Function of Candida albicans Adhesin Hwp1 in Biofilm Formation

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Hwp1 is a well-characterized Candida albicans cell surface protein, expressed only on hyphae, that mediates tight binding to oral epithelial cells. Prior studies indicate that HWP1 expression is dependent upon Bcr1, a key regulator of biofilm formation. Here we test the hypothesis that Hwp1 is required for biofilm formation. In an in vitro model, the hwp1/hwp1 mutant produces a thin biofilm that lacks much of the hyphal mass found in the hwp1/HWP1 reconstituted strain. In a biofilm cell retention assay, we find that the hwp1/hwp1 mutant is defective in retention of nonadherent bcr1/bcr1 mutant cells. In an in vivo rat venous catheter model, the hwp1/hwp1 mutant has a severe biofilm defect, yielding only yeast microcolonies in the catheter lumen. These properties of the hwp1/hwp1 mutant are consistent with its role as a hypha-specific adhesin and indicate that it is required for normal biofilm formation. Overexpression of HWP1 in a bcr1/bcr1 mutant background improves adherence in the in vivo catheter model. This finding provides additional support for the model that Hwp1 is critical for biofilm adhesion. Hwp1 is the first cell surface protein known to be required for C. albicans biofilm formation in vivo and is thus an excellent therapeutic target.

Many microorganisms exist in nature as surface-attached communities called biofilms. This microbial growth state has profound consequences for industry, the environment, and human health (7). Adherence is a critical property for biofilm cells, and one might expect that multiple adhesion molecules would function in successful biofilm formers.

Our studies focus on the fungal pathogen Candida albicans, for which biofilm formation creates a serious medical challenge (5, 8, 9). Implanted medical devices, such as venous catheters, are a serious risk factor for C. albicans infection. They are substrates for the formation of biofilms, which in turn serve as reservoirs of cells to continually seed an infection. The resistance of C. albicans biofilm cells to the commonly used azole antifungals limits therapeutic options. Often, removal of the implanted device is the only means to eliminate the infection. However, an understanding of the mechanisms that support C. albicans biofilm formation may highlight new targets that permit noninvasive diagnostic and therapeutic strategies.

We have recently taken a genetic approach to the analysis of C. albicans biofilm formation. A library of C. albicans mutants defective in individual transcription factor homologs was screened for biofilm defects; these studies indicated that the zinc finger protein Bcr1 is required for biofilm formation in vitro (13). Further analysis with a newly developed rat catheter model has shown that Bcr1 is also required for biofilm formation in vivo: a bcr1Δ/bcr1Δ strain is unable to populate the luminal catheter surface by 48 h after inoculation, whereas the wild-type and bcr1Δ/bcr1Δ+pBCR1-complemented strains create biofilms within 12 to 24 h (11). The mutant is fully virulent in a disseminated infection model (11). Therefore, Bcr1 appears to govern a mechanism that is required for biofilm formation rather than overall growth in vitro and in vivo.

The mechanistic role of Bcr1 was first suggested through microarray analysis (13). Most of the major Bcr1-dependent genes specify known or predicted glycosylphosphatidylinositol (GPI)-linked cell surface proteins, including the adhesins ALS1, ALS3, and HWP1. Mutant and overexpression studies indicate that ALS3 is a pivotal Bcr1 target: an als3/als3 mutant is defective in biofilm formation in vitro, and overexpression of ALS3 permits biofilm formation by a bcr1/bcr1 mutant in vitro and in vivo (11). However, ALS3 is clearly not the sole functional Bcr1 target because an als3/als3 mutant produces a thin biofilm in vivo and is thus an excellent therapeutic target.

In this report, we focus on the role of Hwp1 in biofilm formation. Hwp1 is among the most well understood C. albicans adhesins (19). It is a cell surface protein that is covalently linked to cell wall glucan through a remnant of its GPI anchor (17). Functional analysis has shown clearly that it is required for tight adherence to oral epithelial cells, mediated by a novel mechanism (18). The N-terminal domain of Hwp1 serves as a substrate for mammalian transglutaminases, which cross-link Hwp1 covalently to host cell surface proteins. Whether Hwp1 functions as an adhesin in the absence of host transglutaminase activity is less certain, though the possibility has never been ruled out. Indeed, a possible function for Hwp1 in C. albicans cell-cell adherence comes from the finding that it is induced by mating factor (2, 21) and is deposited on the surface of the bridge between mating partners (3). This localization might be expected for a cell-cell adhesin. Our studies presented here indicate that Hwp1 is required for normal biofilm formation in vitro and in vivo. Our analysis of mutant biofilms in vitro supports the idea that Hwp1 is a cell-cell adhesin in this context. Most importantly, Hwp1 is the first C. albicans cell surface protein that is required for biofilm formation in vivo.
MATERIALS AND METHODS

C. albicans strains and media. All strains used in this study are listed in Table 1. Construction of CAH7-1A1E2 (hwp1/hwp1) (20), CAH3 (hwp1/HWP1) (20), CAI4-URA3 (reference strain) (15), CAYF178U (als3/als3) (11), and CACY2F1U (als1/als1) (6) was described previously. Construction of the Bcr1 target gene mutants FJS2 (bcr1/hyr1), FJS5 (cht2/Tn7-URA3), and FJS6 (ecel/Tn7-URA3) was described previously (11). Construction of strain CJNI1222 (bcr1/bcr1 TEF1-HWP1) was constructed as previously described (11) using PCR products from template plasmid pCN498 and primers HWP1-F-0E-Ag-NAT-Ag-TEF1p (5′-TACATACATGGATGTTATTTGCAATCTACTATAAGCTAACAAATTATCTTTACAAGATGTATACGAGTTGAGCAGTGAATCTCATATTAAATGATATGACCC-3′) and HWP1-R-0E-Ag-NAT-Ag-TEF1p (5′-CTCTTTCGTTTCCACCGTTACGCCGAGTTGGGACAGTGGCCTCCCATGTAATAGGGATAGCAATTGAGCTTTGAGCAGTGAATCTCATATTAAATGATATGACCC-3′). All strains were grown at 30°C in YPD (2% Bacto peptone, 1% dextrose, 1% yeast extract). For biofilm growth, strains were grown at 37°C in Spider medium (10).

In vitro biofilm growth conditions. Our procedure has been described in detail previously (13). Briefly, strains were grown overnight in YPD at 30°C, diluted to an optical density at 600 nm (OD600) of 0.5 in 2 ml Spider medium, and added to a sterile 12-well plate containing a prepared silicone square (1.5 by 1.5 cm cut from cardiovascular instrument silicone sheets PR72034-06N). The silicone square with no cells added. Statistical significance (p values) was calculated from three independent samples after subtracting the mass of a blank assay, except that during the initial adhesion step, various amounts of biofilm-defective mutant strain and test strain were added to the wells. The ratios of the mutant strain to the test strain in each well were 0%, 50%, 62.5%, and 87.5%. Final desiccation was accomplished by critical point drying. Specimens were mounted on aluminum stubs and sputter coated with gold. Samples were imaged in a scanning electron microscope (Hitachi S-5700) in the high-vacuum mode at 10 kV. The images were assembled using Adobe Photoshop 7.0.1.

Biofilm dry mass measurements. For dry mass measurements, each silicone square was weighed prior to inoculation with the strain of interest. Biofilms were grown for 48 h on the silicone square, and the silicone squares containing biofilms were removed from the wells, dried overnight in a fume hood, and weighed the following day. The total biomass of each biofilm was calculated by subtracting the weight of the silicone prior to biofilm growth from the weight of the silicone after biofilm growth. The average total biomass for each strain was calculated from three independent samples after subtracting the mass of a blank silicone square with no cells added. Statistical significance (p values) was calculated with Student’s one-tailed paired t test function in Microsoft Excel.

In vitro biofilm cell retention assays. To assess subtle biofilm defects, we used a biofilm cell retention assay in which increasing amounts of biofilm-defective bcr1/bcr1 mutant were mixed in with test strains to assess the severity of their biofilm defects. Biofilms were prepared as described above in the in vitro biofilm assay, except that during the initial adhesion step, various amounts of bcr1/bcr1 mutant strain and test strain were added to the wells. The ratios of the bcr1/bcr1 mutant strain to the test strain in each well were 0%, 50%, 62.5%, and 87.5%, thus gradually increasing the amount of bcr1/bcr1 mutant strain in each well.
FIG. 1. In vitro biofilm formation. Biofilms were grown under our standard conditions (13) in Spider medium and stained with concanavalin A for CSLM visualization. Artificially colored CSLM depth views, in which blue color represents cells closest to the silicone and red color represents cells farthest from the silicone, are shown in panels A and B, in which blue represents 0 μm and red represents 300 μm (panel A) or 500 μm (panel B). CSLM side views are shown in lower panels C and D, in which the scale bars represent 50 μm. Cells in the surrounding medium of the \textit{hwp1}/\textit{hwp1} biofilm were visualized through phase-contrast microscopy at \( \times 400 \) magnification (panel E).
Hwp1 IS REQUIRED FOR C. ALBICANS BIOFILM FORMATION

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RESULTS

Hwp1 requirement for biofilm integrity in vitro. We reported recently that an hwp1/hwp1 mutant produces biofilms in vitro with a slight reduction in biomass compared to the wild-type strain (11). To determine whether this defect is caused by the hwp1 mutation, we compared biofilm biomasses (means ± standard deviations of results from quadruplicate samples) from the wild-type (0.0089 ± 0.0023 g), mutant (0.0024 ± 0.0002 g), and reconstituted (0.0077 ± 0.0016 g) strains. The hwp1/hwp1 mutant produced a biofilm with threefold less biomass than the reconstituted strain (P = 0.006). The mutant biomass was also significantly reduced from that of the wild-type strain (P = 0.005). The reconstituted strain and wild-type control strain (CAI4-URA3) produced similar levels of biofilm biomasses. These results indicate that Hwp1 is required for normal biofilm formation.

Biofilm visualization through CSLM confirmed the biofilm defect of the hwp1/hwp1 mutant (Fig. 1). The mutant produced a biofilm of ~100 μm in depth that contained few hyphae (Fig. 1A and C). Both hyphae and yeast cells were found in the medium surrounding the biofilm (Fig. 1E). Reconstitution with a wild-type HWP1 allele permitted production of a biofilm of 200 to 300 μm in depth in which hyphae were readily apparent (Fig. 1 B and D). Therefore, the mutant defect in biofilm biomass is similar in magnitude to its defect in biofilm depth. The finding that cells are present in the biofilm supernatant suggests that Hwp1 may be required to retain cells within a biofilm.

To assay the capacity of strains to retain cells in a biofilm, we turned to a biofilm cell retention assay. The assay employed a bcr1/bcr1 mutant strain, which is defective in biofilm formation and fails to fully express several surface adhesins (13). Thus, inoculation of the mutant onto a silicone square in growth medium and incubation for 48 h yields few cells adhering to the square and a turbid supernatant (13). This biofilm defect was reflected in the OD600 of the surrounding medium, which was ~9.0 (Fig. 2, 100% bcr1ΔΔ cells). In contrast, the wild-type reference strain DAY185 yields extensive biofilm growth on the silicone square and little growth in the surrounding medium (13); its surrounding medium OD600 was 0.1 (Fig. 2). Previous studies show that inoculation of the silicone square with the wild type and bcr1/bcr1 mutant together results in incorporation of mutant cells into the biofilm (13). We observed that a 1:1 mixed inoculum of the two strains yielded a surrounding medium OD600 of 0.3 (Fig. 2). As the fraction of mutant cells in the initial inoculum was increased, there was little increase in the surrounding medium OD600 until 87.5% of the inoculum was bcr1/bcr1 mutant cells. This mixture yielded a surrounding medium OD600 of 1.6 (Fig. 2). The behavior of the hwp1/hwp1 mutant strain in this assay was quite different from that of the wild-type strain. The hwp1/hwp1 mutant alone yielded a surrounding medium OD600 of 1.45. A 1:1 mixed inoculum of hwp1/hwp1 and bcr1/bcr1 cells yielded a surrounding medium OD600 of 1.6; when 87.5% of the inoculum was bcr1/bcr1 mutant cells, the surrounding medium OD600 was 6.0 (Fig. 2). The hwp1/HWP1 reconstituted strain behaved similarly to the wild-type strain in these assays (Fig. 2). These observations support the idea that Hwp1 is required to retain cells within a biofilm, as expected if Hwp1 functions as a biofilm adhesin.

We extended the biofilm cell retention assays to test additional Bcr1 target gene mutants for this relatively subtle biofilm formation defect (Fig. 3). The als3/als3 mutant had a severe
FIG. 4. Hwp1 requirement for biofilm formation in vivo. Central venous catheters were introduced into rats, inoculated with C. albicans strain CAH7-1A1E2 (hwp1/hwp1) (A), CAHR3 (hwp1/HWP1) (B), or CJN1222 (bcr1/bcr1 TEF1-HWP1/HWP1) (C), and then flushed and incubated (1). Catheters were then removed, and their contents were visualized by scanning electron microscopy after 24 h.
biofilm formation defect in this assay without the addition of bcr1/bcr1 cells, as documented in other assays previously (11). The als1/als1 mutant had a mild defect in this assay, in keeping with our previous observation that its biofilms had reduced biomass compared to the wild-type strain (11). The hwp1/hwp1, cht2/cht2, ecc1/ecc1, and rbt5/rbt5 strains had little if any measurable defect in this assay.

**Hwp1 function in biofilm formation in vivo.** To determine whether Hwp1 may function in biofilm formation in vivo, we compared mutant and complemented strains in a rat venous catheter model (1). Biofilm formation was permitted to proceed for 24 h, and then catheters were removed. The luminal surfaces were examined for presence of a biofilm, as visualized by scanning electron microscopy (Fig. 4). The hwp1/hwp1 mutant had a severe biofilm defect: only sparse microcolonies were observed on the catheter, and the microcolonies were devoid of hyphae (Fig. 4A). The reconstituted strain produced a biofilm (Fig. 4B) that was similar in appearance to that of wild-type strains DAY185 (11) and K1 (1) in this model. These results indicate that Hwp1 is required for biofilm formation in vivo.

A complementary strategy provided additional evidence that Hwp1 promotes biofilm formation in vivo. We found previously that a bcr1/bcr1 mutant strain is severely defective in biofilm formation in vitro; it fails to populate a catheter surface even after 48 h of incubation (11). Overexpression of the adhesin gene ALS3 in the bcr1/bcr1 mutant restores biofilm formation in vivo, as expected if the bcr1/bcr1 mutant is defective in adherence (11). If Hwp1 functions as a biofilm adhesin, we reasoned that overexpression of HWP1 in the bcr1/bcr1 mutant may also rescue biofilm formation. We observed that a bcr1/bcr1 pTEF1-HWP1 strain was able to populate the catheter surface with microcolonies and that both yeast cells and hyphae were apparent (Fig. 4C). Thus, overexpression of HWP1 does not fully restore biofilm formation by the bcr1/bcr1 mutant, but it does clearly improve its ability to adhere to a catheter surface in vivo. These results support the conclusion that Hwp1 functions as a biofilm adhesin in vivo.

**DISCUSSION**

Hwp1 is well known for its role in host cell attachment and is among the most well characterized C. albicans cell surface proteins (19). Here we have provided clear evidence that Hwp1 has a critical role in biofilm formation as well. We have extended the finding that an hwp1/hwp1 mutant has a slight defect in biofilm formation in vitro through both CSLM and a new biofilm cell retention assay for biofilm cell adherence. We have also used a reconstituted strain to verify that the hwp1 mutation is the cause of the biofilm defects observed. In contrast to the somewhat subtle role of the protein in vitro, our analysis in the rat catheter model indicates that Hwp1 has a major role in biofilm formation in vivo. As the first cell C. albicans surface protein shown to be required for biofilm formation in vivo, Hwp1 may be useful as a therapeutic target for catheter-related C. albicans infections.

Prior studies have shown that Hwp1 is not expressed during yeast phase growth but is strongly expressed on germ tubes and hyphal surfaces (19). The biofilm defects of the hwp1/hwp1 mutant in vitro and in vivo are entirely consistent with the idea that expression of Hwp1 is hypha-specific in the context of a biofilm. Biofilms formed by the mutant in our in vitro assays almost exclusively contain yeast cells, and their depth (~100 μm) corresponds to that of the yeast-specific layer (~80 μm) we have observed previously in wild-type biofilms in this system (13). In vivo, the mutant fails to form a biofilm at all, but the few adherent cells on the catheter surface are primarily yeast cells. These observations are consistent with our understanding that the adherence properties of hyphae, not of yeast, are dependent upon Hwp1.

Although the hwp1/hwp1 mutant seems to form only the yeast layer of a biofilm in vitro, we observe yeast cells as well as hyphae in the medium surrounding the mutant biofilm. This observation could be interpreted to mean that yeast cell adherence is slightly defective in the mutant. A second possibility is that hyphae within a biofilm help to retain yeast cells. This second explanation is consistent with our previous finding that wild-type cells, which produce hyphae, can retain green fluorescent protein-labeled hypha-defective mutant cells within a mixed biofilm (13, 14). Indeed, the published image of a mixed wild-type and suv3/suv3 biofilm shows green fluorescent protein-labeled mutant yeast cells adhering to unlabeled wild-type hyphae (14). Therefore, we favor the interpretation that hyphae provide an adherent scaffold (12) for retention of yeast cells and that Hwp1 supports hyphal adherence.

**HWP1** is induced by pheromone (2, 21), so the finding that it serves as a biofilm adhesin could help to explain the recent observation that induction of the mating response facilitates biofilm formation (4). However, we note that the most surprising observation in that report—that pheromone induces biofilm formation by mating-inefficient white phase cells—cannot be explained by our observations. The reason for this limitation is that pheromone does not induce **HWP1** in white cells (4). However, Hwp1 may well function as a biofilm adhesin when opaque cells are included in the mating mixtures that form biofilms (4).

Hwp1 permits binding of *C. albicans* to host epithelial cells by serving as a transglutaminase substrate, thus permitting covalent cross-linking of *C. albicans* germ tubes to host cell surfaces (17, 18). Transglutaminase activity is produced by mammalian cells and not by *C. albicans* (17, 18). Thus, it is possible that host transglutaminases promote biofilm formation in the rat catheter model. However, in the in vitro biofilm model, Hwp1 must contribute to adherence without involvement of a transglutaminase. These observations suggest that Hwp1 can bind noncovalently to *C. albicans* surface features to function as an adhesin.

We reported recently that Als3 is also a major biofilm adhesin (11), and it may be useful to compare the properties of Hwp1 and Als3. Both are members of the GPI-linked cell surface protein family, with an internal serine-threonine-rich region and an N-terminal domain involved in binding to at least some ligands (16, 17, 19). Thus, it is reasonable to suggest that they function as adhesins in the context of a biofilm. In terms of functional analysis, their properties are remarkably complementary. Als3 is required for biofilm formation in vitro but not in vivo, and its overexpression fully rescues the biofilm defect of a bcr1/bcr1 mutant in vitro and in vivo. Hwp1 is only partially required for biofilm formation in vitro but is absolutely required in vivo, and its overexpression only partially
rescues the bcr1/bcr1 mutant in vitro and in vivo. It is possible that the distinction between null mutant phenotypes is not an accurate reflection of protein function because other Als family members may compensate for the absence of Als3 under some conditions (16, 22). Similarly, the distinction in overexpression phenotypes may be artificial, since we do not know the actual numbers of Als3 and Hwp1 protein molecules that end up on the cell surface, nor do we know that they are properly modified in the overexpressors. Regardless of those limitations, our findings raise the question of whether and how the roles of Hwp1 and Als3 may differ in biofilm formation and whether additional biofilm adhesins, such as possible cell surface ligands for Als3 or Hwp1, remain to be discovered.

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