Virulence of the Fungal Pathogen *Candida albicans* Requires the Five Isoforms of Protein Mannosyltransferases

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The *PMT* gene family in *Candida albicans* encodes five isoforms of protein mannosyltransferases (Pmt proteins Pmt1p, Pmt2p, Pmt4p, Pmt5p, and Pmt6p) that initiate O mannosylation of secretory proteins. We compared virulence characteristics of *pmt* mutants in two complex, three-dimensional models of localized candidiasis, using reconstituted human epithelium (RHE) and engineered human oral mucosa (EHOM); in addition, mutants were tested in a mouse model of hematogenously disseminated candidiasis (HDC). All *pmt* mutants showed attenuated virulence in the HDC model and at least one model of localized candidiasis. The *pmt5* mutant, which lacks in vitro growth phenotypes, was less virulent in the EHOM and HDC assays but had no consistent phenotype in the RHE assay. In contrast, the *pmt4* and *pmt6* mutants were less virulent in the RHE and HDC assays but not in the EHOM assay. The results stress the contribution of all Pmt isoforms to the virulence of *C. albicans* and suggest that the importance of individual Pmt isoforms may differ in specific host niches. We propose that Pmt proteins may be suitable targets for future novel classes of antifungal agents.

Sugar-lectin and protein-protein interactions are very important during contact of the human fungal pathogen *Candida albicans* with epithelial cells of the human host (reviewed in references 4 and 31). α 1,2- and β 1,3-Mannosyl structures present in mannoproteins or phospholipomannan at the fungal surface bind to cognate lectin receptors on human enterocytes (7, 21). On the other hand, fungal mannoprotein adhesins bind to blood group glycolipids of human buccal epithelial cells (3). In the related species *Candida glabrata* the Epa family of adhesins, which is missing in *C. albicans*, mediates adhesion to *N*-acetyl lactosamine determinants on glycosphingolipids of epithelial cells (6). Members of the Als family (11, 14) and the Eap1 protein (16) of fungal cells also contribute to host cell attachment. Fungal surface hydrophobicity may be a general or specific property that enhances host cell interactions; this characteristic is directly influenced by the degree of surface mannosylation (18, 22, 29). An initial reversible interaction between *C. albicans* and epithelial cells is fixed subsequently by human transglutaminase activity, which forms a link between as-yet-undefined host proteins and the Hwp1 protein in the fungal cell wall; the latter interaction appears to be especially important during infection of mucosal surfaces (30, 31). In spite of the plethora of data, the relative importance of these and other adhesion mechanisms (4) during *C. albicans* infection remains unclear. Clearly, although adhesion to host cells is an important first step during infection, successful colonization and, consequently, damage of the host by the pathogen depend on subsequent processes. Such processes include efficient phagocytosis or penetration of cells or tissues, dissemination to other infection sites, and colony or biofilm formation. Hypha

formation helps the pathogen to penetrate epithelial monolayers and the extracellular matrix, to migrate into deep tissues, to enter the blood, to cross the vascular lining, and to invade organs. Individual cells may be invaded by hyphae, or hyphae may help *C. albicans* exit epithelial cells and macrophages (17, 35, 38).

We recently completed characterization of the Pmt family of *C. albicans*, which comprises five isoforms of protein mannosyltransferases (Pmt1p, Pmt2p, Pmt4p, Pmt5p, and Pmt6p) that initiate protein O glycosylation (22, 32, 33). Pmt proteins influence various aspects of host interactions and virulence (9). While Pmt2p is essential for growth, the presence of Pmt1p and Pmt6p is required for the full adhesive potential and virulence of *C. albicans* (22, 26, 32, 33), similar to phenotypes of *mnt* mutants, in which extension of O-glycosyl chains is defective (2). All *pmt* mutations except *pmt5* cause a partial defect in hyphal morphogenesis. Interestingly, the *pmt6* phenotype can be corrected by overexpression of components of known signaling pathways that trigger morphogenesis (33), suggesting that an upstream component, possibly a membrane sensor, is activated by Pmt6-mediated O glycosylation. Some secreted proteins and wall or cytoplasmic membrane proteins, including chitinase, Als1p, Kre9p, and Sec20p, have been shown to be targets of Pmt proteins (22, 32, 33). Finally, *pmt* mutants are supersensitive to a range of antifungal agents, particularly aminoglycosides (22, 32).

In this study we evaluated the relevance of the five Pmt isoforms for host interaction and virulence. For this purpose we characterized *pmt* mutants in two complex, three-dimensional models of tissue infection rather than analyzing adhesion with individual cells or monolayers. In the reconstituted human epithelium (RHE) model, differentiation of the TR146 cell line generates a multilayer cell system resembling the human buccal mucosa (27). The second model used was derived

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TABLE 1. *C. albicans* strains

Strain	Genotype	Reference(s)
CAF2-1	<i>ura3Δ::imm434/URA3</i>	10
CAI4	<i>ura3Δ::imm434/ura3Δ::imm434</i>	10
<i>URA3</i> -reconstituted strains		
SPCa2, SPCa3	As CAI4, but <i>pmt1Δ::hisG/pmt1Δ::hisG ura3Δ::imm434/URA3</i>	22
SPCa4, SPCa5	As CAI4, but <i>pmt2Δ::hisG/PMT2 ura3Δ::imm434/URA3</i>	22
SPCa6, SPCa7	As CAI4, but <i>pmt4Δ::hisG/pmt4Δ::hisG ura3Δ::imm434/URA3</i>	22
SPCa10, SPCa11	As CAI4, but <i>pmt5Δ::hisG/pmt5Δ::hisG ura3Δ::imm434/URA3</i>	22
SPCa7, SPCa8	As CAI4, but <i>pmt6Δ::hisG/pmt6Δ::hisG ura3Δ::imm434/URA3</i>	22
<i>PMT</i> -reconstituted strains and controls		
CAP1-3121(pCT30)	As CAI4, but <i>pmt1Δ::hisG/pmt1Δ::hisG (PMT1-URA3::LEU2)</i>	32, this study
CAP1-3121(pRC18)	As CAI4, but <i>pmt1Δ::hisG/pmt1Δ::hisG (URA3::LEU2)</i>	32, this study
CAP4-2164(pSP1)	As CAI4, but <i>pmt4Δ::hisG/pmt4Δ::hisG (PMT4-URA3::LEU2)</i>	22
CAP4-2164(pRC18)	As CAI4, but <i>pmt4Δ::hisG/pmt4Δ::hisG (URA3::LEU2)</i>	22
P5-5744(pBK1)	As CAI4, but <i>pmt5Δ::hisG/pmt5Δ::hisG (PMT5-URA3::LEU2)</i>	22
P5-5744(pRC18)	As CAI4, but <i>pmt5Δ::hisG/pmt5Δ::hisG (URA3::LEU2)</i>	22
CAP2-2391(pCT34)	As CAI4, but <i>pmt6Δ::hisG/pmt6Δ::hisG (PMT6-URA3::LEU2)</i>	34, this study
CAP2-2391(pRC18)	As CAI4, but <i>pmt6Δ::hisG/pmt6Δ::hisG (URA3::LEU2)</i>	34, this study

from primary cells that reconstitute a multilayer engineered human oral mucosa (EHOM) (23). Finally, we investigated the virulence of *pmt* mutants in a hematogenously disseminated candidiasis (HDC) model. The results demonstrate that all five *Pmt* isoforms make a significant contribution to the virulence of *C. albicans*.

MATERIALS AND METHODS

***C. albicans* strains and growth conditions.** The strains used are listed in Table 1.

PMT-carrying plasmids (22, 32, 33) and control plasmid pRC18 (33) were linearized by KpnI and integrated into the *LEU2* locus of appropriate mutant strains by transformation (22). Strains were grown routinely in liquid or on solid YPD or SD medium (28).

To assay protease production, plates containing bovine serum albumin as the sole source of nitrogen were used (1). *C. albicans* colonies were grown at 37°C for 7 days, and the plates were flooded with 20% trichloroacetic acid, washed with phosphate-buffered saline (PBS), stained with 0.6% amido black in methanol-acetic acid-distilled water (45:10:45), and destained with the same solvent. As a measure of protease activity, the diameters of clearing zones were divided by the corresponding colony diameters.

In vitro model of oral candidiasis. The human epithelium used for the in vitro model of oral candidiasis was supplied by Skinethic Laboratory (Nice, France). Human keratinocytes derived from a squamous cell carcinoma of the buccal mucosa (cell line TR146) were cultured on an inert supporting membrane (25). A defined serum-free medium based on MCDB-153 medium (Clonetics, San Diego, CA) supplemented with 5 µg per ml insulin was deposited below a 0.5-cm² microporous polycarbonate filter (pore size, 0.5 µm) and incubated for 7 days at 37°C with 100% humidity and 5% CO₂ to generate a human reconstituted epithelium. No antibiotics or antimycotics were used.

C. albicans strains were cultured for 24 h at 37°C on Sabouraud dextrose agar (Difco). Part of the culture was suspended in a 0.9% sodium chloride solution and centrifuged. After the suspension was washed three times, 2 × 10⁵ cells were suspended in 10 ml YPD. The suspension was cultured for 16 h at 25°C, and 4 × 10⁶ cells were suspended in fresh medium and incubated for 24 h at 37°C with shaking. After three washes with PBS, the inoculum size was adjusted with PBS.

A 0.5-cm² RHE surface was infected with 2 × 10⁶ cells of each *C. albicans* strain in 50 µl of PBS; as a control 50 µl of PBS alone was used. The RHE was incubated at 37°C with 5% CO₂ at 100% humidity for 12, 24, and 29 h. Infected RHE were examined by light microscopy, as described previously (27).

The release of lactate dehydrogenase (LDH) from epithelial cells into the surrounding medium was monitored as a measure of epithelial cell damage. LDH release into the maintenance media of the cultures containing uninfected and infected epithelial cells was measured at 12, 24, and 29 h. LDH activity was analyzed spectrophotometrically by measuring the rate of NADH disappearance

at 340 nm during the LDH-catalyzed conversion of pyruvate to lactate by using the Wróblewski-La Due method (37). The LDH activity was expressed in U/liter at 37°C, and the statistical significance of differences between groups was analyzed with an unpaired *t* test and by calculating two-tailed *P* values (GraphPad Prism 4 program).

Preparation and infection of engineered human oral mucosa. Using normal primary oral epithelial cells and oral fibroblasts, EHOM were produced as previously described (23). Briefly, oral fibroblasts were mixed with bovine skin collagen (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) to produce the lamina propria. Tissues were grown in Dulbecco-Vogt modified Eagle medium supplemented with 5% fetal calf serum for 4 days. The oral epithelial cells were then seeded on the lamina propria, and tissues were grown in a 3:1 mixture of the Dulbecco-Vogt modification of Eagle's medium and Ham's F12 (H) medium (DMEH; Invitrogen Life Technologies, Burlington, Ontario, Canada) supplemented with 24.3 µg/ml adenine, 10 µg/ml human epidermal growth factor (Chiron Corp., Emeryville, CA), 0.4 µg/ml hydrocortisone (Calbiochem, La Jolla, CA), 5 µg/ml bovine insulin, 5 µg/ml human transferrin, 2 × 10⁻⁹ M 3,3',5'-triiodo-L-thyronine, 10⁻¹⁰ M cholera toxin (Schwarz/Mann, Cleveland, OH), 100 U/ml penicillin, 25 µg/ml gentamicin (Schering, Pointe-Claire, Quebec, Canada), 0.5 µg/ml amphotericin B (Fungizone), and 10% fetal calf serum (NCS, fetal clone II; HyClone, Logan, UT). Once the epithelial cells reached confluence, the EHOM were raised to the air-liquid interface for 5 days to allow the epithelium to stratify (23).

The *C. albicans* strains were grown in 10 ml of Phytone-peptone broth (Becton-Dickinson) supplemented with 0.1% glucose. Each culture was grown for 17 h at 25°C in a shaking water bath. The cells were collected, washed three times with PBS, and counted using a hemacytometer (24). Then 2 × 10⁵ cells were suspended in 10 ml of YPD medium and cultured for 17 h at 25°C, and 4 × 10⁶ cells were taken from the culture and suspended in fresh medium. The cell suspension was incubated for 17 h at 37°C with shaking. The cells were then washed three times with PBS. The cell concentration was adjusted with PBS so that each EHOM was in contact with 4 × 10⁶ cells/cm² in 100 µl PBS. Forty-eight hours before infection, tissues were grown in a serum-free and amphotericin B-free DMEH medium. Infected and uninfected tissues were cultured for 6, 12, and 22 h in the serum- and amphotericin B-free medium. At the end of each incubation time, biopsies were collected, fixed with Bouin's solution, and then embedded in paraffin. To evaluate the tissue structure, thin cryostat sections (thickness, 4 µm) of the paraffin-embedded biopsies were stained with Masson trichrome (23).

Infection of mice with *C. albicans*. Female CD2F1 mice purchased from Charles River Breeding Laboratories (Calco, Lecco, Italy) were used when they were 4 to 6 weeks old. *C. albicans* yeast cells taken from a 24-h culture on Sabouraud agar were washed with sterile endotoxin-free physiological saline (Fresenius Kabi Italia SPA, Verona, Italy), counts were determined with a hemacytometer, and the concentration was adjusted to the appropriate value for intravenous injection. The number of viable yeast cells injected was confirmed by culturing dilutions of the inoculum on Sabouraud agar. A blood-borne infection

TABLE 2. Damage of reconstituted human epithelium by *C. albicans* strains

Expt	Type of strains	<i>C. albicans</i> strain	Relevant genotype	Amt of LDH released (U/liter) ^a
A	<i>URA3</i> reconstituted	Uninfected		10 ± 0
		CAF2-1	Wild type	460 ± 10
		SPCa2	<i>pmt1/pmt1</i>	90 ± 0 ^b
		SPCa4	<i>pmt2/PMT2</i>	180 ± 10 ^b
		SPCa6	<i>pmt4/pmt4</i>	190 ± 10 ^b
		SPCa10	<i>pmt5/pmt5</i>	320 ± 10 ^b
		SPCa8	<i>pmt6/pmt6</i>	140 ± 10 ^b
B	PMT, reconstituted and controls	Uninfected		20 ± 0
		CAI4	Wild type	250 ± 10
		CAP1-3121(pRC18)	<i>pmt1/pmt1</i>	150 ± 10
		CAP1-3121(pCT30)	<i>pmt1/pmt1</i> (PMT1)	210 ± 0 ^b
		CAP4-2164(pRC18)	<i>pmt4/pmt4</i>	80 ± 0
		CAP4-2164(pSP1)	<i>pmt4/pmt4</i> (PMT4)	240 ± 10 ^b
		P5-5744(pRC18)	<i>pmt5/pmt5</i>	270 ± 0
		P5-5744(pBK1)	<i>pmt5/pmt5</i> (PMT5)	270 ± 0
		CAP2-2391(pRC18)	<i>pmt6/pmt6</i>	110 ± 10
		CAP2-2391(pCT34)	<i>pmt6/pmt6</i> (PMT6)	270 ± 10 ^b

^a Mean ± standard deviation of three measurements.

^b $P < 0.0001$ for a comparison of a *pmt* mutant and the wild-type strain in experiment A and for a comparison of a *pmt* mutant and the corresponding PMT-reconstituted strain in experiment B.

was created by injecting 0.5 ml of a prewarmed yeast cell suspension (1×10^6 yeast cells/0.5 ml) through a 27-gauge needle into the tail vein. The outcome of infection was evaluated in terms of the median survival time, the number of dead animals compared with the total number, and the fungal burden in the kidneys, expressed in CFU.

At designated times after infection mice were sacrificed, and their kidneys and brains were removed and homogenized. Serial 10-fold dilutions of each sample were plated in duplicate on Sabouraud agar plates and incubated for 24 h, and then CFU were counted. For in vivo experiments the statistical significance of differences between groups was analyzed with the Mann-Whitney U test.

RESULTS

In previous studies we characterized *C. albicans pmt1* and *pmt6* mutants (32, 33). Mutagenesis and characterization of the entire PMT family were completed recently by construction of *pmt2*, *pmt4*, and *pmt5* mutants (22). To clarify and ascertain the roles of individual Pmt isoforms in *C. albicans* virulence, we decided to perform side-by-side virulence tests of all *pmt* mutants in two complex, three-dimensional models of oropharyngeal candidiasis, as well as in a mouse model of hematogenously disseminated candidiasis. Because ectopic expression of *URA3* at disrupted gene loci may differ and may influence the outcome of virulence tests (5, 15), these experiments were done initially with homozygous *pmt* mutants in which *URA3* had been reconstituted at its authentic locus, and phenotypes were reconfirmed using PMT-reconstituted strains (Table 1). In addition, because PMT2 is essential for growth and a homozygous *pmt2/pmt2* mutant is not viable, a heterozygous *pmt2/PMT2* strain was examined in some virulence tests.

Interaction of *pmt* mutants with reconstituted human epithelium. Human keratinocytes derived from a squamous cell carcinoma of the buccal mucosa (cell line TR146 [25]) were allowed to differentiate on an inert supporting membrane into a multilayer epithelium (RHE); this epithelium model has been used to study several key processes in *C. albicans* epithelial infection, including adherence, penetration, and keratinocyte cellular damage (13, 27). The RHE system was infected

with *pmt* mutants for 12, 24, and 29 h, and the damage to RHE cells caused by *C. albicans* infection was assessed by determining the release of the intracellular marker enzyme LDH from keratinocytes into the culture fluid and by microscopy of the RHE structure.

It was observed that the amount of LDH released differed greatly between series of experiments. To reduce the variability due to the stage of RHE differentiation, the amount of RHE cells, and the physiological state of *C. albicans* cells, control strains were always tested along with *pmt* mutants, and all tests were performed in triplicate. Infection of RHE with the control strains CAF2-1 and CAI4 led to significant increases in LDH in the medium compared to the results obtained for uninfected RHE, which was indicative of damaged keratinocytes (Table 2). The abilities of all *pmt* mutants in the *URA3*-reconstituted series of strains to damage the RHE were decreased, although the reduction was relatively minor in the case of the *pmt5* mutant (Table 2, experiment A). Comparisons of *pmt* mutant strains reconstituted by chromosomal integration of plasmids carrying the appropriate PMT gene or a control plasmid confirmed that LDH release was reduced in the case of *pmt1*, *pmt4*, and *pmt6* mutations (Table 2, experiment B). We concluded that mutations in all PMT genes except PMT5 significantly decreased the ability of *C. albicans* to damage epithelial cells.

For all *pmt* mutants, the levels of LDH released from epithelial cells were correlated with the degree of damage to the overall RHE structure. Microscopic inspections of the infected RHE revealed an intact multilayer epithelium in the absence of *C. albicans* infection. In the presence of control strain CAI4 (which had a phenotype identical to that of strain CAF2-1 in this assay) and during infection by the *pmt5* mutant SPCa10, the RHE was vacuolized and showed edema accompanied by invasion of all epithelial layers (Fig. 1 and data not shown). In contrast, a *pmt1* mutant carrying a control vector [strain CAP1-3121(pRC18)] caused little epithelial damage, while reintro-

duction of the *PMT1* gene into the *pmt1* mutant background [strain CAP1-3121(pCT30)] restored RHE damage. Similar comparisons between *pmt* mutants and *PMT*-reconstituted mutants confirmed that mutations in *PMT4* and *PMT6* significantly reduced damage to the RHE (Fig. 1). In additional experiments, we verified that *pmt* mutants containing *URA3* at its authentic locus generated epithelial phenotypes identical to those generated by *pmt* mutants carrying *URA3* on an integrated control plasmid (data not shown), indicating that the lack of virulence of any *pmt* mutant in this assay was not caused by lower levels of *URA3* expression (5, 15). In this series of experiments we also showed that the *pmt2/PMT2* heterozygous strain SPCa4 caused little damage to the RHE (data not shown).

We concluded that in the RHE assay all *pmt* mutants except the *pmt5* mutant had significantly reduced abilities to cause host cell damage and to damage the overall epithelial structure.

Interaction of *pmt* mutants with engineered human oral mucosa. The RHE system relies on differentiation of cell line TR146, which generates a multilayer epithelium representing important features, but not all features, of an authentic epithelium (25, 27). To confirm the results in a more complex system in which the conditions approach the conditions in normal human mucosa, we used a model system based on differentiation of primary cells (fibroblasts and keratinocytes), which form a stratified epithelium (23). The EHOM had a well-organized epidermal structure at all times examined (6, 12, and 22 h), which was characterized by the presence of the major strata (germinativum, spinosum, granulosum, and corneum), as well as a collagen-containing dermis found in normal mucosa (Fig. 2). The epithelial cells in the basal layer were characterized by their small size, their cuboidal shape, the presence of a small nucleus, and a lack of vacuoles (Fig. 2A). In the dermis, whose thickness varied according to the source of the primary cells used for construction of the EHOM, fibroblasts were detected occasionally in the collagen matrix.

When an engineered mucosa was infected with *C. albicans* CAF2-1, changes were observed as soon as 6 h (data not shown). The epithelium was thinner and not well organized, and it contained a significant number of cells that had undergone aberrant differentiation (i.e., large vacuolated cells containing a large nucleus) (Fig. 2A). At this time the basal layer could still be distinguished from the other layers, and the dermis remained well organized and contained fibroblasts that were the normal size. After 12 h the dermis was notably thinner, and the epithelium contained higher numbers of large differentiated cells that also occurred even in the basal layer (data not shown). After 22 h the epithelium was significantly thinner compared to the uninfected EHOM; also, the basal layer and stratum corneum contained many large differentiated cells. Fewer but larger fibroblasts were observed in the dermis. Similar to an EHOM infected with control strain CAF2-1, EHOM infected with *pmt4* mutants (SPCa6 and SPCa7) or with *pmt6* mutants (SPCa8 and SPCa9) were significantly damaged (Fig. 2A).

When the EHOM was infected with the *URA3*-reconstituted *C. albicans* *pmt1* mutant SPCa2 or SPCa3, no significant changes had occurred after 6 h of incubation compared to the uninfected control; after 12 h, the epithelium had a slightly

disorganized basal layer, and some large differentiated cells were observed in the adjacent strata (data not shown). After 22 h the EHOM had a relatively normal appearance and the major strata, including the basal layer with cuboidal proliferative cells, although the epidermis appeared to be thinner compared to the uninfected control. Thus, the *pmt1* mutant was defective in the ability to massively damage the EHOM structure. The *pmt5* mutants SPCa10 and SPCa11 also had reduced effects on the EHOM, although after 22 h of infection significant numbers of large differentiated cells were visible (Fig. 2A).

To confirm the finding that mutations in *PMT1* and *PMT5* genes significantly reduce damage in the EHOM model, we compared the virulence of *pmt1* mutants (or *pmt5* mutants) transformed by a *PMT1*-containing plasmid, a *PMT5*-containing plasmid, or an empty control plasmid (Fig. 2B). The results clearly revealed that the *PMT1*-reconstituted mutant strain, but not the strain transformed with the empty control plasmid, was able to significantly damage the EHOM structure. Similarly, an EHOM infected with the *pmt5* mutant carrying the control plasmid had a relatively normal appearance, while the EHOM was highly disorganized in the case of infection by the *PMT5*-reconstituted strain. The latter result is of particular interest, because in previous in vitro growth tests (22) and in the RHE assay described above the *pmt5* mutant did not reveal any mutant phenotypes.

Thus, in the EHOM model system the *pmt1* and *pmt5* mutants showed markedly reduced virulence, while the *pmt4* and *pmt6* mutants were almost as virulent as the control strain and caused important damage to the epithelial structure.

Virulence of *pmt* mutants in a model of hematogenously disseminated candidiasis. The *URA3*-reconstituted series of *pmt* mutants were injected intravenously into CD2F1 mice, and their survival was monitored. Control strain CAF2-1 killed the infected mice rapidly (the median survival time was 2 days). At the other extreme, mice infected with the homozygous *pmt1* mutant or the heterozygous *pmt2/PMT2* strain survived for at least 60 days (Table 3). Compared to the CAF2-1 control strain, all other *pmt* mutants showed attenuated virulence, leading to median survival times between 3 and 6 days. Thus, the results for the HDC model reflected the defective virulence behavior in either one or both oropharyngeal candidiasis models.

Using BALB/c mice, we previously did not observe any phenotype of the *pmt5* mutant in an HDC model, while the *pmt4* mutant completely lacked virulence (22); these results are at variance with the results described above obtained with *URA3*-reconstituted mutants and CD2F1 mice. To confirm these results and to identify differences in the degree of virulence due to the type of mouse strain used, we carried out comparative infection experiments with CD2F1 mice. In these experiments, *pmt4* and *pmt5* mutants that were reconstituted or were not reconstituted with *PMT4*- and *PMT5*-expressing plasmids were used (Fig. 3A). The results confirmed that mutation of *PMT4* leads to a strong defect and that mutation of *PMT5* leads to a moderate defect in *C. albicans* virulence; as expected, introduction of the corresponding *PMT* gene into the mutant background fully restored the killing efficiency. We concluded that the reduced virulence of *pmt5* mutants was confirmed by comparisons of strains containing the *PMT5* gene and strains not

uninfected

PMT/PMT

pmt1/pmt1

pmt1/pmt1[PMT1]

pmt4/pmt4

pmt4/pmt4[PMT4]

pmt6/pmt6

pmt6/pmt6[PMT6]

FIG. 1. Infection of RHE by *C. albicans*. A total of 2×10^6 *C. albicans* cells were used to infect the RHE, which was examined at 24 h by light microscopy. The strains used for infection were CAI4 (*PMT/PMT*), CAP1-3121(pRC18) (*pmt1/pmt1*), CAP1-3121(pCT30) (*pmt1/pmt1[PMT1]*), CAP4-2164(pRC18) (*pmt4/pmt4*), CAP4-2164(pSP1) (*pmt4/pmt4[PMT4]*), CAP2-2391(pRC18) (*pmt6/pmt6*), and CAP2-2391(pCT34) (*pmt6/pmt6[PMT6]*). Note that epithelial invasion caused by *C. albicans* CAI4 and *PMT*-reconstituted strains (dark spheres and filaments) is accompanied by vacuolization and edema of all keratinocyte layers.

containing the *PMT5* gene. This finding and the residual killing activity of the *pmt4* mutant, which was not observed using BALB/c mice (22), suggest that the outcome of the HDC assay may vary to some degree depending on the mouse strain used.

Because in the HDC model the *pmt1* and *pmt2* mutants were completely avirulent, we tested if, nevertheless, kidneys or brains of infected mice were colonized by these *C. albicans* strains. The results revealed that significant numbers of *C. albicans* cells were present for about 6 days after infection, especially in the kidney, but that by day 9 fungal cells had disappeared completely from the organs (Fig. 3B). We concluded that both the *pmt1* homozygous mutant and the *pmt2* heterozygous mutant are able to grow within and colonize the host, causing no symptoms of disease, and that host defenses clear these infections eventually.

Virulence in relation to other phenotypes of *pmt* mutants. We previously established that *PMT2* in *C. albicans* is an essential gene and that the homozygous *pmt1* mutant grows at moderately lower growth rates (about 30% lower) than *PMT* control strains (22). This growth defect was also observed at 37°C in the media used for the virulence assays described above, while the other *pmt* mutants grew similar to the control strains. In addition, the homozygous *pmt1* mutant and to a lesser degree the *pmt4* mutant displayed increased surface hydrophilicity, which presumably was caused by a decreased mannoprotein content of the cell wall (22). Other relevant phenotypes of *C. albicans* known to affect the outcome of virulence tests include the ability to form hyphae and the ability to secrete proteases (8, 17, 19, 27).

We showed previously that hyphal morphogenesis of the *pmt1* mutant was severely blocked in most inducing conditions (22, 32, 33), while a partial morphogenetic defect, apparent only in a subset of inducing conditions, was characteristic for the *pmt2/PMT2* heterozygous mutant and the *pmt4* and *pmt6* homozygous mutants; in contrast, the *pmt5* mutant did not show a morphogenetic defect (22). Microscopic inspection revealed that in the RHE assay, all *pmt* mutants (including the *pmt1* mutant) were able to form hyphal filaments, suggesting that defective hypha formation is not the reason for the diminished ability of *pmt* mutants to damage the RHE. In the EHOM assay, however, close microscopic examination of infected tissue revealed significant hypha formation by *C. albicans* CAF2-1 and *pmt4* and *pmt6* mutants, while the *pmt1* mutant in particular grew mostly in the yeast form and the *pmt5* mutant produced only a few filaments. Thus, in the EHOM assay the ability to form hyphae appeared to be related to virulence. In agreement with this result, the kidneys of mice infected with strain CAF2-1 contained all fungal morphological forms (yeast cells, pseudohyphae, hyphae), while the kid-

TABLE 3. Virulence of *pmt* mutants in a mouse model of hematogenously disseminated infection

<i>C. albicans</i> strain ^a	Relevant genotype	Mean survival time (days)	No. of dead mice/total no. ^b
SPCa2	<i>pmt1/pmt1</i>	>60 ^c	0/6
SPCa3	<i>pmt1/pmt1</i>	>60 ^c	0/6
SPCa4	<i>pmt2/PMT2</i>	>60 ^c	0/6
SPCa5	<i>pmt2/PMT2</i>	>60 ^c	0/6
SPCa6	<i>pmt4/pmt4</i>	3.5 ^d	6/6
SPCa7	<i>pmt4/pmt4</i>	6.5 ^c	6/6
SPCa10	<i>pmt5/pmt5</i>	4 ^d	6/6
SPCa11	<i>pmt5/pmt5</i>	3.5 ^d	6/6
SPCa8	<i>pmt6/pmt6</i>	3 ^d	6/6
SPCa9	<i>pmt6/pmt6</i>	3 ^d	6/6
CAF2-1	Wild type	2	6/6

^a CD2F1 mice were infected intravenously with 1×10^6 *C. albicans* cells.

^b The results were determined after 60 days.

^c $P < 0.01$ for a comparison of a *pmt* mutant and the wild-type strain.

^d $P < 0.05$ for a comparison of a *pmt* mutant and the wild-type strain.

neys of *pmt1* mutant-infected animals contained mostly yeast cells, which were rapidly cleared (Fig. 3 and data not shown).

To examine if protease secretion was defective in *pmt* mutants, we used growth plates containing bovine serum albumin and measured the zones of protease digestion around colonies. The diameters of digestion zones were divided by the colony diameters as a quantitative measure of protease secretion, and the data resulted in the following ratios (means of duplicate measurements from three experiments, with standard deviations of <5%): for no protease secretion, 1; for wild-type CAF2-1, 2.4; for *pmt1* mutant SPa2, 1.2; for *pmt2/PMT2* mutant SPa4, 1.7; for *pmt4* mutant SPa6, 1.8; for *pmt5* mutant SPa10, 2.2; and for *pmt6* mutant SPa7, 2.3. Thus, especially the *pmt1* mutant and to a lesser degree also the *pmt2* and *pmt4* mutant strains were significantly defective in protease activity, which may have affected the results of virulence assays (19).

DISCUSSION

Our results stress the importance of *PMT* genes and the main process that they determine, protein O glycosylation, for the virulence of *C. albicans*. Each of the five *Pmt* isoforms is involved in virulence, but the isoforms appear to have different roles depending on the environment of the infection, which is probably different in different host niches.

In both models of localized candidiasis (RHE and EHOM) and in the systemic model of candidiasis (HDC) tested here, the *pmt1* mutant was severely attenuated in virulence. The *pmt1* mutant was not able to significantly damage individual epithelial cells, as measured by LDH release in the RHE assay,

FIG. 2. Histological appearance of EHOM not infected or infected with *C. albicans* strains. An uninfected EHOM has an epidermis (Ep) containing an outmost stratum corneum layer (S.C.) and a basal layer (B. L.), as well as a collagen-containing dermis (D) that contains some fibroblasts (F). Cuboidal cells in the basal layer are indicated by asterisks, and large differentiated cells are indicated by white arrows. Following 22 h of infection, biopsies were taken from each EHOM, and Masson trichrome staining was performed. Sections of stained tissues were observed with an optical microscope and photographed. Representative photographs from three different experiments are shown (magnification, $\times 250$). (A) Comparison of *URA3*-reconstituted *pmt* mutants with control strain CAF2-1. Mutants SPCa2 (*pmt1*), SPCa6 (*pmt4*), SPCa10 (*pmt5*), and SPCa8 (*pmt6*) were used for infection. (B) Comparison of *PMT*-reconstituted mutants and unreconstituted mutants. Strains CAP1-3121(pRC18) (*pmt1/pmt1*), CAP1-3121(pCT30) (*pmt1/pmt1[PMT1]*), P5-5744(pRC18) (*pmt5/pmt5*), and P5-5744(pBK1) (*pmt5/pmt5*) were compared.

FIG. 3. Virulence of *C. albicans* in an HDC model. CD2F1 mice were infected with *C. albicans* strains (1×10^6 cells/mouse). (A) Survival curves determined by the Kaplan-Meier method. Strains CAF2-1 (●), CAP4-2164(pSP1) (*pmt4/pmt4* [*PMT4*]) (■), CAP4-2164(pRC18) (*pmt4/pmt4*) (▲), P5-5744(pBK1) (*pmt5/pmt5* [*PMT5*]) (▼), and P5-5744(pRC18) (*pmt5/pmt5*) (◆) were tested. (B) Viable *C. albicans* cells in the kidneys and brain. Mice were treated with *C. albicans* strains (1×10^6 cells/mouse). At different times, mice were killed, and viable yeasts (CFU) in the kidneys (grey bars) and brain (black bars) were counted by plating samples of homogenized tissues on Sabouraud agar. The data are the means $\pm$ standard deviations for five mice.

nor did it affect the overall epithelial structure in either model. Reduced virulence of the *pmt1* mutant was expected because of its significant defect in hyphal morphogenesis, its leakage of cell wall proteins, including chitinase, its supersensitivity to antifungal agents directed against wall components, its lack of adhesion to Caco-2 cells, and its lower virulence in an HDC model and in a model assessing damage to epithelial cells (26, 32). We note, however, that virulence defects of *C. albicans* mutants detected in our epithelial model systems are not necessarily predicative of the HDC assay results; the *sit1* mutant, which lacks a siderophore transporter, is fully virulent in the HDC model, but it is not able to migrate into deep layers in the RHE model (13) and also causes less damage in the EHOM model (unpublished results). Furthermore, the outcome of the

HDC assay is influenced by the strain of mice used (see below). The *pmt1* mutant formed filaments normally in the RHE assay, but it did not form hyphae in the EHOM assay; because in both assays the virulence of this mutant was reduced, we concluded that virulence and hypha formation are not closely correlated in these models.

In a previous study we reported that a *C. albicans* strain lacking both alleles of *PMT2* is not viable and that even a heterozygous *pmt2/PMT2* strain has severe growth defects in stress conditions (22). Deletion of a single *PMT2* allele also resulted in significantly reduced virulence in the RHE model and in the systemic model of infection. In the latter model, the *pmt2/PMT2* heterozygous mutant and the *pmt1* homozygous mutant did not cause killing, although both strains persisted in the kidneys and even the brains of infected mice for 6 days. Thus, a partial loss of Pmt2p function is equivalent to a complete loss of Pmt1p function with regard to systemic virulence, and reduced virulence is not caused by a lack of colonization.

The *pmt4* and *pmt6* mutants were significantly less virulent than the wild-type strain in the RHE assay, while in the EHOM assay they caused massive damage, similar to the wild-type strain. Thus, for these *pmt* mutants the results in the RHE and EHOM assays did not match, and the reasons for the difference, indicating that Pmt isoforms have an environment-specific role, remain to be elucidated. Because the *pmt4* and *pmt6* mutants formed hyphae in both the RHE and EHOM assay yet their virulence appeared to be different in these systems, we concluded that hypha formation per se is not indicative of virulence in these epithelial models, as described above.

The finding that the virulence of the *pmt5* mutant was attenuated in the RHE and HDC assays was completely unexpected, because the *pmt5* mutant had not shown any in vitro growth phenotype or decreased virulence in the HDC model previously (22). On the other hand, we showed previously that *PMT5* is transcribed, and we recently showed that deletion of *pmt5* has a significant impact on the transcriptomal pattern (unpublished results). Thus, it appears that the *pmt5* phenotype becomes apparent only in some conditions that approach the conditions in the environment of the infected host, which is a unique finding not commonly observed for other mutants of *C. albicans*. Unexpectedly, in the EHOM assay we observed defective hypha formation by the *pmt5* mutant, which established that there are as-yet-undefined environmental conditions in which defective morphogenesis of a *pmt5* mutant can be observed. This defect was not apparent previously in any of the known hyphal induction protocols (22).

The systemic HDC model mirrors the interaction of *C. albicans* with host cells and host components in multiple host niches, and therefore, the virulence observed is a composite result of all host interactions. Given the strong effects in at least one of the localized infection models, it is not surprising that all *pmt* mutations significantly affected systemic virulence. In our initial characterization of *PMT* genes, we established that the *PMT1*, *PMT4*, and *PMT6* genes are required for full virulence in the HDC model (22, 32, 33), and this was largely confirmed here using the *URA3*-reconstituted strains. However, at variance with results reported in the present report, virulence was completely abolished in a *pmt4* mutant, while a *pmt5* mutant remained fully virulent (22). These differences may be due to (i) the position of *URA3* at the *LEU2* locus in

the previous study, which may have decreased virulence (5, 15); (ii) a lower infection dose in the previous study (5×10^5 CFU, compared to the 1×10^6 CFU used in this study), which may have amplified differences in virulence; and (iii) the use of different mouse host strains (BALB/c in the previous study and CD2F1 in the present study) (12). The first hypothesis is not likely to explain the results, because *URA3*-reconstituted and plasmid-reconstituted derivatives of *pmt4* and *pmt5* mutants were similarly defective in virulence, if CD2F1 mice were used for the assay. We instead suggest that the *pmt5* mutant is a remarkable example of a strain which has a virulence defect in CD2F1 mice but not in BALB/c mice.

Specific Pmt target proteins involved in virulence characteristics remain to be established. Pmt1p contributes to construction of the fungal cell wall, since *pmt1* mutants underglycosylate cell wall proteins (Als1p, Pir2p), underglycosylate the Kre9 protein involved in β 1,6-glucan biosynthesis, release chitinase, and are supersensitive to cell wall-damaging agents (22, 32). Also, *pmt1* and *pmt4* mutants underglycosylate an essential component of the secretory apparatus, Sec20p (34), and have a significantly lower mannoprotein content in the cell wall than the wild-type strain or the other *pmt* mutants (22). Possibly, the lack of Pmt1p- and/or Pmt4p-mediated O glycosylation prevents linkage of mannoproteins such as Als proteins to the glucan in the cell wall, causing reduced adhesion to epithelial cells (14). Alternatively, defective O glycosylation of certain wall, membrane, and/or secreted proteins may compromise other functions relevant for host cell interactions. We demonstrate here that *pmt1* mutants and, to a lesser extent, *pmt4* mutants and *pmt2* heterozygous strains are partially defective in protease secretion. This defect may limit their ability to use proteins as sources of nitrogen in a host, slowing fungal growth, or it may prevent invasion of tissues and cells (19).

The results reported here strengthen the view that fungal Pmt proteins may be suitable targets for future novel classes of antifungal agents. All Pmt isoforms affect fungal virulence, and the bulk of mammalian O glycosylation occurs by a mechanism different from the mechanism in other fungi. A novel class of antifungal agents directed toward Pmt1p has indeed already been reported (20). Although the concept of Pmt inhibition may still be valid, it should be considered that there are human Pmt homologues, POMT1 and POMT2, and that they are involved in rare mannosylation of some important human proteins, including α -dystroglycan (36). Thus, specific Pmt inhibitors should be developed on the basis of the significant structural differences between fungal Pmt isoforms and human Pmt proteins.

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