Multiple Functions of Pmt1p-mediated Protein O-Mannosylation in the Fungal Pathogen Candida albicans*

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Protein mannosylation by Pmt proteins initiates O-glycosylation in fungi. We have identified the PMT1 gene and analyzed the function of Pmt1p in the fungal human pathogen Candida albicans. Mutants defective in PMT1 alleles lacked Pmt in vitro enzymatic activity, showed reduced growth rates, and tended to form cellular aggregates. In addition, multiple specific deficiencies not known in Saccharomyces cerevisiae (including defective hyphal morphogenesis; supersensitivity to the antifungal agents hygromycin B, G418, clotrimazole, and calcofluor white; and reduced adherence to Caco-2 epithelial cells) were observed in pmt1 mutants. PMT1 deficiency also led to faster electrophoretic mobility of the Als1p cell wall protein and to elevated extracellular activities of chitinase. Homozygous pmt1 mutants were avirulent in a mouse model of systemic infection, while heterozygous pmt1/pmt1 strains showed reduced virulence. The results indicate that protein O-mannosylation by Pmt proteins occurs in different fungal species, where PMT1 deficiency can lead to defects in multiple cellular functions.

Candida albicans is the leading cause of superficial and systemic fungal infections of humans. Virulence of C. albicans is a composite of several characteristics, including adaptation to body temperature, adhesion, and penetration of epithelial cells as well as its ability to undergo morphogenetic changes, especially between a yeast and a filamentous growth form (1, 2). Conceivably, defects in protein glycosylation may have profound effects on the physical stability and function of the fungal cellular surface and may also affect the secretion efficiency and the activity of secreted soluble proteins.

Present evidence suggests that O-glycosylation proceeds differently in fungal and in higher eukaryotic cells. The first O-glycosylation step in the yeast Saccharomyces cerevisiae, which is catalyzed by protein mannosyltransferases (Pmt)1 (3), occurs in the endoplasmic reticulum and consists of the co-translational transfer of mannose from Dol-P-Man to serine or threonine residues; further mannosylation extension reactions occur in the Golgi (reviewed in Refs. 4 and 5). In C. albicans, the majority of O-glycosyl chains consist of two or three mannoses, but they can extend up to about seven mannoses in α-glycosidic linkages (6). Dol-P-Man-dependent O-glycosylation of secreted proteins has been observed in other yeast species and filamentous fungi (7–9). In contrast to fungi, most O-glycosylation reactions in higher eukaryotic cells including human cells proceed in the Golgi and commence with the attachment of N-acetylgalactosamine to proteins (10). However, some mammalian proteins also appear to be O-mannosylated (11, 12).

A total of seven homologous PMT genes have been identified in the genome of S. cerevisiae (13–17). The PMT1 and PMT2 gene products appear to form a heterodimer that is necessary for enzymatic activity in vitro and in vivo (18). Deletion of PMT1 does not affect viability but leads to cells that tend to aggregate, especially in a pmt2 genetic background (16, 17). Inactivation of both PMT1 and PMT2 causes defects in growth and resistance to killer toxin K1 (16), while some combinations of Pmt triple mutants are inviable (19). Recently, a genomic sequence of Schizosaccharomyces pombe has been reported, which encodes a Pmt homologue (GenBankTM accession number Z99126). In Drosophila melanogaster, a gene encoding a protein with high homology to the yeast Pmt1 proteins has been described (20). Furthermore, several “expressed sequence tags” deposited at GenBankTM are derived from genes encoding putative Pmt proteins of human, mouse, rat, Caenorhabditis elegans, and rice cells.

In this report, we describe the C. albicans homologue of the S. cerevisiae PMT1 gene and characterize its function. PMT1 deletion in C. albicans leads to a lack of in vitro Pmt activity and affects two secreted proteins, Als1p and chitinase. In addition, more drastic in vivo phenotypes compared with S. cerevisiae are observed, including a complete block of morphogenesis in some inducing conditions, an increased sensitivity to some antifungal compounds, and a decreased adherence to host cells. Furthermore, PMT1 function is shown to be essential for the virulence of C. albicans in a mouse model of infection.

MATERIALS AND METHODS

Strains and Growth Conditions—PMT alleles of C. albicans and S. cerevisiae are designated CaPMT and ScPMT respectively, whenever necessary for their distinction. Strains and plasmids are listed in Table I. C. albicans strain CAH4 (21) was used for transformations and gene disruptions. Strains were grown in YPD or SD medium (22), which for Ura strains was supplemented with 20 μg/ml uridine, YPGal medium is identical to YPD except that it contains 2% galactose and 0.2% glucose as carbon sources. Growth was monitored by measuring the optical density at 600 nm of cultures (A600) using a Novaspec II photometer (Amersham Pharmacia Biotech); cells were dispersed by sonication (1 min) in a bath sonifier (Bandelin Sonorex TK52) before measurements. Transformations were performed using the spheroplast

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM | EBI Data Bank with accession number(s) AF000252 (for CaPMT1).

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‡ The abbreviations used are: Pmt, dolichyl-phosphate-D-mannose: protein O-D-mannosyltransferase (EC 2.4.1.190); PAGE, polyacrylamide gel electrophoresis; SD, yeast synthetic minimal medium with dextrose; kb, kilobase pair(s); bp, base pair(s); PCR, polymerase chain reaction; Dol, dolichyl.
C. albicans strains

| Strain or plasmid | Genotype or description | Reference/Source |
|-------------------|------------------------|------------------|
| SC5314            | Prototrophic           | 21               |
| CAI4              | ura3::imm434/ura3::imm434 | 21               |
| CAI4-1            | ura3::imm434/ura3::imm434 | 21               |
| CAP1–3            | As CAI4, but PMT1::hisG-URA3-hisG | This work |
| CAP1–5            | As CAI-1·3·5           | This work        |
| CAP1–312          | As CAI4, but pmt1Δ::hisG | This work        |
| CAP1–312          | As CAP1–312            | This work        |
| CAP1–315          | As CAP1–315            | This work        |
| CAP1–3121         | As CAI4, but pmt1Δ::hisG/pmt1Δ::hisG | This work |

S. cerevisiae strains

| Strains and plasmids | Strains and plasmids |
|----------------------|----------------------|
| B76                  | MATa/trp1 ura3–52 put2–57 | 24 |
| B76/4                | MATa/MATa trp1/trp1 ura3–52/ura3–52 put2–57/ put2–57 | 24 |
| B76/pmt1             | As B76, but pmt1Δ::URA3 | This work |
| B76/pmt1            | As B76/4, but pmt1Δ::URA3/pmt1Δ::URA3 | This work |

S. cerevisiae transformation vectors

| pRC18               | As pRC2312, but containing pUC18 multiple cloning site | 24 |
| pCT29, pCT30        | URA3-marked CaARS vector containing CaPMT1 | This work |

TABLE I

| Strains and plasmids | Strains and plasmids |
|----------------------|----------------------|
| YEpplac112-HO        | TRP1-marked 2-μm vector containing HO | A. Sonnenborn, unpublished |
| YEpplac22RAS2°       | TRP1-marked centromeric vector containing RAS2° | Rademaker et al., manuscript in preparation |
| pCT28                | TRP1-marked centromeric vector containing ScPMT1 | This work |
| pCT31, pCT32         | TRP1-marked 2-μm vector containing CaPMT1 | This work |
| pCH1                 | TRP1-marked centromeric vector containing ScGAL1 | This work |
| pCH2                 | TRP1-marked 2-μm vector containing ScGAL1 | This work |

G protein O-Mannosylation in C. albicans

Cloning and Sequencing of PMT1—A partial genomic DNA library of C. albicans CAI-1 (21) was constructed by digesting DNA with HindIII and insertion of 6–8-kb fragments into YEpplac195 (23). E. coli DH5α transformants carrying the genomic library were analyzed by colony hybridization using a 5.5-kb EcoRI-XhoI fragment containing the S. cerevisiae PMT1 gene (17) as a probe. Plasmid pCT1 was found to strongly hybridize to ScPMT1 and to contain a 5.5-kb C. albicans insert. Subfragments of the pCT1 insert were ligated into pUC19 and sequenced from both ends using forward and reverse primers or by using insert-specific oligonucleotides. The 5.5-kb HindIII CaPMT1 fragment was inserted into the HindIII site of pRC18 (24) to generate replicating pCT23 (25) (which was cut with BglII and HindIII to ligated the 4-kb SalI-BglII “URA blaster” fragment of plasmid p5921 (26). From the resulting plasmid, pCT23, a 6.7-kb Asp718–SpeI isolated that was used to transform strain CAI4 to prototrophy. Correct insertion of the URA blaster into one of the two PMT1 alleles was verified by Southern blotting on DNA of transformants, which was cut with HindIII and probed with a 0.7-kb XbaI fragment derived from the PMT1 promoter region or a 1.3-kb NheI–BamHI fragment from p5921 carrying hisG. A pmt1Δ::hisG-Ura3::hisG/PMT1 strain was plated out on media containing 0.02% 5-fluoroorotic acid. Spontaneous 5-fluoroorotic acid-resistant strains were analyzed for loss of the URA3 sequence by Southern blotting. One of several identified strains, CAP1–31, of the genotype pmt1Δ::hisG/PMT1 was used for a second round of gene disruption using the original URA blaster fragment. Several transformants had the genotype pmt1Δ::hisG/pmt1Δ::hisG/PMT1, as described (24). Strain CAI-312 is a representative of mutant strains of the genotype pmt1Δ::hisG/pmt1Δ::hisG/PMT1. The CaPMT1 gene was reintroduced in CAP1–312 by transformation with plasmid pCT29 or pCT30.

To disrupt ScPMT1, we transformed S. cerevisiae strain B76 (24) with an XbaI–SacI fragment containing pmt1Δ::URA3, as described (13, 17). Correct replacement of the genomic ScPMT1 gene was verified by Southern blotting (13). The resulting strain, B76Δpmt1::URA3, was diploidized by transformation with YEpplac112–HO, which contains the HO gene (27). One diploid transformant was grown repeatedly under nonselective conditions inYPD, and a plasmid-free derivative, B76Δpmt1, was identified. To induce pseudohyphal growth, B76Δpmt1 was transformed with YEpplac22RAS2° and cells were grown on low nitrogen SLAHD plates as described (28). As a control, the diploid strain B76/4 was analyzed (24).

or lithium acetate methods (22). Hyphae were induced by diluting washed cells growing exponentially at 30 °C in 5% serum at 37 °C; alternatively, washed cells were first starved in salt base (SB) (0.45% NaCl, 0.35% yeast nitrogen base without amino acids (Difco); 10 mM NaCl; 0.335% yeast nitrogen base without amino acids (Difco)); 10 mM GlcNAc at 37 °C. Subfragments of the pCT1 insert were ligated into pUC19 and sequenced from both ends using forward and reverse primers or by using insert-specific oligonucleotides. The 5.5-kb HindIII CaPMT1 fragment was inserted into the HindIII site of pRC18 (24) to generate replicating pCT23 (25) to construct a high copy (pCT31) or a low copy vector (pCT32) for construction of S. cerevisiae. To place CaPMT1 under transcriptional control of a defined S. cerevisiae promoter, the CaPMT1 coding region was amplified by PCR, using the primers pS1–9Bam (5’-TTAGATCTTTAGACCTTACCAATCATTGGCAAA-3’) and pP1–10Hin (5’-TAAAGCTTTAGTTTATCATCATACAC-3’), by which a BamHI site (italic type) is placed 9 bp upstream of the ATG start codon (underlined), and a HindIII site (italic type) is placed 17 bp downstream of the stop codon. The BamHI–HindIII CaPMT1 fragment was placed downstream of the GAL1 promoter (the EcoRI–BamHI fragment of pBM150 (25)), and the promoter region was inserted, as an EcoRI–HindIII fragment, between the EcoRI and HindIII sites of YEpplac122 or YEpplac112 (26) to generate plasmids pCH1 or pCH2. pCt28 contains the 7.1-kb BamHI–XhoI genomic ScPMT1 fragment (17) as an Ssc–SpI insert in YEpplac22.

Gene Disruptions—In preparation for disruption of PMT1, a pUC19 derivative (pCT9) containing 2 kb of the PMT1 promoter region and 2 kb of the coding region on a HindIII–HindIII fragment was used as template for “divergent” PCR using the oligonucleotides A (5’-TTAGATCTTTAGACCTTACCAATCATTGGCAAA-3’) and B (5’-TTAGATCTTTAGACCTTACCAATCATTGGCAAA-3’). Oligonucleotide A corresponds to nucleotides –1–15 relative to the ATG in the PMT1 sequence and also a SalI site to the PCR product, while oligonucleotide B corresponds to nucleotides 975–990 and adds a BglII site. The 6.7-kb PCR fragment was cut with SstI and BglII and ligated to the 4-kb SalI–BglII “URA blaster” fragment of plasmid p5921 (26). From the resulting plasmid, pCT23, a 6.7-kb Asp718–SpeI was isolated that was used to transform strain CAI4 to prototrophy. Correct insertion of the URA blaster into one of the two PMT1 alleles was verified by Southern blotting on DNA of transformants, which was cut with HindIII and probed with a 0.7-kb XbaI fragment derived from the PMT1 promoter region or a 1.3-kb NheI–BamHI fragment from p5921 carrying hisG. A pmt1Δ::hisG-Ura3::hisG/PMT1 strain was plated out on media containing 0.02% 5-fluoroorotic acid. Spontaneous 5-fluoroorotic acid-resistant strains were analyzed for loss of the URA3 sequence by Southern blotting. One of several identified strains, CAP1–31, of the genotype pmt1Δ::hisG/PMT1 was used for a second round of gene disruption using the original URA blaster fragment. Several transformants had the genotype pmt1Δ::hisG/pmt1Δ::hisG/PMT1, as described (24). Strain CAP1–312 is a representative of mutant strains of the genotype pmt1Δ::hisG/pmt1Δ::hisG/PMT1. The CaPMT1 gene was reintroduced in CAP1–312 by transformation with plasmid pCT29 or pCT30.

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2 A. Sonnenborn and J. Ernst, unpublished results.
3 F. Rademaker and J. F. Ernst, manuscript in preparation.
Assay of Pmt1p Enzyme Activity—Cells were grown in 50 ml of YPD or SD to an A590 of 0.5–1.0. 40 OD units of cells were harvested and washed once with 10 ml of ice-cold TM buffer (50 mM Tris-HCl, pH 7.5, 0.3 mM MgCl2). The cells were resuspended in 100 μl of TM buffer containing protease inhibitors (1 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 50 mM N-tosyl-phenylalanylchloromethyl ketone, 0.25 mM N-p-tosyl-L-Lysine chloromethyl ketone, 20 μg/ml antipain, 1 μM benzamidine) and transferred to a 1.5-ml microcentrifuge tube. An equal volume of glass beads was added, and the cells were lysed by vortexing eight times in intervals of 1 min with a 1-min incubation on ice. The lystate was collected into a fresh microcentrifuge tube. Cells were pelleted by centrifugation (5,000 x g, 30 °C) and supernatant was transferred to a new tube. Membranes were collected by centrifugation at 48,000 x g for 30 min at 4 °C and were then resuspended in 100 μl of 50 mM bicine, pH 7.7, 1 mM EDTA, 33% glycerol, and protease inhibitors. Microsomal membranes were stored in liquid nitrogen.

The in vitro peptide assay for Pmt1p activity was performed according to Westen et al. (30). The assay was optimized using Del-P-(14C)mannose (approximately 30,000 cpm; specific activity of 305 Ci/mmol) and the acceptor peptide Ac-YATAV-NH2 (final concentration of 4 μM). Up to 25 μl of C. albicans microsomal membranes (protein concentration of 3 mg/ml) were added. Assay mixtures were incubated at 25 °C for 20 min.

Adherence to Epithelial Cells—Human colon carcinoma cells (Caco-2) (30, 31) were grown to confluency in Dulbecco’s modified Eagle’s medium containing 20% fetal calf serum, 0.45% glucose, 292 μg/ml glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 50 μg/ml streptomycin, and 50 μg/ml penicillin G (Life Technologies, Inc.) at 37 °C (5% CO2). Monolayers established in culture dishes (well diameter of 60 mm) were used 2–4 days after confluency for adhesion studies. Adhesion was determined according to Rotrosen et al. (32) and Pratti et al. (33). Briefly, monolayers were washed twice with 2 ml of phosphate-buffered saline, followed by the addition of approximately 200 C. albicans cells in 1 ml of phosphate-buffered saline. Cells were incubated at 37 °C for 45 min in an atmosphere containing 5% CO2. Following the incubation, monolayers were washed once with 5 ml of phosphate-buffered saline to remove nonadhering cells; the monolayer in each well was then covered by 2 ml of YPD agar (1% agar), and the supernatant was transferred to a new tube. Membranes were collected by centrifugation at 48,000 x g for 30 min at 4 °C and were then resuspended in 100 μl of 50 mM bicine, pH 7.7, 1 mM EDTA, 33% glycerol, and protease inhibitors. Microsomal membranes were stored in liquid nitrogen.

Animal Experiments—Virulence studies were performed as described, using 8-week-old, male CFW-1 mice (Halan-Winkelmann, Paderborn, Germany) (34). Because of the aggregation of Capmt1 mutants, all strains were preincubated for 5–10 min in a bath sonifier (Bandelin Sonorex TK52) before injection; as determined by plating efficiency before and after sonication, viability was not affected by this treatment. Survival curves were calculated according to the Kaplan-Meier method using the Prism (TM) program (GraphPad Software Inc., San Diego) and compared using the log-rank test. A p value <0.05 was considered significant. To quantify colony-forming C. albicans units in kidneys, mice were sacrificed 48 h after injection, and kidneys were homogenized in 5 ml of phosphate-buffered saline, serially diluted, and plated.

Other Procedures—S. cerevisiae PMT genes were used as heterologous probes in Southern blots with C. albicans genomic DNA. Chromosomal DNA of C. albicans CAF3–1 was cut with BamHI (lane 1), EcoRI (lane 2), HindIII (lane 3), and EcoRV (lane 4) and analyzed in a Southern blot using pCT5 (A) or PMT2 (B) of S. cerevisiae as probes. Plasmid pUC21/Pmt1 containing ScPMT1 (cut with EcoRV and XhoI; A, lane 5) and YEp352/Pmt2 containing ScPMT2 (cut with XhoI; B, lane 5) were used as controls. The migration of standard fragments is indicated.

Chitinase was assayed essentially as described by McCreath et al. (41) using 4-methylumbelliferyl-β-D-N-tetraacetylchitotrioside as substrate. 20 μl of medium or cells contained in 20 μl of culture were hydrolyzed at 37 °C for 60 min. The reaction was stopped by the addition of 1.9 ml of 0.5 M glycine/NaOH buffer (pH 10.4), and fluorescence was determined in a Kontron SFM25 fluorometer.

RESULTS

Sequence of CaPMT1 Each of the seven S. cerevisiae PMT genes was used to detect homologous genes in C. albicans genomic DNA by low stringency Southern hybridization. With genomic DNA cut by different restriction enzymes, only two patterns of hybridization were obtained. PMT1 and PMT5 probes yielded one pattern (Fig. 1A), while use of the PMT2, PMT3, and PMT6 genes resulted in an alternative pattern (Fig. 1B). Since only a single hybridizing band was obtained in each case, these results suggested that only two PMT genes, designated PMT1 and PMT2, exist in C. albicans, although the possibility of the presence of less homologous genes could not be excluded. Previously, we had pointed out the sequence-relatedness of the S. cerevisiae PMT1 and PMT5 genes as well as the PMT2, PMT3, and PMT6 genes (13).

Based on the Southern results, we constructed a partial C. albicans genomic bank and cloned CaPMT1 by hybridization to the S. cerevisiae PMT1 gene. A positive clone contained a 5.5-kb fragment, which was sequenced (GenBank™/EMBO accession number AF000232). The DNA sequence revealed an open reading frame with the potential to encode a protein of 877 amino acids (Fig. 2). Because of N- and C-terminal extensions, CaPmt1p is longer than any other Pmt proteins. The
deduced Pmt1 protein has 49% identical residues compared with Pmt1p of *S. cerevisiae* (17). Identities to the ScPmt2–7 proteins were lower, amounting to 28, 27, 25, 42, 23, and 19% identical residues, respectively. As determined by the TMpred program, CaPmt1p is a likely integral membrane protein containing 10 possible transmembrane regions. Asparagine residues at positions 83, 195, 395, 400, and 721 are potential sites for *N*-linked glycosylation.

**CaPMT1 Complements the *S. cerevisiae* pmt1 Mutation**—To test if CaPmt1p functions in *S. cerevisiae*, we constructed a low copy centromeric and a high copy *S. cerevisiae* vector containing CaPMT1 (plasmids pCT32 and pCT31, respectively). In addition, the CaPMT1 coding region was placed under transcriptional control of the GAL1 promoter in low and high copy expression vectors (pCH1 and pCH2, respectively). All plasmids were transformed into the *S. cerevisiae* pmt1 mutant B76Δpmt1, and transformants were grown in YPGal (to induce the GAL1 promoter). Chitinase in the culture medium was adsorbed to chitin, separated by SDS-PAGE, and silver-stained (42). As expected, chitinase of the B76Δpmt1 strain showed a faster electrophoretic mobility compared with the parental wild-type strain B76 (Fig. 3), which is due to a partial defect in O-glycosylation (17, 42). In transformants carrying either the *S. cerevisiae* or the *C. albicans* PMT1 genes on plasmids, the wild-type migration of chitinase was restored. A complementation of the pmt1 defect was obtained by CaPMT1 present on low or high copy vectors and either containing the authentic promoter or the strong *S. cerevisiae* GAL1 promoter. Thus, these results indicate that CaPMT1 is efficiently expressed in *S. cerevisiae* and that CaPmt1p is able to fully substitute the functions of ScPmt1p in *S. cerevisiae*.

**Disruption of CaPMT1 Alleles**—To assess the function of CaPMT1, we constructed strains lacking one or both PMT1 alleles in *C. albicans* strain CAI4. According to an established protocol (21), one allele in strain CAI4 was disrupted by a hisG-URA3-hisG cassette, generating strain CAP1–3 (Fig. 4, top). A Ura2 derivative, strain CAP1–31, with the genotype PMT1/pmt1Δ::hisG was identified, and its remaining wild-type allele was again deleted to construct strain CAP1–312 lacking *CaPMT1* wild-type alleles (pmt1Δ::hisG-URA3-hisG/pmt1Δ::hisG). Finally, a Ura− derivative of this strain, CAP-3121, was isolated (pmt1Δ::hisG-URA3-hisG/pmt1Δ::hisG).
**Protein O-Mannosylation in C. albicans**

The incorporation of \[^{14}C\]mannose from added Dol-P-[^14]C]mannose (approximately 30,000 cpm) into the pentapeptide Ac-YATAV-NH₂ (final concentration of 4 mM) was determined using 5, 10, and 20 μl of microsomal membranes (3 mg/ml protein). Background values in control assay without peptide have been subtracted. The mean values of at least three independent experiments (± S.D.) are shown. The activity of the wild-type strain SC5314 was assigned the value 100%.

| Genotype | Strain | \[^{14}C\]mannose transferred | Activity |
|----------|--------|------------------------------|----------|
| pmt1/PMT1 | SC5314 | 4005 ± 143 | 100 |
| pmt1/PMT1 | CAP1–3 | 2350 ± 60 | 58.7 |
| pmt1/PMT1 | CAP1–312 | 147 ± 76 | 3.67 |
| pmt1/pmt1 | CAP1–312 | 37 ± 25 | 0.9 |
| pmt1/pmt1 | CAP1–3121 (pCT30) | 2742 ± 159 | 68.5 |

**FIG. 3.** C. albicans PMT1 complements a S. cerevisiae pmt1 mutant. Chitinase secreted by different S. cerevisiae strains was purified by adsorption to chitin, separated by SDS-PAGE, and silver-stained. A wild type (B76; lane 1), a pmt1 derivative (B76Δpmt1; lane 2), and transformants of B76Δpmt1 (lanes 3–9) were compared. B76Δpmt1 carried pCT28 (S-PMT1; lane 3), Yeplac122 (control; lane 4), pCT32 (CaPMT1; lane 5), pCH1 (GAL1p::CaPMT1; lane 6), Yeplac112 (control; lane 7), pCT31 (CaPMT1; lane 8), and pCH2 (GAL1p::CaPMT1; lane 9). All cells were pregrown in YPGal media.

**FIG. 4.** Disruption of CaPMT1 alleles. Top, structure of different alleles. The wild-type PMT1 gene (gray arrow) and the alleles disrupted by the hisG-URA3-hisG cassette or by hisG alone are shown. H, HindIII; X, XbaI; V, EcoRV. Bottom, Southern blots were performed on genomic DNA of the following strains digested by HindIII–EcoRV (A) or HindIII (B): SC5314 (PMT1/PMT1; lanes 1 and 6), strain CAP1–3 (PMT1/pmt1Δ::hisG-URA3-hisG; lanes 2 and 7); strain CAP1–31 (PMT1/pmt1Δ::hisG; lanes 3 and 8); strain CAP1–312 (pmt1Δ::hisG-URA3-hisG/pmt1Δ::hisG; lanes 4 and 9), strain CAP1–3121 (pmt1Δ::hisG/pmt1Δ::hisG; lanes 5 and 10. In A, the fragment marked by the asterisks in the schematic diagram (top) was used as probe; in B, a hisG probe was used.

Δ::hisG/pmt1Δ::hisG. For each strain, the expected genetic configuration at the PMT1 locus was verified by Southern blotting (Fig. 4, bottom). In genomic DNA cut with HindIII and EcoRV (Fig. 4A, bottom) the wild-type allele yielded a 5.5-kb band (lane 1), and the disrupted allele resulted in a 3.4-kb band (lanes 2–5). To distinguish pmt1 alleles containing the full-length disruption cassette from alleles only containing hisG

**TABLE II**

| Genotype | Strain | \[^{14}C\]mannose transferred | Activity |
|----------|--------|------------------------------|----------|
| pmt1/PMT1 | SC5314 | 4005 ± 143 | 100 |
| pmt1/PMT1 | CAP1–3 | 2350 ± 60 | 58.7 |
| pmt1/PMT1 | CAP1–312 | 147 ± 76 | 3.67 |
| pmt1/pmt1 | CAP1–312 | 37 ± 25 | 0.9 |
| pmt1/pmt1 | CAP1–3121 (pCT30) | 2742 ± 159 | 68.5 |

Membrane proteins of the wild-type strain SC5314 showed high levels of mannosylation activity, while the activity in the homozygous pmt1 mutant (CAP1–312) was low in comparison, amounting to 1–4% (Table II). Reintroduction of CaPMT1 into the pmt1 background restored in vitro O-mannosylation activity. The pmt1/PMT1 heterozygous strain showed an activity intermediate between the activities in the pmt1 mutant and wild-type cells, indicating an effect of gene dosage on enzymatic activity. Thus, the results of these in vitro enzymatic tests and the in vivo complementation (see above) strongly suggest that CaPMT1 encodes a functional Pmt protein.

**CaPmt1p Affects Growth and Secreted Proteins—Strains carrying deleted PMT1 alleles were viable and did not show gross cytological defects. However, in contrast to the wild-type strain SC5314, the heterozygous strain CAP1–3, and the retransformed mutant CAP1–3121(pCT30), cells of the homozygous mutant CAP1–312, lacking both functional PMT1 alleles, formed aggregates (Fig. 5), which at culture densities A₆₀₀ < 1 could be separated into single cells by mild ultrasonic treatment using a bath sonifier. Once disaggregated, however, pmt1 cells remained separate and did not reaggregate, suggesting that aggregates of pmt1 strains arise because of a defect in cell separation after cell division rather than by autoaggregation of individual cells. Growth of the homozygous disruptive strain CAP1–312 was reduced compared with the other strains. For strains SC5314, CAP1–3, and CAP1–312, doubling times in SD or YPA medium were 100 or 70 min, 100 or 70 min, and 150 or 90 min, respectively. This phenotype differs from pmt1 mutants of S. cerevisiae, which are not compromised for growth and do not form aggregates in several genetic backgrounds (e.g. B76Δpmt1). Simultaneous deletion of both PMT1 and PMT2 are required to generate a comparable aggregation phenotype in S. cerevisiae (16, 19) as in the C. albicans pmt1 strains.

The Als1 protein of C. albicans has high homology to α-agglutinin of S. cerevisiae and is predicted to be O- and N-glyco-
with the assumption that Als1p is a partially O-band (the homozygous pmt1
masses between 130 and 230 kDa; none of these proteins mi-
clonal antibody 1B12 showed five proteins with molecular
ognize major cell wall proteins (36). In our immunoblots, mono-
shown). The monoclonal antibody 1B12 was described to rec-
surface components are modified by CaPmt1p (data not
led to prolonged growth on "spider" plates (Fig. 7, B and D). In
omogeneous in cell wall components may impair retention of chitinase in the cell wall. C. albicans chitinase
cannot be adsorbed to chitin (41), and specific antibodies
availability, which at present does not allow a more
detailed analysis of this phenotype.
We used immunoblots to determine if other described cell
components are modified by CaPmt1p (data not shown). The monoclonal antibody 1B12 was described to rec-
ize major cell wall proteins (36). In our immunoblots, mono-
clonal antibody 1B12 showed five proteins with molecular
masses between 130 and 230 kDa; none of these proteins mi-
generated differently in the Capmt1 mutant. An antibody against
the S. cerevisiae gp115 cell wall protein, which also reacts with

![Figure 5. Microscopic appearances of C. albicans strains. Strains were grown in SD medium to the logarithmic phase and visualized by microscopy using a Zeiss Axioscop with Nomarski optics. A, SC5314 (PMT1/FMT1); B, CAP1–3 (PMT1/pmt1); C, CAP1–312 (pmt1/pmt1).](Image)

| Genotype | Strain                  | Medium Cells |
|----------|-------------------------|--------------|
| PMT1/PMT1| SC5314                  | 0.32 ± 0.04  |
| pmt1/PMT1| CAP1–3                  | 0.43 ± 0.01  |
| pmt1/PMT1| CAP1–5                  | 0.37 ± 0.07  |
| pmt1/pmt1| CAP1–312                | 0.72 ± 0.07  |
| pmt1/pmt1| CAP1–313                | 0.73 ± 0.02  |
| pmt1/pmt1| CAP1–312 (pCT30)        | 0.47 ± 0.01  |
| pmt1/pmt1| CAP1–312 (pCT29)        | 0.44 ± 0.03  |

Chitinase activity associated with whole cells contained in 20 μl of culture fluid (A_w = 5) or in 20 μl of the culture medium was deter-
Activity is expressed as nmol of 4-methylumbelliferyl released from 4-methylumbelliferyl chitotetraoside. Values represent the mean of two independent measurements (±S.D.).

- **Table III**
  - **Chitinase enzymatic activity**
  
  | Genotype | Strain                  | Chitinase activity |
  |----------|-------------------------|--------------------|
  | PMT1/PMT1| SC5314                  | 0.32 ± 0.04        |
  | pmt1/PMT1| CAP1–3                  | 0.43 ± 0.01        |
  | pmt1/PMT1| CAP1–5                  | 0.37 ± 0.07        |
  | pmt1/pmt1| CAP1–312                | 0.72 ± 0.07        |
  | pmt1/pmt1| CAP1–313                | 0.73 ± 0.02        |
  | pmt1/pmt1| CAP1–312 (pCT30)        | 0.47 ± 0.01        |
  | pmt1/pmt1| CAP1–312 (pCT29)        | 0.44 ± 0.03        |

**Fig. 6. The Als1 cell wall protein is affected by a pmt1 mutation.** Strains were grown in SD medium to an A_w = 3–5, and proteins in cell extracts were separated by SDS-PAGE (5%). Blots were developed with anti-Als1 antibody (1:10,000), followed by anti-rabbit IgG
grouped to horseradish peroxidase. Strains tested were SC5314 (wild-
type, lane 1), CAP1–3 (PMT1/pmt1; lane 2), CAP1–312 and CAP1–313 (pmt1/pmt1; lanes 3 and 4), and CAP1–3121(pCT30) (pmt1/
pmt1/PMT1; lane 5). The positions of the glycosylated and undergly-
cosylated Als1 proteins are indicated by the filled and open triangles, respectively.

- **Table III**
  - **Chitinase enzymatic activity**
  
  | Genotype | Strain                  | Chitinase activity |
  |----------|-------------------------|--------------------|
  | PMT1/PMT1| SC5314                  | 0.32 ± 0.04        |
  | pmt1/PMT1| CAP1–3                  | 0.43 ± 0.01        |
  | pmt1/PMT1| CAP1–5                  | 0.37 ± 0.07        |
  | pmt1/pmt1| CAP1–312                | 0.72 ± 0.07        |
  | pmt1/pmt1| CAP1–313                | 0.73 ± 0.02        |
  | pmt1/pmt1| CAP1–312 (pCT30)        | 0.47 ± 0.01        |
  | pmt1/pmt1| CAP1–312 (pCT29)        | 0.44 ± 0.03        |
Hyphal formation induced in YP medium at 37 °C proceeded similarly in strain CAP1–312 as in the wild-type strain SC5314. These results suggest that the molecular machinery of hyphal formation is not affected in pmt1 mutants but that a component of a signaling pathway that is operative on spider medium is defective. Because a similar phenotype (no hyphal formation on spider medium, but normal hyphal formation on serum) was obtained for strains defective in components of a conserved mitogen-activated protein kinase pathway (34, 48), we assume that this pathway is defective in pmt1 mutants.

Because of these results, we examined if the PMT1 gene in S. cerevisiae is also necessary for pseudohyphal growth of this organism. To test this possibility, we disrupted the SpmT1 gene in a haploid strain (B76), which was subsequently diploidized by transformation with a plasmid containing the HO gene (27). After the loss of the HO plasmid, we had generated strain B76dΔpmt1 (pmt1Δ::URA3/pmt1Δ::URA3). This strain and strain B76/4 as a control (24) were each transformed with plasmid YCplac22RAS2Val19 containing the dominant RAS2Val19 allele (49), and transformants were tested on low nitrogen media as described by Gimeno et al. (28). All tested transformants of strains B76/4 and B76dΔpmt1 had an identical phenotype, in that vigorous pseudohyphal growth was observed.

Thus, the ability to undergo filamentous morphogenesis is significantly affected by deletion of the Pmt1p-encoding gene in C. albicans, while lack of Pmt1p in S. cerevisiae has no effect.

**FIG. 7.** Hyphal morphogenesis of C. albicans strains. Shown are sections of colonies grown for 2 days on spider medium at 37 °C. Wild-type strain SC5314 (A), the pmt1 strain CAP1–312 (B), the heterozygous strain CAP1–3 (C), and the transformant CAP1–3121(pCT30) (D) were tested.

**FIG. 8.** Increased sensitivities of pmt1 mutants to antifungal agents. Strains were grown on YPD-media at 30 °C containing substances at the indicated concentrations; hygromycin B was added at 100 μg/ml, and G418 was added at 1.2 mg/ml. Sensitivity to calcofluor white was tested by spotting decreasing cell counts (from left to right: 50,000, 5,000, and 500 cells) on YPD plates containing 0 or 10 μg/ml calcofluor white.

**Pmt1 Mutants Are Supersensitive to Some Antifungals—** Multiple pmt mutations are known to cause increased antifungal sensitivities in S. cerevisiae (19). Therefore, we tested susceptibility to several antifungal compounds of the C. albicans wild-type and the constructed heterozygous and homozygous pmt1 mutant strains (Fig. 8).

Hygromycin B at 100 μg/ml completely blocked growth of the homozygous CAP1–312 mutant on solid media, while the heterozygous strain CAP1–3 (PMT1/pmt1) was affected partially at this concentration, leading to smaller colonies. An effect of heterozygosity was confirmed in liquid YPD at 150 μg/ml hygromycin B, where the wild-type strain (SC5314), the heterozygous strain (CAP1–3), and the homozygous strain (CAP1–312) had doubling times of 130, 150, and >360 min, respectively. Another aminoglycoside antibiotic, G418, mainly prevented growth of the homozygous strain at 1.2 mg/ml G418, while the heterozygous strain was not affected significantly. Similarly, clotrimazole at 2 μg/ml was found to significantly block growth of the homozygous disruptant strain while affecting the heterozygous and the wild-type strain less strongly. S. cerevisiae strains defective in cell wall biosynthesis are known to be supersensitive to calcofluor (51). Disruption of both PMT1 alleles was found to cause reduced plating efficiency on media containing calcofluor white (Fig. 8) and led to an inability to grow on YPD media containing low levels (0.06%) of the deter-
gent SDS; in contrast, the heterozygous strain and the wild-type strain were not supersensitive (data not shown). No differences in susceptibilities of all strains were observed for nystatine (1–15 \( \mu \)g/ml), for amphotericin B (0.5–2 \( \mu \)g/ml), for a combination of methotrexate and sulfanilamide (0–150 \( \mu \)g/ml and 5 mg/ml, respectively (52)), for fluconazole (5 \( \mu \)g/ml), for fluphenazine (50 \( \mu \)g/ml), and for caffeine at up to 20 mM (19).

Thus, deletion of \( PMT1 \) causes a significant increase in the susceptibility of \( C. albicans \) to various antifungal compounds and compounds perturbing the cell wall. Supersensitivity for hygromycin B is observed even with the heterozygous \( CaPMT1/CaPmpt1 \)-strain, indicating a gene dosage effect. These results contrast with the findings in \( S. cerevisiae \), where deletions of several \( PMT \) genes are required to alter antifungal susceptibilities.

**Capmpt1p Is Required for Adherence to Epithelial Cells**—Conceivably, membrane and cell wall proteins that are O-glycosylated by CaPmt1p could be essential for \( C. albicans \) virulence, by mediating adherence to and migration across epithelial cell layers. To test this possibility, we assayed adherence of \( C. albicans \) strains to monolayers of Caco-2 epithelial cells (30, 31) (Table IV). In this assay, fungal cells, which had been briefly dispersed by a mild ultrasonification, were placed on the epithelial monolayer for 45 min, after which nonadhering cells were removed by washing; numbers of tightly adhering cells were determined by growth in a YPD agar overlay.

The results demonstrate that the homozygous \( pmt1 \) mutant is significantly impaired in adhesion to the epithelial cells, while the heterozygous strain is not affected. Reintroduction of \( CaPMT1 \) into the \( pmt1 \) mutant background restores adhesion. Thus, a lack of Pmt1 activity has an important effect on a trait considered essential for virulence of \( C. albicans \).

**Both \( CaPMT1 \) Alleles Are Required for Virulence of \( C. albicans \)**—To determine directly, if \( CaPMT1 \) is involved in pathogenicity of \( C. albicans \), we infected mice (\( n = 15 \)) intravenously with wild-type and mutant strains, and the survival of animals was tested. Because of the aggregation behavior of \( C. albicans pmt1 \) mutants, strains were briefly sonicated prior to injection to ensure that animals were infected with equal cell numbers. Determination of plating efficiencies before and after sonification indicated that this treatment did not affect cellular viability.

Infection with the wild-type strain SC5314 resulted in rapid death (mean survival of 4 days), while deletion of both \( PMT1 \) copies (strain \( CAP1–3 pmt1/pmt1 \)) led to complete loss of virulence (Fig. 9). These characteristics correlated with kidney colonization by \( C. albicans \) strains, which amounted to 5.0 \( \times \) 10\(^6\) and 0 colony-forming units/kidney in the wild type and \( pmt1 \) mutant, respectively. Interestingly, although the heterozygous \( CAP1–3 \) strain almost showed wild-type kidney colonization (9.5 \( \times \) 10\(^6\) colony-forming units), its survival was increased significantly (mean survival of 7 days; \( p < 0.0001 \)). Thus, deletion of \( PMT1 \) alleles, in a dosage-dependent manner, decreases the virulence of \( C. albicans \). The lack of kidney colonization suggests that strains lacking both \( PMT1 \) alleles are defective in systemic spread or that they are quickly eliminated by defense mechanisms, e.g. by phagocytosis.

**DISCUSSION**

The \( PMT1 \) gene of \( C. albicans \) is the first described \( PMT \) gene in a species other than \( S. cerevisiae \) whose function has been characterized. Other \( ScPTM \) homologues of unknown function are known in fungi. Recently, a genomic sequence of \( S. pombe \) has been reported, which encodes a putative Pmt protein (GenBank \( ^{TM} \) accession number Z99126). Also, a \( PMT1 \) homologue appears to exist in the yeast \( K. lactis \). Because we also obtained evidence for a homologue of the \( SpMT2 \) gene in \( C. albicans \), it appears that protein O-mannosylation by Pmt-type proteins is a reaction occurring generally in other fungi; this process may require two or more Pmt proteins. The sequence of the \( C. albicans PMT1 \) gene predicts a protein with a hydrophilic center and membrane-spanning termini, in agreement with the structures of the \( S. cerevisiae \) Pmt1 protein (17). Because we were unable to detect \( C. albicans \) homologues of the \( S. cerevisiae PMT3–7 \) genes, it is possible that \( C. albicans \) only contains two \( PMT \) genes. Conceivably, the two alleles of \( PMT1 \) and \( PMT2 \) provide sufficient genetic redundancy in the diploid yeast \( C. albicans \), a function that is secured by multiple \( PMT \) loci in \( S. cerevisiae \), which has a haploid or a diploid genome. The existence of only two \( PMT \) genes in \( C. albicans \) could also explain the drastic phenotypes of \( PMT1 \) deletion in \( C. albicans \), as discussed below. Although individual deletions of the \( S. cerevisiae PMT1 \) and \( PMT2 \) genes do not cause significant growth phenotypes, their simultaneous mutation significantly retards growth and causes cells to aggregate strongly (16). This phenotype is obtained in \( C. albicans \) by merely deleting both \( PMT1 \) alleles. Similarly, while \( S. cerevisiae pmt1 \) mutants do not show increased sensitivity toward hygromycin B, \( C. albicans pmt1 \) mutants are supersensitive, as is observed for \( S. cerevisiae pmt1 pmt2 \) double mutants.\(^6\) Unlike defects in outer chain addition to O-glycosyl chains in \( S. cerevisiae \), which also lead to increased hygromycin B sensitivity (53), the \( pmt \) mutations did not cause resistance to vanadate. Surprisingly, deletion of a single \( PMT1 \) allele in \( C. albicans \) sufficed to increase hygromycin B sensitivity, providing evidence that the dosage of \( PMT1 \) expression determines the wild-type phenotype. Haplo-insufficiency has been observed for other genes in \( C. albicans \) (48).\(^7\) Supersensi-

\(^5\) C. C. Marcireau, personal communication.

\(^6\) S. Wickert and J. F. Ernst, unpublished results.

\(^7\) F. Rademacher and J. F. Ernst, unpublished results.

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**TABLE IV**

Adhesion of \( C. albicans \) strains to Caco-2 epithelial cells

| Strain (genotype) | Adhesion (%) |
|------------------|-------------|
| SC5314 (\( PMT1/PMT1 \)) | 42 ± 5.9 |
| \( CAP1–3 (PMT1/pmt1) \) | 37 ± 5.8 |
| \( CAP1–31 (pmt1/pmt1) \) | 19 ± 2.8 |
| \( CAP1–313 (pmt1/pmt1) \) | 26 ± 3.1 |
| \( CAP1–3121 (pCT30 pmt1/pmt1 PMT1) \) | 42 ± 1.7 |
Activity for calcofluor white is another example of the strong consequence of \textit{PMT1} deletion in \textit{C. albicans}, which compares to the phenotype of \textit{S. cerevisiae pmt1 pmt2} double mutants (19). The calcofluor white phenotype suggests that O-glycosylation of unknown proteins by Pmt1p stabilizes the \textit{C. albicans} cell wall. In our study, we identified the Als1 protein (44), which recently has been recognized as a potential adhesion factor for \textit{C. albicans} (45), as the first likely O-glycosylated protein in the cell wall of \textit{C. albicans}. Recent results suggest that \textit{PMT1} activity is required to incorporate several proteins into the cell wall of \textit{S. cerevisiae} (54). O-Glycosylated proteins may also be involved in an unknown system of resistance to various agents, such as aminoglycoside antibiotics, \textit{e.g.} by promoting their export (39). However, a defect in Pmt1 function does not cause a general or an equal increase in the susceptibility toward damaging agents, since the sensitivity toward clotrimazole is increased only slightly and is absent for nystatin, amphotericin B, and methotrexate/sulfanilamide. Despite the multiple drastic phenotypes of the \textit{pmt1} mutation in \textit{C. albicans}, a significant effect on osmotic sensitivity, as has been observed for multiple \textit{pmt} mutations in \textit{S. cerevisiae} (19), was not detected. Thus, although \textit{Pmt1} fulfills similar basic functions in both fungal species, the resulting phenotypes differ significantly.

Depending on environmental conditions, \textit{C. albicans} and \textit{S. cerevisiae} are able to either grow as a budding yeast or in a multicellular filamentous form (reviewed in Ref. 2). Dimorphism of \textit{C. albicans} is considered an important virulence trait. Hyphal formation of this pathogen can be induced either on certain depauperated solid media (spider media), a process requiring components of a conserved mitogen-activated protein kinase cascade (46); alternatively, positive stimuli, such as serum, are able to induce morphogenesis. Deletion of \textit{PMT1} completely abolished the ability of \textit{C. albicans} to form hyphae on spider media. Surprisingly, even the heterozygous \textit{pmt1}/\textit{PMT1} strain had a severe defect in morphogenesis, confirming the gene dosage effect observed for hygromycin B sensitivity and virulence in the animal model. Because serum still stimulated hyphae formation, we assume that not morphogenesis per se but rather sufficient levels of a component required for a signaling component operative on spider media requires O-glycosylation by Pmt1 for its function. A similar phenotype (block of hyphae formation on spider medium and normal hyphae formation on serum) has been reported for mutants defective in elements of a conserved mitogen-activated protein kinase pathway (34, 48). Therefore, it is possible that an unidentified O-glycosylated protein of this pathway, \textit{e.g.} a membrane protein involved in signal transduction, is defective in \textit{pmt1} mutants. In contrast to the results obtained in \textit{C. albicans}, \textit{pmt1} deletion in \textit{S. cerevisiae} did not affect its ability to form pseudohyphae on low nitrogen media. Thus, even with regard to morphogenesis, \textit{PMT1} has a more important function in \textit{C. albicans} compared with \textit{S. cerevisiae}.

Despite the relatively minor growth defect in most media, \textit{pmt1} mutants were completely avirulent in the mouse model of systemic infection. Even the \textit{pmt1}/\textit{PMT1} heterozygous strain showed a significant decline in virulence, although this strain grew identically to the wild-type strain and could be distinguished only by increased sensitivity to certain agents and by reduced morphogenesis (see above). Thus, even a 2-fold drop in Pmt1 activity (assuming equal contribution of each allele to overall \textit{PMT1} expression) interferes with pathogenicity. It remains to be determined experimentally which of the described various phenotypes of \textit{pmt1} mutants is responsible for attenuation of virulence. Conceivably, the alteration of cell surface properties and the morphogenesis defect of \textit{pmt1} strains cause defects in adhesion and penetration of epithelial and endothelial cell layers. We obtained experimental support for this possibility by demonstrating that \textit{pmt1} mutants adhere less strongly than wild-type cells to a Caco-2 epithelial cell layer. Thus, it appears that the ability to penetrate endothelial and epithelial layers, which is the precondition to infect organs (1), does not occur with \textit{pmt1} mutants. In agreement with this hypothesis, kidneys of infected animals were free of \textit{C. albicans pmt1} cells. Recently, the Als1 protein has been described as a likely adhesion factor for \textit{C. albicans} (45). It appears possible that reduced O-glycosylation of this protein in \textit{pmt1} mutants, which is suggested here, reduces the adhesive properties of Als1p and thus affects virulence. Alternatively, the aggregation phenotype of \textit{pmt1} mutants could contribute to lower fungal dissemination in the infected animal. A third possibility is an increased host activity, \textit{e.g.} phagocytosis, against \textit{pmt1} strains.

In conclusion, the characterization of the \textit{C. albicans PMT1} gene not only demonstrates that Pmt proteins exist and function in O-glycosylation in fungal species other than \textit{S. cerevisiae}; the results also show that CaPmt1p assumes novel functions that are not observed (in hyphal morphogenesis and antifungal resistance) or are not considered relevant (in adherence and virulence) in \textit{S. cerevisiae}. O-Glycosylation reactions mediated by Pmt proteins appear to be possible targets for antifungal agents for the following reasons. (i) The bulk of O-glycosylation occurs by different mechanisms in mammalian and fungal cells. (ii) Even a 2-fold reduction in \textit{PMT1} gene dosage causes defects in morphogenesis, as well as a significant drop in virulence. (iii) Reduced Pmt1p activity leads to an increased sensitivity to antifungal agents, thereby potentially amplifying any response to antifungals and offering the prospect for a combination therapy. (iv) Cells lacking Pmt1p activity show reduced adherence to host cells and reduced colonization of organs; this characteristic is in contrast to other avirulent \textit{C. albicans} strains, which still show kidney colonization (55, 56). On the other hand, rare O-mannosylation of some human proteins has been reported (11, 12), and expressed sequence tags deposited at GenBank\textsuperscript{TM} contain sequences encoding possible Pmt homologs derived from human (accession numbers AA670164, AA425494, AA496399, AA099791, N42494, and N35574), mouse (accession numbers AA274738, AA277592, AA274738, and Z31210), rat (accession number H33465), \textit{C. elegans} (accession numbers C41029, C47075, C42623, C66447, and C69714), and rice (accession number C73075) cells. It is possible (although this remains to be established) that the corresponding genes perform essential functions in the respective cell types. In \textit{D. melanogaster}, a gene encoding a Pmt homologue appears to be required for muscle development (20).

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