pES10 (pHIS/DFG5-1001-V5), which expresses the V5 epitope-tagged DFG5 allele, was generated as follows. The plasmid pDTC1001 was digested with PmlI, which cleaves within the 15-bp insertion. Then, the V5-His6 5′ and V5-His6 3′ oligonucleotides were annealed and ligated into the PmlI site. Sequence analysis verified the fidelity and orientation of the inserted sequence. To express DFG5 under the MET3 promoter (2), the primers 5′DFG5-5DR and 3′DFG5-5DR were used to amplify the DFG5-MET3 were used to amplify the DFG5-PHR1 were used to amplify the DFG5-PHR1 5′ and 3′ representing the DFG5 ORF nucleotide number immediately 5′ to each insertion. Plasmid pES10 (pHIS/DFG5-1001-V5), which expresses the V5 epitope-tagged DFG5 allele, was generated as follows. The plasmid pDTC1001 was digested with PmlI, which cleaves within the 15-bp insertion. Then, the V5-His6 5′ and V5-His6 3′ oligonucleotides were annealed and ligated into the PmlI site. Sequence analysis verified the fidelity and orientation of the inserted sequence. To express DFG5 under the MET3 promoter (2), the primers 5′DFG5-5DR and 3′DFG5-5DR were used to amplify the DFG5-MET3 promoter sequence from −1615 to −1; to express DFG5 under the PHRI promoter (33), the primers 5′DFG5-PHRI and 3′DFG5-PHRI were used to amplify the PHRI promoter sequence from −98 to −1. In vivo recombination in S. cerevisiae was used to integrate the PCR products into PmlI-linearized plasmid pPDB14, yielding the plasmids pES18 (PHIS1/DFG5-MET3-PHRI) and pES16 (PHIS1/DFG5-PHRI). Media and growth conditions. C. albicans was routinely grown in YPD + Uri (2% Bacto Peptone, 1% yeast extract, 2% dextrose, and 80 μg of uridine per ml, with 2% Bacto agar for solid media). Selection following transformation was done on SD minimal medium (6.7% yeast nitrogen base plus ammonium sulfate and without amino acids, 2% dextrose, and 2% Bacto agar for solid media) supplemented with amino acids and nucleotides as required. M199 medium (Gibco BRL) was buffered at either pH 4.0 or pH 8.0 with 150 mM HEPES, supplemented if necessary with 80 μg of uridine per ml, and solidified if necessary with 2% agar. (We previously [reference 8] called this medium TC199, but we have found that it is more commonly called M199.) Cell densities were determined by light scattering at 600 nm. For filamentation tests, C. albicans strains were grown overnight in YPD + Uri at 30°C and then subcultured at an optical density of 0.05 into buffered M199 medium or 4% saline liquid prewarmed to 37°C. For tests on solid media, 5 μl of the overnight cultures was plated on M199 medium or 4% serum plates (4% [vol/vol] calf serum [Sigma] added to 2% agar) and incubated at 37°C.

For MET3-DFG5 shutoff experiments, C. albicans strains were grown overnight in SD minimal medium and then subcultured in SD medium with or without 5 mM methionine (Met) and 2 mM cysteine (Cys). For PHR1-DFG5 shutoff experiments, the cells were grown overnight in SD medium at pH 7 (buffered with 150 mM HEPES) and subcultured in SD media at pHs of 7 and 4 (each containing 150 mM HEPES).

Preparation of cell wall and membrane protein fractions. We followed the S. cerevisiae fractionation procedure of Lu et al. (21) with minor modifications. Mid-axialonal-phase 30°C YPD cultures were pelleted, washed, and broken in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2% protease inhibitor cocktail [Sigma]) with glass beads as described previously (32). The lysate was cleared by centrifugation at 1,000 × g for 10 min at 4°C. The 1,000 × g pellet (cell wall fraction) was frozen at −80°C, and the supernatant, containing the membrane and the soluble protein fractions, was centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was removed, and the 10,000 × g pellet (membrane fraction) was resuspended in 1 ml of 0.1 M sodium acetate (pH 5.5) containing 2% sodium dodecyl sulfate (SDS) and protease inhibitors and then boiled for 5 min. The SDS membrane extract was separated from the insoluble material by spinning at 10,000 × g for 10 min at 4°C, dialyzed overnight against 2 liters of 0.1 M potassium phosphate buffer (pH 5.5) at 4°C, and lyophilized.

The cell wall fraction was resuspended in lysin buffer containing 2% SDS, 2% β-mercaptoethanol, and protease inhibitors and then boiled for 5 min and centrifuged at 10,000 × g for 5 min. Boiling and centrifugation were repeated twice, and the pellet was washed three times with deionized water containing protease inhibitors. The washed pellet was digested with the recombinant β-1,3-glucanase yeast lytic enzyme (ICN Biomedicals, Aurora, Ohio) (1,500 U [wet weight of cell walls]) at room temperature overnight. The lytic enzyme-treated cell walls were centrifuged, and the supernatant, containing the glucanase extracts, was lyophilized into a vacuum pump.

The dry pellet from membranes and the cell wall fraction was resuspended in approximately 10 times the pellet volume of 0.1 M sodium acetate (pH 5.5) buffer containing protease inhibitors. Where indicated, samples were treated with en-
FIG. 1. Alignments of Dcw1p and Dfg5p and positions of Dfg5p insertions. A Clustal alignment of the *C. albicans* and *S. cerevisiae* proteins Dcw1p and Dfg5p (Ca and Sc, respectively) is shown. Identical residues in at least two of the proteins are shaded black; conservative substitutions are shaded gray. The triangles indicate the positions of Dfg5p mutant insertions; under each triangle is the allele designation and the deduced sequence of the mutant protein with inserted residues in italics. The four insertion sequences that are underlined are partially or fully functional. The extent of filamentation promoted by each construct in strain DAY280, after incubation in pH 8 M199 medium for 6 h, is as follows: p*HIS1*::H11021, 0.25%; p*HIS1*::DFG5, 80%; p*HIS1*::DFG5-14, 82%; p*HIS1*::DFG5-286, 389, 323, 361, -703, -761, -888, -1193, and -1196, all 0.25%; p*HIS1*::DFG5-680, 41%; p*HIS1*::DFG5-918, 70%; p*HIS1*::DFG5-1001, 83%. Numbers represent the mean percent filamentation for six transformants, with standard deviations within 10% of the mean.
doglycosidase H (8 mU/µg of extract protein [Sigma]) for 2 h at room temperature.

Western blot analysis. Membrane and cell wall fractions were boiled in 1× Laemml reducing buffer and centrifuged at 10,000 × g for 5 min. Samples were analyzed by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose. The blot was then incubated with a blocking solution (3% bovine serum albumin in TBST [Tris-buffered saline plus Tween]) for 60 min at room temperature and washed four times with 1× TBST. Thereafter, the membrane was probed for 2 h at room temperature with horseradish peroxidase-coupled anti-V5 antibody (Invitrogen; 1:2,500 dilution in TBST). Immunoreactivity was detected with an enhanced chemiluminescence kit (Amersham) according to the manufacturer’s instructions. As a control, each blot was probed for DFG5/DFG5 after, the membrane was probed for 2 h at room temperature with horseradish peroxidase-coupled anti-V5 antibody (Invitrogen; 1:2,500 dilution in TBST). Immunoreactivity was detected with an enhanced chemiluminescence kit (Amersham) according to the manufacturer’s instructions. As a control, each blot was probed for DFG5/DFG5.

Northern blot analysis. Preparation of RNA, Northern analysis, and probes have been described previously (8).

RESULTS

Analysis of Dfg5p and Dcw1p function. A search of the C. albicans genome sequence revealed two genes that specify proteins similar to S. cerevisiae Dfg5p and Dcw1p (Fig. 1). Based on reciprocal Blastp searches, the predicted C. albicans ORF 19.2075 product and the allelic ORF 19.9622 product are more similar to ScDfg5p than to ScDcw1p, and so we designated the C. albicans gene DFG5. The predicted C. albicans ORF 19.1989 product and the identical allelic 19.9540 product are more similar to ScDcw1p, and we designated the C. albicans gene DCW1. The two C. albicans genes lie approximately 150 kbp apart on allelic sequence contigs 19-1019 and 19-2019.

S. cerevisiae Dfg5p promotes agar invasion, a form of filamentous growth (25). To determine whether C. albicans Dfg5p or Dcw1p may promote filamentous growth, we examined the phenotype of homozygous deletion mutant strains. The dfg5Δ mutation replaces nucleotides −150 to +409 with a marker cassette; the dcw1Δ mutation replaces nucleotides +600 to +818. These deletion endpoints correspond to the regions of greatest sequence certainty from C. albicans sequence assembly 4, which was current when this work was initiated. Each mutation removes a significant portion of the respective ORF and separates putative signal sequences from putative GPI addition sequences (3), so it is likely that these mutations abolish gene function. Filamentation ability was tested after induction either by alkaline conditions or by the presence of serum. We observed that a dfg5Δ/dfg5Δ strain was defective in filamentation when tested in alkaline (pH 8) liquid or solid medium (Fig. 2, strain ES51). Introduction of a functional DFG5 allele restored filamentation of the dfg5Δ/dfg5Δ strain (Fig. 2, strain ES55), thus indicating that the dfg5Δ mutation is recessive and is the cause of the filamentation defect. The dfg5Δ/dfg5Δ strain had no defect in filamentation in response to serum (Fig. 2). A dcw1Δ/dcw1Δ strain had no filamentation defect under several conditions (Fig. 2) (data not shown). Our results indicate that C. albicans Dfg5p is required for filamentation under alkaline conditions.

Because Dfg5p and Dcw1p are highly related (>50% identity), it seemed possible that they contribute to the same function. This model predicts that the phenotype of a dfg5Δ/dfg5Δ strain may be exacerbated by reduction or elimination of Dcw1p activity. To test this prediction, we first created a dfg5Δ/dfg5Δ strain with a deletion of one DCW1 allele. Like other dfg5Δ/dfg5Δ strains, the dfg5Δ/dfg5Δ dcw1Δ/DCW1 strain was defective in filamentation under alkaline conditions. However, this strain was also defective in filamentation on solid serum-containing medium (Fig. 2, strain ES1). Filamentation ability was restored by introduction of a wild-type DFG5 allele (Fig. 2, strain ES5). The fact that a dfg5Δ/dfg5Δ dcw1Δ/DCW1 strain has a more severe filamentation defect than dfg5Δ/dfg5Δ DCW1/DCW1 strains provides one line of evidence that Dfg5p and Dcw1p have overlapping functions.

Many gene products are required for C. albicans to produce hyphae in response to alkaline growth conditions. Known mutants that grow but fail to form hyphae in pH 8 medium are defective in signal transduction in some way, as indicated by their failure to express hypha-specific genes (7, 8, 28, 29), such

FIG. 2. Filamentation defects caused by dfg5 and dcw1 mutations. Strains indicated across the top of the figure were incubated on 4% serum agar (top row), pH 8 M199 agar (middle row), or pH 8 M199 broth culture (bottom row) to induce filamentation (see Materials and Methods). Photographs of colonies or cells are presented.
as HWP1 (36). We used Northern analysis to determine whether Dfg5p is also required for HWP1 expression in pH 8 medium. In a DFG5/DFG5 DCW1/DCW1 control strain, the HWP1 transcript was derepressed in pH 8 medium and repressed in pH 4 medium, as expected (Fig. 3, lanes 3 and 7, respectively). In a dfg5δ/dfg5δ dcw1Δ/DCW1 mutant, the HWP1 transcript was barely detectable under either growth condition (lanes 1 and 5). Introduction of an ectopic copy of DFG5 restored regulated HWP1 expression in the dfg5δ/dfg5δ dcw1Δ/DCW1 mutant (lanes 2 and 6) and did not alter HWP1 expression in a DFG5/DFG5 DCW1/DCW1 control strain (lanes 4 and 8). Hybridization to a control probe for TEF1 RNA verified the integrity of the RNA samples. These results indicate that Dfg5p is required for expression of the haplo-specific HWP1 gene in pH 8 medium.

**Synthetic lethality of dfg5 and dcw1 mutations.** We sought to create a dfg5δ/dfg5δ dcw1Δ/dcw1Δ double homozygote to obtain additional evidence for Dfg5p-Dcw1p functional overlap. Attempts to create such a strain through sequential transformation were unsuccessful, which can indicate that the double mutant is inviable. We tested this idea through use of an HT test (10). This test determines whether selection from a heterozygous mitotic segregants (Fig. 4A). In contrast, control strain ES5 lost the wild-type DCW1 allele in 18 of 30 independent Arg+ Ura+ mitotic segregants (Fig. 4B). These observations support the model that Dfg5p and Dcw1p share an essential function, so that cells that lack both gene products are inviable.

To test this model directly, we examined the growth of strains homozygous for deletions of the native DFG5 and DCW1 loci that carried an ectopic copy of a conditionally expressed DFG5 gene. For strain ES195, the DFG5 ORF and 3′ region were fused to the MET3 promoter, which is repressed in the presence of methionine and cysteine (2). In the absence of methionine and cysteine, strain ES195 grew as well as control strain ES187, in which an ectopic copy of DFG5 was expressed from its native promoter (Fig. 4C and Table 3). However, strain ES195 failed to grow in the presence of methionine and cysteine, while control strain ES187 grew well. Two independently constructed strains with the same genotype as strain ES195 also displayed a growth defect in the presence of methionine and cysteine (data not shown). We also created strain ES218, in which the DFG5 ORF and 3′ region were fused to the PHR1 promoter, which is expressed in neutral and alkaline media and is repressed in acidic media. Strain ES218 grew as well as control strain ES187 in pH 7 medium, but it displayed a quantitative growth defect in pH 4 medium (Table 3). These results support the model that cells require at least one of the proteins Dfg5p and Dcw1p for growth, so that dfg5 and dcw1 are synthetic lethal mutations.

**Evidence that Dfg5p is a membrane and cell wall protein.** We sought to determine the localization of Dfg5p through subcellular fractionation. To identify Dfg5p in extracts, we wished to introduce an epitope tag. Dfg5p presents two difficulties for epitope tagging. First, introduction of an epitope at the N or C terminus may alter localization by blocking recognition of the signal sequence or GPI-anchor addition signal. Sequence inspection confirmed presence of these hydrophobic sequences (3) at the Dfg5p N and C termini (Fig. 1). Also, these segments are generally cleaved during maturation of surface proteins, so an appended epitope would be lost. Second, because Dfg5p is highly conserved throughout the length of the protein (Fig. 1), few internal sites are logical choices for insertion of an epitope. Thus, we used insertional mutagenesis to identify tolerant sites in Dfg5p, introduced an epitope tag at a tolerant internal site, and analyzed membrane and cell wall fractions for the presence of the epitope-tagged protein.
Insertion mutants were created and analyzed as follows. Transposon Tn7-GPS-LS was inserted into a DFG5 plasmid through in vitro transposition, and insertions within the ORF were identified through restriction digestion and sequence analysis. The bulk of each transposon was removed via restriction enzyme digestion and ligation to yield a set of 15-bp insertion mutants. Some of these insertions introduced chain-terminating nonsense codons and were not analyzed further. The remaining 12 DFG5 insertion alleles were subjected to functional analysis (Fig. 1). Each allele, carried on a HIS1 vector, was transformed into strain DAY280 (dfg5/dfg5·H9004/dfg5·H9004·dcw1·H9004/DCW1) with integration directed to the HIS1 locus through cleavage within the C. albicans HIS1 vector sequences. Six independent transformants were then tested for filamentation ability in pH 8 liquid medium (Fig. 1). Most of the insertions (8 of 12) interfered with DFG5 function, because the transformants were as defective in filamentation as control transformants carrying the pHIS1 vector. Four insertion alleles retained considerable function: DFG5-14, -680, -918, and -1001 (underlined in Fig. 1). DFG5-14 is within the putative signal sequence; this site is not suitable for epitope introduction, as discussed above. DFG5-680 is at the junction between two sequence blocks that are highly conserved among C. albicans.
and S. cerevisiae Dfg5p and Dcw1p. Nearby insertions (DFG5-670, -703, and -761) abolish detectable function, thus suggesting that this region has limited tolerance for sequence alteration. Therefore, we considered this region less than optimal for epitope introduction. DFG5-918 lies within a well-conserved sequence block; DFG5-1001 lies at the end of the same sequence block. The fact that these two nearby insertions are both functional suggests that this region may be relatively tolerant of sequence alterations. Therefore, we inserted sequences specifying an in-frame V5-His6 cassette into the DFG5-1001 insertion site. Complementation tests verified that epitope-tagged Dfg5-1001-V5p retained function (data not shown).

To determine the subcellular distribution of Dfg5p, we prepared membrane and cell wall fractions from two strains, one expressing tagged Dfg5-1001-V5p and one expressing untagged Dfg5-1001p. Samples from the two strains were compared on anti-V5 immunoblots (Fig. 5). The anti-V5 antibody detected a 72-kDa protein present in both membrane and cell wall fractions of the strain expressing Dfg5-1001-V5p (lanes 2 and 10) and not the strain expressing Dfg5-1001p (lanes 1 and 9). Some Dfg5-1001-V5p was also detectable as a heterogeneous ~83-kDa species (lanes 2 and 10). The membrane fractions had cross-reacting proteins of 53 and 45 kDa; the presence of these species in both untagged and tagged extracts (lanes 1 and 2) indicates that they are not derived from Dfg5p. Reprobing of the blots with concanavalin A verified that samples from both strains had comparable amounts of total glycoprotein (lanes 5, 6, 13, and 14). These results support the idea that Dfg5p is a 72-kDa protein present in the cell membrane and cell wall.

Many cell surface proteins are modified by N-linked glycosylation, and Dfg5p contains seven potential N-glycosylation sites (NXT/T) at amino acid positions 86, 111, 135, 203, 243, 268, and 402. Thus, we determined whether the apparent molecular weight of Dfg5-1001-V5p is altered by digestion with endoglycosidase H, which removes N-linked carbohydrate residues. We observed that the mobility of Dfg5-1001-V5p in both membrane and cell wall fractions was increased after digestion with endoglycosidase H, yielding a 55-kDa protein species (Fig. 5, lanes 4 and 12). Parallel digestion of samples containing untagged Dfg5-1001p (lanes 3 and 11) indicated that the 55-kDa protein is Dfg5-1001-V5p and not a cross-reacting protein. Reprobing with concanavalin A indicated that total glycoprotein mobility was increased by endoglycosidase H treatment (lanes 7, 8, 15, and 16). These results indicate that Dfg5p undergoes N-linked glycosylation. This finding is consistent with the model that Dfg5p is a cell surface protein.

**DISCUSSION**

The C. albicans cell wall is a participant in dramatic morphological changes, a contributor to virulence, and a source of prospective targets for therapeutic strategies. Our studies here have focused on a pair of closely related proteins, Dfg5p and Dcw1p, with structural features of cell membrane and cell wall proteins. Our findings argue that C. albicans Dfg5p and, by analogy, Dcw1p are localized in the cell membrane and cell wall and that they are together essential for growth. These properties are similar to those of S. cerevisiae Dfg5p and Dcw1p (14). Dfg5p is required for hypha formation under some conditions in C. albicans, a feature that we expected based on the requirement for S. cerevisiae Dfg5p in agar invasion (25). Surprisingly, we find that C. albicans Dfg5p is required for expression of a hypha-specific gene, which suggests that Dfg5p functions in some way as a regulator of hypha development, as discussed below.

Two lines of evidence indicate that Dfg5p is a cell membrane and cell wall protein. First, we find that epitope-tagged Dfg5-1001-V5p is associated with cell membrane and cell wall fractions. The epitope-tagged protein is functional, because it complements the filamentation defect of a dfg5Δ/dfg5Δ mutant. This observation argues that its localization does not arise from misfolding or artificial channeling of the protein into the secretory system. Second, endoglycosidase H digestion shows that the protein is N glycosylated. This modification occurs during transit of proteins to the cell surface and membrane-bound organelles (15). Most or all detectable Dfg5-1001-V5p migrates at 72 kDa, thus arguing that most or all Dfg5p is N glycosylated. Therefore, we conclude that Dfg5p is largely associated with membranes and the cell wall or en route to those destinations.

We are unaware of other reports of Tn7 mutagenesis for epitope tagging in C. albicans, and aspects of this strategy may prove generally useful. The N and C termini of proteins are simple sites for epitope introduction for technical reasons, but epitope attachment can disrupt protein function. Such problems are expected in the case of secreted and surface proteins. Therefore, an epitope must be introduced at an internal site. Although suitable internal sites can be deduced from conservation or structural information, our experience has been that epitope introduction at such sites often compromises protein function. Identification of tolerant sites is thus a trial-and-error exercise. Tn7 mutagenesis simplifies sampling of many insertion sites and permits introduction of a variety of epitopes, functional domains, or other sequences. For exam-
ple, here we used Tn7 insertions both for introduction of an epitope tag and for fusions to regulated promoters.

Our studies indicate that C. albicans Dfg5p and Dcw1p share a function that is required for growth, as indicated by both an HT test and the consequences of Dfg5p depletion. Depletion experiments were conducted with strains lacking native functional DFG5 and DCW1 loci and carrying instead fusions of the DFG5 ORF to either the MET3 or PHR1 promoter. Growth of the strain carrying MET3-DFG5 was blocked on repressing medium. This observation argues that Dfg5p is required for growth in the absence of Dcw1p function. Growth of the strain carrying PHR1-DFG5 was impaired but not blocked on repressing medium. This result is consistent with the conclusion that Dfg5p and Dcw1p are required for growth; we infer that PHR1-DFG5 is expressed to some extent under our repression conditions. MET3-DFG5 strains provide the clearest demonstration of Dfg5p-Dcw1p essentiality, but PHR1-DFG5 strains might be useful in screening for inhibitors of Dfg5p activity.

The insertional mutagenesis of Dfg5p reported here was not extensive, but supports the model that Dfg5p and Dcw1p have similar biochemical functions. First, most mutations (8 of 12) impaired Dfg5p function, as expected given the extensive sequence conservation of Dfg5p and Dcw1p. Second, two of the four functional insertion mutations (DFG5-14 and -680) affected regions of low conservation. Sequence conservation within the Dfg5p-Dcw1p protein family thus appears to be significant for function.

S. cerevisiae Dfg5p and Dcw1p have been proposed to function in cell wall biogenesis, as indicated by several observations (14). First, a dcw1Δ mutant is hypersensitive to β-glucan hydrolysis by zymolase. Second, the dcw1Δ dfg5Δ genotype is lethal, and depletion of Dcw1p in a strain lacking Dfg5p causes cells to acquire properties typical of cell wall-defective mutants. These properties include an enlarged, rounded appearance, accumulation of delocalized chitin, and release of the GPI-linked cell wall protein Cwp1p into the medium. The parallels between C. albicans and S. cerevisiae Dfg5p and Dcw1p functions and properties are substantial. All four proteins share considerable sequence identity, and S. cerevisiae Dcw1p is an N-glycosylated protein found in the cell membrane and cell wall, as we report here for C. albicans Dfg5p. Moreover, Dfg5p is required for forms of filamentous growth in both S. cerevisiae (25) and C. albicans. Thus, the inference that C. albicans Dfg5p and Dcw1p also function in cell wall biogenesis is reasonable, although we have not addressed this question directly.

We note one difference between observations in S. cerevisiae and C. albicans. Our strains in which Dfg5p is depleted through repression of MET3-DFG5 do not acquire aberrant morphology and remain viable for several days. This observation stands in contrast to the results of Dcw1p depletion in a dcw1Δ dfg5Δ S. cerevisiae strain, which yielded an aberrant cell morphology and cell death. It is possible that C. albicans is more tolerant of cell wall aberrations than S. cerevisiae. A second explanation is that a low-repressed level of MET3-DFG5 expression is sufficient to permit stasis but not growth.

If Dfg5p functions in cell wall biogenesis, how can it be required for hypha development? One simple model is that a defect in cell wall integrity may block initiation of hypha formation. Three prior studies support this model. First, a kre9/ kre9 mutant, which has reduced β1,6-glucan levels, grows as yeast cells rather than hyphae in serum (22). Second, a gpi7/ gpi7 mutant, which has abnormal cell wall structure and composition, grows as yeast cells rather than hyphae in Spider medium (30, 31). Third, the C. albicans mitogen-activated protein kinase Mkc1p is required for both cell wall integrity and filamentation (26). Our study extends this model with the observation that Dfg5p is required for full expression of the hypha-specific gene HWP1. According to this model, the cell wall defect of a dfg5Δdfg5 mutant does not simply cause a structural impediment to hyphal growth, but causes a regulatory response that reduces expression of a hypha-specific gene. Thus, there may be a cell wall integrity “checkpoint” that is required for the hyphal growth and gene expression program. A second model is that Dfg5p has a more direct role in sensing the environmental signals that induce hypha formation. Analysis of double mutants has not allowed us to place Dfg5p clearly in either the Rim101p or Mds3p pH-response regulatory pathways (8), but a Dfg5p-dependent signal could act downstream of both pathways (M. Kim and A. P. Mitchell, unpublished results). This model may seem unlikely, because Dfg5p has no obvious transmembrane domain for signal transduction across the plasma membrane. However, there are precedents for transmembrane signaling by GPI-linked cell surface proteins (5, 13). It is also possible that Dfg5p may promote signaling through interaction with or modification of a protein that has a transmembrane domain. Our work here lays the foundation for elucidation of this signaling mechanism.

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