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 HWJ1 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 genitourinary 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 mannoprotein (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, 35, 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
TABLE 1. Genotypes of the C. albicans strains used in this study

| Genotype* | DAY210          | DAY229          | DAY254          | DAY280          | ES1           | ES5           | ES5           | ES7           | ES9           | ES187          | ES190          | ES193          | ES195          | ES218          |
|-----------|-----------------|-----------------|-----------------|-----------------|---------------|---------------|---------------|---------------|---------------|----------------|----------------|---------------|---------------|---------------|
| dfg5::HIS1 DFA5 AR4G       | dcf1::HIS1       | dfg5::dpl200    | dfg5::dpl200    | dfg5::dpl200    | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         |
| dfg5::HIS1 DFA5 AR4G       | dcf1::HIS1       | dfg5::dpl200    | dfg5::dpl200    | dfg5::dpl200    | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         |
| dfg5::HIS1 DFA5 AR4G       | dcf1::HIS1       | dfg5::dpl200    | dfg5::dpl200    | dfg5::dpl200    | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         |
| dfg5::HIS1 DFA5 AR4G       | dcf1::HIS1       | dfg5::dpl200    | dfg5::dpl200    | dfg5::dpl200    | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         |
| dfg5::HIS1 DFA5 AR4G       | dcf1::HIS1       | dfg5::dpl200    | dfg5::dpl200    | dfg5::dpl200    | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         | pHHSI         |

* All strains have the genotype ura3Δ::kanm434/ura3Δ::kanm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG and the additional genetic alterations indicated (underlined) and are derived from strain BWP17. Plasmid integration was targeted to the his1::hisG locus by digestion with NrdI.

its surface localization. The role of S. cerevisiae Dfg5p in invasive growth is not understood. We have found unexpectedly that C. albicans Dfg5p is required for expression of a hypha-specific gene, thus arguing that Dfg5p or its dependent biological process has a regulatory role in hypha development.

MATERIALS AND METHODS

Strains and plasmids. The C. albicans strains used in this study (Table 1) are derivatives of strain BWP17 (ura3Δ::kanm434/ura3Δ::kanm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG) and the additional genetic alterations indicated. PCR product-directed gene disruption methods (38, 39) were used to generate this strain. The C. albicans strains DAY210 and DAY254, using primers DFG5-5DR and DFG5-3DR. (All primer sequences are listed in Table 2.) The Dfg5p deletion removes nucleotides 150 to +409, which includes residues 1 to 136 of the predicted Dfg5p protein. Correct integration was demonstrated by PCR with the primers DFG5 5′ Del and DFG5 3′ Del, which flank the site of integration. The same methods were used to construct dcf1::dcw1::URA3 strain DAY229, using primers YKL046 5′ Exon and YKL046 3′ Exon for disruption and primers 5′ YKL046 Del and 3′ YKL046 Del for detection. The dcf1::dcw1::URA3 strain DAY280 was constructed from dfg5::dfg5::dpl200 strain DAY254 by transformation with the dfg5::dpl200 disruption cassette (10) amplified with primers YKL046 5′ Del and YKL046 3′ Del. Integration was demonstrated by PCR with the primers YKL046 5′ CL2 and YKL046 3′ CL2 to detect the dcf1::dcw1::URA3 allele.

For the homzygote-triplication (HT) test (10), strain DAY280 was transformed with plasmid pDDB78 (a vector carrying HIS1) and pMMS (pDDB78 carrying DFG5) with selection for His′ transformants to generate strains ES1 and ES5, respectively. Thirty independent His′ transformants were grown overnight on YPD at 30°C, and one Arg′ Ura′ mitotic segregant was selected from each transformant.

For DFG5 insertion mutant analysis, strain DAY280 was transformed with pDCTs insertion mutagenesis plasmids (described below). Six independent transformants with each plasmid were tested for filamentation ability in pH 8 liquid M199 medium. The experiment included transformants carrying control plasmids pDDB78 (pHIS1) and pMMS (pHIS1:DFG5).

For analysis of epitope-tagged Dfg5p, strain DAY280 was transformed with plasmids pDCT1001 (pHIS1:DFG5-1001) or pES10 (pHIS1:DFG5-1001-V5), and His′ transformants were selected to generate the strains ES193 and ES190, respectively. Restoration of filamentation ability was verified on pH 8 M199 medium plus uridine.

For DFG5 shutoff experiments, strain DAY280 was transformed with plasmids pMMS (pHIS1:DFG5), pES16 (pHIS1:MET3-DFG5), and pES16 (pHIS1:PHR1-DFG5) with selection for His′ transformants. The dcf1::dcw1::URA3 recombinants were then selected from the transformants after overnight growth in YPD. We used Arg′ Ura′ selection conditions in which we expected the MET3-DFG5 and PHR1-DFG5 hybrid genes to be expressed (2, 33): SD minimal medium (lacking methionine and cysteine) for the MET3-DFG5 and SD medium titrated to pH 7 for PHR1-DFG5. Arg′ Ura′ mitotic segreagates were genotypically identified by PCR with the primers YKL046 5′ CL2 and YKL046 3′ CL2 to detect the DCW1 allele and primers ARG4 and YKL046 3′ to detect the dcf1::dcw1::URA3 allele. We characterized the growth properties of three independent Arg′ Ura′ mitotic segreagates that had lost the DCW1 allele for each plasmid. These strains were ES187, ES188, and ES189 (dfg5::dfg5::dcw1::dcw1::URA3 pMMS); ES195, ES269, and ES270 (dfg5::dfg5::dcw1::dcw1::URA3 pES18); and ES218, ES219, and ES220 (dfg5::dfg5::dcw1::dcw1::URA3 pES16).

Plasmid pDDB78, a HIS1 vector, was generated by in vivo recombination in S. cerevisiae (23) of NgoMI-linearized pRS314 (34) with a His1 selection cassette (10) inserted into vector pGEM-HIS (39). Plasmid pDCT1001 (pHIS1:DFG5) was constructed from plasmid pGEM-HIS (39) with primers prs/pgemt-5 and prs/pgemt-3. It has a unique His1 allele. We characterized the growth properties of three independent Arg′ Ura′ mitotic segreagates that had lost the DCW1 allele for each plasmid. These strains were ES187, ES188, and ES189 (dfg5::dfg5::dcw1::dcw1::URA3 pMMS); ES195, ES269, and ES270 (dfg5::dfg5::dcw1::dcw1::URA3 pES18); and ES218, ES219, and ES220 (dfg5::dfg5::dcw1::dcw1::URA3 pES16).

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Plasmid pDDB100 was constructed by ligation of a DFG5 PCR product, generated with primers DFG5-759 and DFG5-1619, into vector pGEM- Easy (Promega). Plasmid pMMS (pHIS1:DFG5) was constructed as follows. Plasmid pDDB100 was digested with PnuI, plasmid pDDB78 was digested with EcoRI and NcoI, and both were agarose gel purified. In vivo recombination of the DFG5-containing fragment into pDDB78 was carried out through S. cerevisiae cotransformation (23) to generate plasmid pMMS.

To construct DFG5 insertion-bearing plasmids, the Tn7 transposon GPS-LPS (New England Biolabs) was used to mutagenize the DFG5 insert of plasmid pDDB100 according to the manufacturer's protocol. Each insertion-bearing plasmid was screened through NcoI digestion to identify DFG5 open reading frame (ORF) inserts, which were then sequenced with primer S (New England Biolabs), which primes from one end of the transposon. Plasmids were then digested with PmeI and religated to remove the bulk of Tn7 sequences.
leaving behind a 15-bp insertion. Each plasmid insert was released with PvuI and moved by in vivo recombination into vector pDDB78. The resulting plasmids were designated pDTCx, with “x” representing the DFG5 ORF nucleotide number immediately 5’ to each insertion. Plasmid pES10 (pHIS1/DFG5-1001-V5), which expresses the V5 epitope-tagged DFG5 allele, was generated as follows. The plasmid pDTC1001 was digested with Pmel, which cleaves within the 15-bp insertion. Then, the V5-His6 5’ and V5-His6 3’ oligonucleotides were annealed and ligated into the Pmel site. Sequence analysis verified the fidelity and orientation of the inserted sequence.

The plasmids pES10 (pHIS1/DFG5-1001-V5), which expresses the V5 epitope-tagged DFG5 allele, was generated as follows. The plasmid pDTC1001 was digested with Pmel, which cleaves within the 15-bp insertion. Then, the V5-His6 5’ and V5-His6 3’ oligonucleotides were annealed and ligated into the Pmel site. Sequence analysis verified the fidelity and orientation of the inserted sequence.

To express DFG5 under the MET3 promoter (2), the primers 5’DFG5-MET3 and 3’DFG5-MET3 were used to amplify the MET3 promoter sequence from −1615 to −1; to express DFG5 under the PHR1 promoter (33), the primers 5’DFG5-PHR1 and 3’DFG5-PHR1 were used to amplify the PHR1 promoter sequence from −989 to −1. In vivo recombination in S. cerevisiae was used to incorporate the PCR products into Pmel-linearized plasmid pDTC14, yielding the plasmids pES18 (pHIS1/DFG5-MET3) and pES16 (pHIS1/DFG5-PHR1).

### 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% serum 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-exponential-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 lysis 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 g⁻¹ [wet weight of cell walls]) at room temperature overnight. The lytic enzyme-treated cell walls was 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: pHIS1::H1102, 0.25%; pHIS1::DFG5, 80%; pHIS1::DFG5-14, 82%; pHIS1::DFG5-296, -389, -670, -703, -761, -888, -1193, and -1196, all 0.25%; pHIS1::DFG5-680, 41%; pHIS1::DFG5-918, 70%; pHIS1::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× Laemmli 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 filter 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 stripped and reprobed with a concanavalin A-horseradish peroxidase conjugate (Sigma), which was again detected by chemiluminescence.

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 kb 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
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 hypha-specific HWP1 gene in pH 8 medium.

**Synthetic lethality of dfg5 and dcw1 mutations.** We sought to create a dfg5Δ/dfg5 Δ dcw1Δ/DCW1 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 mutant strain yields homozygous mutant mitotic segregants. The test employs a gene disruption cassette called UAUI, which can express either ARG4 or, after intracassette recombination, URA3. Thus a strain that is heterozygous for a UAUI insertion is Arg⁺ Ura⁻ (of the genotype dcw1::UAUI/DCW1, for example) and can undergo recombination to yield mitotic segregants that are Arg⁻ Ura⁺ (of the genotype dcw1::URA3/DCW1). Rare mitotic segregants arise that are Arg⁺ Ura⁻, and these include mitotic recombinants that have become homozygous for the UAUI insertion mutation and have undergone recombination in one cassette (of the genotype dcw1::UAUI::UR3/DW1). Other Arg⁺ Ura⁻ mitotic segregants have three copies of the relevant locus (dcw1::UAUI::UR3/DW1): such mitotic segregants are the only type found from heterozygous UAUI insertions in essential genes (10). In practice, we select independent Arg⁺ Ura⁺ derivatives of a strain that is heterozygous for a UAUI insertion after growth in rich medium and assay the presence of the wild-type copy of the locus. In the present case, the analysis was conducted with two strains that were derived by transformation of the same progenitor, strain DAY280, of the genotype dfg5Δ/dfg5Δ dcw1Δ/DCW1, and these include mitotic segregants that have a wild-type DCW1 allele. The control strain, ES5, had a copy of DFG5 integrated at the HIS1 locus. This strain should be able to yield mitotic segregants that lack a wild-type DCW1 allele regardless of the Dfg5p-Dcw1p functional relationship. We observed that experimental strain ES1 failed to lose the wild-type DCW1 allele among 30 independent Arg⁺ Ura⁺ 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 ES118, 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 ES118 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 ES118 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 dfg5Δ and dcw1Δ 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/H9004/dfg5/H9004/dcwl/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.

**TABLE 3. Effects of conditional DFG5 expression on cell growth**

| Strain | Relevant genotype | OD<sub>600</sub> at 24 h<sup>a</sup> | Repressing | Nonrepressing |
|--------|------------------|-------------------------------|------------|---------------|
| ES187  | DFG5             | 7.2                           | 7.1        |               |
| ES195  | MET3-DFG5        | 0.05                          | 7.4        |               |
| ES218  | PHR1-DFG5        | 2.4                           | 10.1       |               |

<sup>a</sup> Strains were of genotype dfg5/dfg5 and carried the indicated DFG5 construct integrated at the HIS1 locus.

<sup>b</sup> Strains were inoculated at an OD<sub>600</sub> of 0.05 and grown for 24 h. Each OD value is the mean of three independent cultures, and standard deviations were within 10% of the mean. For strain ES187 and ES218, the repressing medium is pH 4 SD, and the nonrepressing medium is pH 7 SD. For strain ES187, the repressing medium is pH 4 SD, and the nonrepressing medium is pH 7 SD. Cultures of control strain ES187 reached OD<sub>600</sub> values of 6.0 and 8.7 in the latter two media, respectively. In all cases, overnight cultures were grown in nonrepressing medium prior to inoculation.

The presence of a wild-type DCW1 allele and a mutant dcw1::UAU1 allele in each of 30 independent Arg<sup>+</sup> Ura<sup>+</sup> mitotic segregants was detected by PCR (see Materials and Methods). Lane C shows parallel PCRs using DNA of strain BWP17 (DCW1/DCW1) as a control. (C) Consequences of MET3-DFG5 repression. Strains ES187 and ES195 were streaked on SD (left) and SD + Met + Cys (right) solid media. Growth was assayed by visual inspection after 2 days. Both strains have deletions of the native DFG5 and DCW1 loci. Strain ES187 carries an ectopic copy of the intact DFG5 gene, and strain ES195 carries an ectopic copy of the DFG5 ORF and 3′ region fused to the MET3 promoter, which is repressed in SD + Met + Cys medium.

**Fig. 4.** Tests for synthetic lethality of dfg5 and dcw1 mutations. (A and B) HT tests. Strains ES1 (A) and ES5 (B), both of which are dcw1::UAU1/DCW1, were subjected to Arg<sup>+</sup> Ura<sup>+</sup> selection to yield homozygous (dcw1::UAU1/dcw1::URA3) and triplication-bearing (dcw1::UAU1/dcw1::URA3/DCW1) mitotic segregants (10). Strain ES1 has no functional copy of DFG5; strain ES5 has a functional copy of DFG5.
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 artifactual 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 there 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 dfs5Δ 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 formation 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 dfs5Δ 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 hyphae 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/glk1 mutant, which has reduced β1,6-glucan levels, grows as yeast cells rather than hyphae in serum (22). Second, a gpi7/glk1 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 HWPl. According to this model, the cell wall defect of a dfs5Δdfs5 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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