**Candida albicans** Pmr1p, a Secretory Pathway P-type Ca\(^{2+}\)/Mn\(^{2+}\)-ATPase, Is Required for Glycosylation and Virulence*

Received for publication, February 25, 2005, and in revised form, April 20, 2005
Published, JBC Papers in Press, April 20, 2005, DOI 10.1074/jbc.M502162200

Steven Bates, Donna M. MacCallum, Gwyneth Bertram, Carol A. Munro, H. Bleddyn Hughes, Ed T. Buurman‡, Alistair J. P. Brown, Frank C. Odds, and Neil A. R. Gow§

From the School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland, United Kingdom

The cell surface of **Candida albicans** is the immediate point of contact with the host. The outer layer of the cell wall is enriched in highly glycosylated mannoproteins that are implicated in many aspects of the host-fungus interaction. Glycosylation of cell wall proteins is initiated in the endoplasmic reticulum and then elaborated in the Golgi as the protein passes through the secretory pathway. Golgi-bound mannosyltransferases require Mn\(^{2+}\) as an essential cofactor. In **Saccharomyces cerevisiae**, the P-type ATPase Pmr1p transports Ca\(^{2+}\) and Mn\(^{2+}\) ions into the Golgi. To determine the effect of a gross defect in glycosylation on host-fungus interactions of **C. albicans**, we disrupted the **PMR1** homolog, Capmr1. This mutation would simultaneously inhibit many Golgi-located, Mn\(^{2+}\)-dependent mannosyltransferases. The Capmr1Δ null mutant was viable *in vitro* and had no growth defect even on media containing low Ca\(^{2+}\)/Mn\(^{2+}\) ion concentrations. However, cells grown in these media progressively lost viability upon entering stationary phase. Phosphomannan was almost completely absent, and O-mannan was severely truncated in the null mutant. A defect in N-linked outer chain glycosylation was also apparent, demonstrated by the underglycosylation of surface acid phosphatase. Consistent with the glycosylation defect, the null mutant had a weakened cell wall, exemplified by hypersensitivity to Calcofluor white, Congo red, and hygromycin B and constitutive activation of the cell integrity pathway. In a murine model of systemic infection, the null mutant was severely attenuated in virulence. These results demonstrate the importance of glycosylation for cell wall structure and virulence of **C. albicans**.

*Candida albicans* is the most common fungal agent of invasive disease in humans (1, 2). It is responsible for superficial epithelial infections and, in the immunocompromised host, life-threatening systemic infections (3, 4). The cell wall of **C. albicans** is the immediate point of contact between the fungus and host and hence is important in host-fungus interactions. The cell wall is composed of an inner layer of structural polysaccharides, β1,3- and β1,6-glucans and chitin, and an outer layer that is enriched for mannoproteins (5, 6). The highly glycosylated mannoproteins play important roles in adhesion, antigenicity, and modulation of the host immune responses (7–11). Both the carbohydrate epitopes and the protein components have been implicated in these roles (7, 8, 12), although the exact epitopes involved are still unclear. The study of glycosylation in **C. albicans** will therefore increase our understanding of the host-fungus interaction. To determine the role of glycosylation in the virulence of this fungus, we deleted the Golgi P-type ATPase, which transports divalent cations into the Golgi, where they act as essential cofactors for mannosyltransferases.

In **Saccharomyces cerevisiae**, glycosylation is initiated in the endoplasmic reticulum by the transfer of the first mannose residue to serine or threonine in O-linked glycosylation (13) and by the transfer of the N-linked core structure to asparagine residues (14). The construction and transfer of the N-linked core (14, 15) and the initiation of O-linked glycosylation (16) are essential processes. Glycosylation is continued in the Golgi with the extension of the linear O-linked glycans (17) and the extensive elaboration of the branched outer chains of the N-linked glycans (18).

The process of glycosylation has been extensively studied in **S. cerevisiae** (13–15, 17), and this has provided a resource for understanding glycosylation in **C. albicans**. However, key differences exist between the O- and N-glycan structures present in **S. cerevisiae** and **C. albicans**. For example, in **C. albicans**, the terminal O-linked glycans that are attached by α1,2-linkages, as opposed to α1,3-linkages in **S. cerevisiae** (19), and β1,2-linked mannose residues are present in both the acid-labile and acid-stable N-linked glycans (20). Following the completion of the **C. albicans** genome project, it has also become clear that there has been expansion and contraction of the number of genes in different mannosyltransferase gene families of **C. albicans**. Hence, the process of glycosylation has adapted in **C. albicans**, perhaps as a result of selective pressure from its interaction with a mammalian host.

To date, few genes involved in glycosylation in **C. albicans** have been analyzed in detail. Those studied include members of the *PMT* and *MNT* gene families, all of which act in O-glycosylation, and the studies have demonstrated the importance of O-glycans in virulence (19, 21–24). Also, Mnn9p is involved in extension of the N-linked glycan outer chain and hence is required for normal cell wall composition (25). Phosphomannan has been implicated in the interaction of **C. albicans** with macrophages. However, deletion of the **C. albicans** **MNN4** gene, which is required for mannosyl phosphate transfer, demonstrated that phosphomannan is not required for macrophage interactions or virulence of **C. albicans** (26).

---

* This work was supported by Wellcome Trust Grants 063204 and 72263. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: AstraZeneca R&D Boston, Waltham, MA 02451.
§ To whom correspondence should be addressed. Tel.: 44-1224-555-879; Fax: 44-1224-555-844; E-mail: n.gov@ubdn.ac.uk.

1 S. Bates, C. A. Munro, and N. A. R. Gow, unpublished data.
CaVRG4\textsuperscript{2} and CaSRB1, which encode proteins required for supplying the Golgi with GDP-mannose, the mannose donor, are essential in \textit{C. albicans}, indicating the overall importance of glycosylation to cell viability (27–30). However, to date, no single glycosylation event in the Golgi has been shown to be essential.

As well as requiring GDP-mannose as the mannose donor, Golgi-bound mannosyltransferases require manganous ions as essential cofactors. In \textit{S. cerevisiae}, the P-type ATPase Pmr1p is a Ca\textsuperscript{2+}/Mn\textsuperscript{2+} ion pump that supplies the Golgi with these ions (31–33). Homologs of Pmr1p are classed as secretory pathway Ca\textsuperscript{2+}-ATPases and are distinct from the sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPases and plasma membrane Ca\textsuperscript{2+}-ATPases (34). The secretory pathway Ca\textsuperscript{2+}-ATPase class of P-type ATPases has been identified in a wide range of fungal and animal cells, including \textit{S. cerevisiae}. Disruption of \textit{PMR1} in \textit{S. cerevisiae} results in a range of phenotypes, including some protein-sorting defects such as elevated secretion of heterologously expressed proteins (35, 36) and incomplete processing of gously expressed proteins (35, 36) and incomplete processing of

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|l|}
\hline
\textbf{Strain} & \textbf{Parent strain} & \textbf{Genotype} & \textbf{Source} \\
\hline
CAI-4 & & ura3\textsuperscript{Δ}/mmi344/ura3\textsuperscript{Δ}/mmi344 & Ref. 39 \\
NGY152 & CAI-4 & As CAI-4 but RPS1/psl1\textsuperscript{Δ}/Clp10 & Ref. 40 \\
NGY95 & CAI-4 & As CAI-4 but PMR1/psm1\textsuperscript{Δ}/:hisG-URA3-hisG & This study \\
NGY96 & NGY95 & As CAI-4 but PMR1/psm1\textsuperscript{Δ}/:hisG & This study \\
NGY97 & NGY96 & As CAI-4 but pnm1\textsuperscript{Δ}::hisG/pspm1\textsuperscript{Δ}/:hisG-URA3-hisG & This study \\
NGY98 & NGY97 & As CAI-4 but pnm1\textsuperscript{Δ}/:hisG & This study \\
NGY355 & NGY98 & As CAI-4 but pnm1\textsuperscript{Δ}::hisG/pspm1\textsuperscript{Δ}/:hisG, RPS1/psl1\textsuperscript{Δ}/Clp10 & This study \\
NGY356 & NGY98 & As CAI-4 but pnm1\textsuperscript{Δ}/:hisG, RPS1/psl1\textsuperscript{Δ}/Clp10, PMR1/PMR1 & This study \\
\hline
\end{tabular}
\caption{Strains used in this study}
\end{table}

\textsuperscript{2} The abbreviations used are: \textit{Ca}, \textit{C. albicans}; SD, synthetic dextrose; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MAPK, mitogen-activated protein kinase; BEC, buccal epithelial cell(s); cfu, colony-forming units.

testing, strains were grown in NGY medium (0.1% neopeptone, 0.4% glucose, and 0.1% yeast extract) at 30 °C.

\textbf{Construction of \textit{Capmr1}\textsuperscript{Δ} Null Mutant and Re-integrant Strains—} The \textit{Capmr1}\textsuperscript{Δ} gene was disrupted by the standard “ura-blaster” protocol (39). To make the disruption cassette, the 5' and 3'-flanking regions of the gene were amplified by PCR (5'-primer pair 5'-GAAAGACGTACGG-GCAATGTAAATGTTGCG-3' and 5'-GAAGATCTGTTGATGCGTA- AGTGTTACG-3', with the SacI and BglII restriction sites underlined, respectively; and 3'-primer pair 5'-GAAAGACGTACCG-TAGGTGCAGTCA- AGTGTTACG-3' and 5'-GAAGATCTGTTGATGCGTA- AGTGTTACG-3', with the SacI and SpH1 restriction sites underlined, respectively) and cloned into the complementary restriction sites of \textit{pmB-7} (39). The disruption cassette was released by digestion with SacI and SpH1 and contained the ura-blaster cassette flanked by complementary sequences 733 bp upstream and 620 bp downstream of \textit{Capmr1}\textsuperscript{Δ}. \textit{Capmr1}\textsuperscript{Δ} was disrupted by sequential rounds of transformation of strain CAI-4 and the recovery of the \textit{URA3} marker by selection on SD medium plus 5-fluoroorotic acid (1 mg/ml) and uridine (50 μg/ml). To avoid potential problems associated with the ectopic expression of \textit{URA3} (40), the \textit{Ura-}

\textit{Capmr1}\textsuperscript{Δ} null strain was transformed with StuI-digested Clp10 plasmid (41) so that \textit{URAS} was expressed at the \textit{RPS1} locus (orf19.3002, formally referred to as \textit{RPS10}). To create a re-integrant strain to act as a control, the \textit{Capmr1}\textsuperscript{Δ} open reading frame plus 1055 bp of its promoter and 3' end of its terminator were amplified by PCR (primer pair 5'-CAAGCTTGGGTATACTGATGCG-3' and 5'-ATGAAGCAAGTAT- CATTGAGGC-3'), and the 3.6-kb product was cloned into \textit{pGEM-T-Easy} (Promega Ltd., Southampton, UK). The insert was released by NotI digestion and subcloned into the \textit{NotI} site of Clp10. The resulting plasmid was digested with Stul and transformed into the \textit{Ura-}

\textit{Capmr1}\textsuperscript{Δ} null strain. As a positive control for experiments, strain CAI-4 was also transformed with Stul-digested Clp10; hence, all strains analyzed in this work have \textit{URA3} expressed at the \textit{RPS1} locus. The two \textit{RPS1} alleles in strain CAI-4 can be distinguished by the presence of a polymorphism resulting in the presence or absence of an XbaI restriction site downstream of \textit{RPS1}. As an added control, all strains used were confirmed to have Clp10 inserted into the non-XbaI-containing \textit{RPS1} allele.

\textbf{Sensitivity Testing—} To test strains for sensitivity to specific wall-stressing agents, strains were initially grown for 24 h in YEPD medium and then washed with water and resuspended at \textit{A}\textsubscript{560} = 1. These cells were inoculated into YEPD medium at \textit{A}\textsubscript{560} = 0.01, and 95-μl volumes were pipetted into microdilution plate wells. Test agents in 5-μl volumes were added at a range of doubling dilutions. Plates were incubated for 16 h at 30 °C, and absorbance was read at 600 nm. All strains were tested in duplicate. The agents tested were Calcofluor white (100 μg/ml), Congo red (100 μg/ml), SDS (0.1%), hygromycin B (500 μg/ml), a high salt concentration (NaCl\textsubscript{3}, KCl, CaCl\textsubscript{2}, MnCl\textsubscript{2}, or MgCl\textsubscript{2} each at 1 μl), caffeine (50 μM), vanadate (80 μM), and tunicamycin (100 μg/ml). The concentrations listed are the maximal concentration tested for each agent. Antifungal susceptibility testing was carried out by standard methods (42), except that agents were diluted beyond the stated ranges because derivatives of the \textit{C. albicans} SC5314 genetic background are known to be highly susceptible to antifungal agents.

\textbf{Protein Extracts and Western Blotting—} To test for activation of the cell integrity pathway (43), cells were grown in YEPD medium at 30 °C and collected in mid-exponential growth. For positive controls, the strains were stressed by the addition of 100 μg/ml Calcofluor white 2 h before collection. Cells were washed and resuspended in extraction buffer (100 mM Tris-HCl (pH 7.5), 0.1% (w/v) SDS, 1 mM dithiothreitol, 10% (w/v) glycerol, protease inhibitor mixture (Roche Applied Science, Lewes, UK) and then disrupted with glass beads in a FastPrep machine (Qiogene, Inc., Cambridge, UK). The lysate was clarified by centrifugation at 21,500 \( \times \) g for 10 min. Protein extracts were quantified using the Coomassie protein assay reagent (Pierce, Cramlington,
UK. Prior to Western blotting, 50 μg of protein was separated on a 4–12% NuPAGE bis-Tris gel (Invitrogen) before blotting onto a polyvinylidene difluoride membrane. The membrane was blocked in phosphate-buffered saline plus 0.1% Tween 20 and 5% skim milk in phosphate-buffered saline albumin for 2 h at room temperature. Detection was then carried out with the PhosphoPlus p44/42 MAPK antibody kit (New England Biolabs Inc., Hertfordshire, UK) according to the manufacturer’s instructions. This antibody cross-reacts with C. albicans Mkc1p (Sil2p) in its phosphorylated form.

Cell Wall Analysis—Alcian blue binding assays were carried out as described previously (26). Briefly, 1 x 10^7 stationary phase cells were washed with water, resuspended in 1 ml of Alcian blue (30 μg/ml) in 0.02 M HCl, and incubated at room temperature for 10 min. Cells were then spun down, and the absorbance of the supernatant at 620 nm was measured to determine the level of non-bound Alcian blue from a calibration curve. From these values, the specific amount of Alcian blue bound to cells was calculated (1 unit = 1 μg bound per 10^6 cells of cell suspension). Total cell wall carbohydrate composition was analyzed by hydrolyzing cell walls with sulfuric acid and determining the monosaccharide composition by high performance anion-exchange chromatography with pulsed amperometric detection as described previously (44, 45).

Metabolic Labeling of Glycans and TLC—For the analysis of O-linked glycans, yeast cells were initially labeled with [3,2-H]mannose. Cells growing in 2 ml ofYP medium (1% w/v) yeast extract, 2% w/v) mycological peptone) + 0.5% sucrose were incubated with 1.85 MBq of [3,2-H]mannose (555 GBq/mmol; PerkinElmer Life Sciences, Beaconsfield, UK) at 30 °C for 90 min. Cells were then harvested, resuspended in 100 μl of phosphate-buffered saline, and disrupted with glass beads as described above. The cell walls were collected by centrifugation with 1 x NaCl and twice with water. Cell glycans were released by β-elimination with 100 mEq of NaOH for 16 h at room temperature. Cell walls were pelleted at 21,500 x g for 10 min, and the supernatant containing O-linked glycans was retained for TLC analysis. The cell walls were washed twice with water and then boiled for 1 min in 100 mEq of HCl to release the acid-labile glycans. The remaining cell walls were pelleted as described above, and the supernatant was retained for TLC analysis.

Samples for TLC were spotted onto Silica Gel 60 TLC plates (Whatman, Brentford, UK) and allowed to dry. Plates were chromatographed by two ascents of the solvent (3:4:2.5:4 ethyl acetate/butan-1-ol/acetic acid/water). For detection, plates were sprayed with EN‘HANCE (PerkinElmer Life Sciences) and visualized by autofluorography (Eastman Kodak BioMax XLS).

Acid Phosphatase Zymogram—In situ acid phosphatase activity assays were performed following a modified method of Schweingruber et al. (46). Briefly, cells were grown overnight in phosphate-depleted Sabouraud glucose medium to induce acid phosphatase expression. Cells were collected and washed twice with 1 M NaCl, and twice with water, before resuspension in 0.1 M succrose in a lysis buffer (62.5 mM Tris-HCl (pH 6.8), 0.01% bromphenol blue, 15% glycerol) and run on a 6% Tris/glycine-polyacrylamide gel (Invitrogen) under nondenaturing conditions. The gel was stained for 10 min, washed in 100 mM sodium acetate (pH 5.2) for 10 min at room temperature and then incubated with substrate solution (0.05% p-nitrophenyl phosphate and 0.03% fast blue in 100 mM sodium acetate). The absorbance of the supernatant at 620 nm was measured to determine the level of acid phosphatase activity.

RESULTS

Isolation and Analysis of CaPMR1—We initially identified CaPMR1 before the completion of the C. albicans genome project by PCR with primers based on an unpublished sequence showing homology to S. cerevisiae PMR1 and a degenerate primer based on the P-loop found in all P-type ATPases. The regions surrounding CaPMR1 were then cloned by unidirectional Vectorette PCR (Sigma, Haverhill, UK) (48). The CaPMR1 open reading frame of 2754 bp (GenBankTM/EBI accession AJ277111) is predicted to encode a protein of 917 amino acids. Subsequently, the C. albicans genome sequence has been completed, enabling this open reading frame to be confirmed as the only homolog of S. cerevisiae PMR1. As in S. cerevisiae, CaPMR1 is linked to SUA5, which is involved in translation initiation. However, the orientation of the two genes is reversed, and the conservation of gene order does not extend further in either direction along the chromosome.

The deduced amino acid sequence of CaPMR1 demonstrated high homology to other secretory pathway P-type ATPases of subfamily IIA (68.7, 62.4, 62.1, 58.3, 57.6, 51.8, and 51.5% identity to Pmr1p of Pichia angusta, Kluyveromyces lactis, S. cerevisiae, and Yarrowia lipolytica; Schizosaccharomyces pombe Pgap2; and human and rat ATP2C1, respectively). Ten putative transmembrane regions are predicted by hydrophobicity profile analysis (available at www.cbs.dtu.dk/services/TMHMM) and by the similarity of this profile to other P-type Ca2+-ATPases. As with other P-type Ca2+-ATPases, the transmembrane domains are clustered into two groups separated by a central hydrophilic loop. All 10 conserved regions (regions a–j) that characterize the P-type ATPase family (49) are maintained in CaPMR1 (Fig. 1). In particular, region f contains the aspartic acid residue in the D349KTGTLT motif that is the phosphorylation site in P-type ATPases (34). Region c contains the T308GE motif believed to be important in removing the phosphate group from the enzyme intermediate. Regions g and i, responsible for ATP binding, are also well conserved.

Deletion of CaPMR1—CaPMR1 was disrupted in strain CAI-4 by the standard ura-blast marker method (39). This involved the deletion of the central 1803-bp region containing all 10 regions characteristic of P-type ATPases. To avoid potential problems with ectopic expression of URA3, the Ura- derivatives were transformed with plasmid CIp10 so that URA3 was expressed from the neutral chromosome (40, 41). A re-integrant strain was also constructed in which CaPMR1 was introduced under the control of its own promoter into the Capm1Δ null mutant at the RPS1 locus. Strain CAI-4 transformed with CIp10 was used as a control in all experiments.

Deletion of CaPMR1 had no direct effect on growth rate in either YEPD medium or minimal medium (SD medium). However, the yeast cells in liquid medium tended to form small aggregates (Fig. 2A). There was also no obvious defect in hyphal formation in response to serum, RPMI 1640 medium, or Lee’s medium at pH 6.5. However, there was a delay in filament formation on solid Spider medium (Fig. 2B). Both the small cellular aggregates and the delay in filament formation on Spider medium were restored to those of the wild-type strain in the re-integrant strain. Hence, in vitro growth was not markedly affected in the Capm1Δ null mutant.
Calcium-related Growth Defects in Capmr1—As mentioned above, deletion of CaPMR1 had no effect on the growth rate of the strain in laboratory media. Manganese ions are normally present only at trace concentrations below 10⁻⁶ M in laboratory media and cannot be depleted further without chelation. Therefore, growth parameters were initially assessed under Ca²⁺-depleted conditions alone. Even when the Capmr1 mutant was grown in Ca²⁺-depleted medium, there was no obvious effect on growth rate (Fig. 3A). The only source of Ca²⁺ in this medium was from the calcium salt of pantothenoate (at 0.8 M) and contaminants. However, when viable cell counts were measured, it was clear that the null mutant started to lose viability as the cells entered stationary phase. After 72 h of growth in Ca²⁺-depleted medium, the viability of the null mutant dropped by over 1000-fold (Fig. 3B). When the null mutant was grown in standard SD medium ([Ca²⁺] 1 mM), there was also a slight decrease in viability at 72 h. Loss of viability was not detected when the strain was grown in SD medium supple-

FIG. 1. Amino acid conservation in the 10 regions (regions a–j) present in all P-type ATPases. The amino acid positions are indicated. Sc, S. cerevisiae; Yl, Y. lipolytica; Kl, K. lactis; Pa, P. angusta; Sp, S. pombe; Hs, Homo sapiens.
Growth of the null mutant was completely blocked by the addition of 10 mM CaCl$_2$ or MnCl$_2$, but not MgCl$_2$. B, EGTA sensitivity was quantified by $A_{600}$ after 16 h of growth in YEPD medium with varying EGTA concentrations. ■, wild-type strain; □, null mutant; △, re-integrant strain. Errors bars are means ± S.D.

The Capmr1Δ null mutant was also hypersensitive to EGTA. Growth of the null mutant was completely blocked by the addition of 15 mM EGTA, whereas the wild-type and re-integrant strains were unaffected (Fig. 4). This hypersensitivity could be reversed by the addition of 10 mM CaCl$_2$ or MnCl$_2$, but not MgCl$_2$. Unlike in S. cerevisiae, deletion of CapMr1 did not alter sensitivity to the calmodulin inhibitor trifluoperazine or affect the level of manganese toxicity (data not shown).

**Glycosylation Defects in the Capmr1Δ Mutant—CaPMR1**

provides the Golgi with manganese ions that are required as a cofactor for the Golgi-resident mannosyltransferases. We therefore analyzed the extent of N- and O-linked glycosylation in the Capmr1Δ null mutant. We assessed the electrophoretic mobility of secreted acid phosphatase, which is known to be heavily N-glycosylated, by activity staining (50). The mobility of acid phosphatase present in protein extracts from the Capmr1Δ null mutant was increased on the native gel (Fig. 5A). Hence, the null mutant had an N-glycosylation defect. After treatment with endoglycosidase H to remove the N-linked side chains, the acid phosphatase migrated faster through the native gel; however, it still appeared as a diffuse band, presumably due to variations in O-linked glycosylation. In this case, there was no difference among the null mutant and wild-type and re-integrant controls. However, because O-linked side chains are short, this method cannot be used to assess O-glycosylation defects.

N-Linked glycosylated side chains contain the acid-labile fraction phosphomannan, which is incorporated as the protein passes through the Golgi. This fraction provides the cell wall with its negative charge and can be easily detected with the cationic dye Alcian blue. The Capmr1Δ null mutant demonstrated a severe reduction in specific Alcian blue binding from 143 ± 6.7 units in the wild-type strain to 6.7 ± 3.8 units in the null mutant (5% of the wild-type strain). The re-integrant strain had an intermediate Alcian blue binding phenotype of 102 ± 7.5 units (74% of the wild-type strain). We also analyzed the acid-labile phosphomannan fraction directly by TLC. Cells were labeled with $[^2-3]H$mannose, and phosphomannan was isolated by HCl treatment of cell walls. The TLC autoradiogram clearly demonstrated that the Capmr1Δ null mutant was almost completely devoid of phosphomannan (Fig. 5B).

The wild-type and re-integrant strains displayed normal phosphomannan of one to eight β1,2-linked mannose residues (26, 51).

The consequence of loss of CaPMR1 on the O-mannan structure was also assessed by TLC analysis. O-Mannan of C. albicans typically comprises one to five α1,2-linked mannose residues; the first is added in the endoplasmic reticulum, and subsequent residues are added in the Golgi. The TLC analysis clearly demonstrated that O-mannan was truncated in the Capmr1Δ null mutant (Fig. 5C), with almost no detectable Man$_3$ to Man$_6$. The fact that Man$_3$ was present on the O-mannan isolated implies that some Golgi-based mannosyltransferase activity was retained in the Capmr1Δ null mutant. Re-integration of CaPMR1 to the null mutant restored the normal O-mannan structure.

To confirm the gross glycosylation defect in the Capmr1Δ null mutant and to determine the effect on the cell wall, we analyzed wall composition by high performance anion-exchange chromatography with pulsed amperometric detection. This method determines the carbohydrate composition of the wall by analyzing its monosaccharide composition, where glucose, mannose, and glucosamine are detected. These levels related to the presence of glucan, mannan, and chitin in the cell wall, respectively. This demonstrated a clear defect in mannan levels, which dropped from 39% of the cell wall in the wild-type strain to only 8% in the Capmr1Δ null mutant (80% reduction). This decrease was reciprocated with an increase in glucan levels from 60% in the wild-type to 90% in the null mutant. The level of chitin in the strains was unchanged.
The strains were also stressed with 100 μg/ml Calcofluor white and Congo red and displayed increased sensitivity to hygromycin B (Fig. 6), a phenotype commonly seen in glycosylation mutants (52). However, there was no change in the level of sensitivity to other agents such as SDS, caffeine, vanadate, tunicamycin, NaCl, and KCl (not shown).

Cell Wall Sensitivity and Cell Integrity Pathway Activation—To determine the effect of deleting CapMr1 on the integrity of the cell wall, we tested the null mutant for sensitivity to a range of cell wall-perturbing agents and other agents whose effects have been associated with altered cell walls and glycosylation. The Capmr1Δ null mutant was clearly hypersensitive to the cell wall-perturbing agents Calcofluor white and Congo red and displayed increased sensitivity to hygromycin B (Fig. 6), a phenotype commonly seen in glycosylation mutants (52). However, there was no change in the level of sensitivity to other agents such as SDS, caffeine, vanadate, tunicamycin, NaCl, and KCl. Antifungal susceptibility testing demonstrated that the null mutant showed no change in sensitivity to antifungal agents in clinical use, including the azoles (fluconazole, itraconazole, and ketoconazole), flucytosine, amphotericin B, terbinafine, and the β1,3-glucan synthase inhibitor caspofungin (data not shown).

The hypersensitivity of the Capmr1Δ null mutant to cell wall-perturbing agents shows that the wall is altered and is more sensitive to stress. Therefore, we determined whether the cell integrity pathway was induced in the mutant. The cell integrity pathway signals through Pkc1p and results in the cell integrity pathway being induced in the mutant. The cell wall-perturbing agents show that the wall is altered and is hypersensitive to the cell wall integrity pathway (43). We tested the activation of Mkc1p in our strains by Western blotting with a phosphorylation of the MAPK Mkc1p (43). We tested the activation of Mkc1p in our strains by Western blotting with a commercially available antibody that binds to phosphorylated Mkc1p. Mkc1p was activated in the Capmr1Δ null mutant during exponential growth, whereas Mkc1p was not activated in the wild-type and re-integrant controls (Fig. 7). As a control, the strains were also stressed with 100 μg/ml Calcofluor white for 2 h. This again demonstrated activation of the cell integrity pathway. The results of sensitivity testing and the constitutive activation of the cell wall integrity pathway show that the Capmr1Δ null mutant has a defective cell wall.

Adhesion of the Capmr1Δ Null Mutant—Mannoproteins have been implicated in C. albicans adhesion; hence, we tested the ability of the Capmr1Δ mutant to adhere to BEC. Initially, it appeared that there was an increase in adherence from 0.38 ± 0.03 adhered cells/BEC in the wild-type control compared with 1.19 ± 0.03 adhered cells/BEC in the null mutant. However, the null mutant grew as small clumps of cells (Fig. 2A), and this increase in adhesion may reflect the null mutant clumping phenotype. Indeed, the number of BEC with one or more adherent yeast cells did not increase (76 ± 1.4% negative for the wild-type control and 74 ± 4.5% negative for the null mutant), suggesting that adhesion was not significantly altered.

The Capmr1Δ Null Mutant Is Attenuated in Virulence—The virulence of the Capmr1Δ null mutant and relevant control strains was tested in a mouse model of systemic infection. The Capmr1Δ null mutant was highly attenuated in virulence, with all mice surviving until the end of the experiment, whereas challenge with the wild-type control demonstrated a mean survival time of 7 days (log rank test; p < 0.05) (Fig. 8 and Table II). The re-integrant strain demonstrated an intermediate phenotype, with a mean survival time of 13.8 days, which was significantly different compared with the null mutant (log rank test; p < 0.05). The intermediate phenotype is suggestive of a gene dosage effect on virulence. Such effects are typical of other heterozygous and re-integrant strains containing a single wild-type copy of the target gene (53–55). In terms of tissue burdens determined for kidneys and brains of infected mice, the wild-type and re-integrant strains were not significantly different. The Capmr1Δ null mutant demonstrated a clear reduction in tissue burdens, with a >2 log reduction in kidney cfu/g and a 1 log reduction in brain cfu/g (Table II). There were also a significant number of Candida-negative organs: 33% of kidneys and 50% of brains with the Capmr1Δ mutant. Hence, although the Capmr1Δ mutant was not affected in growth in vitro, this null mutant was extremely attenuated in virulence in a mouse model.

**DISCUSSION**

In this study, we have analyzed the effect of a gross defect in glycosylation in C. albicans by creating a null mutant in PMR1, which is required for glycosylation in the Golgi. Pmr1p is a P-type...
ATPase and supplies the Golgi body with calcium and manganese ions. In the Golgi, manganese ions are required as an essential cofactor for mannosyltransferases. Hence, by disrupting PMR1, we could determine the effect of a general defect in glycosylation both at the cellular level and in pathogenesis. The Capmr1Δ null mutant clearly demonstrated a general glycosylation defect and associated cell wall changes and was severely attenuated in virulence in a murine model of systemic infection.

Previous studies have also examined gross glycosylation defects by studying the synthesis (29) and transport (27) of GDP-mannose into the Golgi, where it acts as the mannose donor for mannosyltransferases. Total lack of GDP-mannose would block not only glycosylation in the Golgi, but also the essential processes of O-glycosylation and biosynthesis of the glycosylphosphatidylinositol anchor in the endoplasmic reticulum, as these steps require dolichylphosphomannose, which is synthesized from GDP-mannose. Hence, CaSRB1, which synthesizes GDP-mannose, was found to be essential, and its depletion was found to have pleiotropic effects (29). The Golgi GDP-mannose transporter VRG4 is essential in C. albicans (27) and S. cerevisiae (56, 57). This suggests that a complete lack of glycosylation in the Golgi results in non-viability. The essential nature of both CaSRB1 and CaVRG4, while emphasizing the importance of glycosylation, means that the role of the glycans in host-fungus interactions and virulence cannot be assessed. The Golgi ATPase CaGDA1 has also been studied (30); this enzyme converts GDP to GMP, which is then exported from the Golgi by Vrg4p in an antiport process with GDP-manassone. However, CaGDA1 is non-essential, presumably due to functional redundancy, and does not exhibit a gross glycosylation defect. It does demonstrate a partial defect in O-glycosylation and, as visualized by Alcian blue binding, has a slight reduction in phosphomannan; however, it displays no defect in the acid-stable N-glycans.

In S. cerevisiae, loss of PMR1 results in a strain displaying a slow growth phenotype, which can be alleviated by adding exogenous calcium ions to the medium; however, higher Ca²⁺ concentrations in the medium result in the strain losing viability more quickly upon entering stationary phase (31). In our studies, deletion of CaPMR1 had no effect on the growth rate of the strain, even when grown on calcium-depleted medium. Additionally, the viability phenotype was opposite of that seen in S. cerevisiae, in that the Capmr1Δ null mutant lost viability upon entering stationary phase after growth on Ca²⁺-depleted medium. Therefore, there must be subtle differences in the homeostasis of calcium ions in S. cerevisiae and C. albicans. Unlike in S. cerevisiae, we also saw no change in the level of manganese toxicity to the Capmr1Δ null mutant. Therefore, there are also differences in the tolerance of S. cerevisiae and C. albicans to manganese ions.

The observed calcium-dependent drop in viability of the null mutant after entering stationary phase may affect pathogenicity. Calcium ions are present in serum at 95 μg/ml, of which is available in an accessible ionized form (58). This concentration approximates that present in standard SD medium, where only a slight decrease in long-term viability was seen (~10% drop at 72 h). Therefore, although we cannot rule out that this may have influenced the virulence study, it is likely to be only a minor effect at physiological calcium and manganese ion levels and, as such, could not account for the almost complete loss of virulence in the Capmr1Δ mutant.

Work in Y. lipolytica has shown that loss of Pmr1p can affect secretion. Depending on the marker protein analyzed, secretion is unchanged, enhanced, or decreased (59). Loss of Pmr1p can also affect the secretion of proteins expressed heterologously in S. cerevisiae and K. lactis (35, 36, 60). We tested the level of secretion in the Capmr1Δ null mutant by analyzing the activity of cell-associated acid phosphatase and secreted aspartyl proteinase (data not shown). We detected a slight decrease in the level of cell-associated acid phosphatase activity (70 ± 2.4% of the wild-type strain) and a slight increase in the level of secreted aspartyl proteinase activity (163 ± 3.7% of the wild-type strain). Therefore, the changes in secretion detected in the Capmr1Δ null mutant were not as high as those reported in other organisms. However, it was clear from these published reports that the levels of change detected are dependent on the protein that is secreted. Therefore, we cannot rule out that proteins other than cell-associated acid phosphatase and secreted aspartyl proteinase may be more severely affected in the Capmr1Δ null mutant.

The Capmr1Δ null mutant had a clumpy phenotype, with most cells present in small aggregates. This may be due to changes in the cell wall composition resulting in flocculation. However, examination of the clumps suggests that they may be the result of a cell separation defect. This suggests that daughter cells are delayed in cell wall separation, perhaps due to a decrease in the activity of cell wall-hydrolyzing enzymes. A similar clumping phenotype has been reported for other C. albicans glycosylation mutants such as the Capmt1Δ/Camtt1Δ double mutant (19). A more severe clumping phenotype was apparent in the Cammt9Δ and Cavrg4Δ mutants (25, 27). A severe cytokinesis defect in an S. pombe pmr1Δ mutant has also been reported recently (61). The clumping phenotype posed technical difficulties with in vitro assays of adhesion. However, it was clear that the overall number of epithelial cells adhered to was unaffected in the mutant, suggesting that loss of Pmr1p does not have a marked effect on adhesion.

Evidence was found for a gross defect in glycosylation in the Capmr1Δ null mutant, with N-glycosylation severely affected as demonstrated by the under-glycosylation of acid phosphatase and the almost complete absence of phosphomannan (Fig. 5). O-Glycosylation was also affected such that O-mannan was truncated with a marked reduction in Man₃ to Man₅ (Fig. 5). The observation that Man₃ was still present in O-mannan isolated from the Capmr1Δ null mutant implies that some mannosyltransferases are still active in the Golgi. This may reflect the localization of specific mannosyltransferases within the Golgi, where enzymes of the cis-Golgi may receive manganese from vesicles derived from the endoplasmic reticulum. Therefore, in the Capmr1Δ null mutant, Man₃ may have been present because it was added in an early Golgi compartment that received manganese ions from endoplasmic reticulum-derived vesicles. This also suggests that phosphomannan, which is almost completely absent in the Capmr1Δ null mutant, is a component added later in the Golgi network and, as such, is more sensitive to the loss of Pmr1p. Alternatively, the presence of Man₂ in the O-mannan of the Capmr1Δ null mu-
tant may reflect the affinity of specific mannosyltransferases for manganese ions. Those that have the highest cation affinity may remain most active in a manganese-limited compartment.

The cell wall was significantly altered in the Capmr1 null mutant, exemplified by the 80% drop in mannose content correlating with the gross glycosylation defect. The consequences of the loss of Pmr1p were significant enough to result in the constitutive activation of the cell integrity pathway acting to compensate for the loss of mannan. The null mutant was hyper-sensitive to the cell wall-perturbing agents Calcofluor white and Congo red, which bind chitin and glucan and interfere with their synthesis and cross-linking. Increased sensitivity to hygromycin B, a phenotype characteristic of null mutant was highly attenuated in virulence, with all mice target both cell wall proteins and the plasma membrane, or to high salt conditions, indicating that the mutant was not osmotically fragile.

The Capmr1 null mutant displayed no significant growth defect in vitro, yet had an altered cell wall due to the gross glycosylation defect. This allowed us to assess the importance to virulence of glycosylation events that occur in the Golgi. The null mutant was highly attenuated in virulence, with all mice surviving the course of the experiment and displaying a significant drop in organ burdens. The low organ burdens and the number of Candida-negative organs suggest either that the host was able to effectively prevent colonization or that the mutant was unable to reach deep-seated infection sites. These results underline the importance of glycosylation to both the cell wall architecture and the pathogenesis of C. albicans disease. Questions still remain regarding the identification of specific epitopes involved in host-pathogen interactions, although, in terms of carbohydrate epitopes, recent work has confirmed the importance of O-glycans (19) and down-played the role of O-glycans (19) and down-played

References

1. Pappas, P. G., Rex, J. H., Lee, J., Hamilton, R. I., Lassen, R. A., Powderly, W., Kaufman, C. A., Hyslop, N., Mangino, J. E., Chapman, S., Horowitz, R. W., Edwards, J. E., and Dismukes, W. E. (2003) Clin. Infect. Dis. 37, 634–643
2. Sandven, P. (2000) Res. Respir. Med. 17, 73–81
3. Odds, F. C. (1988) Candida and Candidiasis, 2nd Ed., Bailliere-Tindall, London
4. Calderone, R. A. (2002) Candida and Candidiasis, ASM Press, Washington, D.C.
5. Klis, F. M., de Groot, P., and Heilingefelt, K. (2001) Mol. Microbiol. 39, Suppl. 1, 1–8
6. Chaudhuri, H., Li, L., Singh, P., Calderone, R., and Krippa, M. (2002) Candida and Candidiasis, pp. 159–175, ASM Press, Washington, D.C.
7. Calderone, R., and Gow, N. A. R. (2002) Candida and Candidiasis, pp. 67–86, ASM Press, Washington, D.C.
8. Calderone, R. A. (1995) Trends Microbiol. 3, 55–58
9. Wang, Y., Li, S. P., and Sommer, S. A., Bost, K. L., and Butcher, J. E. (1998) Infect. Immun. 66, 1384–1391
10. Sundstrom, P. (2002) Cell Microbiol. 4, 461–469
11. Romani, L. (2002) Candida and Candidiasis, pp. 233–242, ASM Press, Washington, D.C.
12. Fukazawa, Y., and Kagaya, K. (1997) J. Med. Vet. Mycol. 35, 87–99
13. Strahl-Bolsinger, S., Gentzsch, M., and Tannen, W. (1999) Biochim. Biophys. Acta 1426, 297–307
14. Kauer, R., and Lehle, L. (1999) Biochim. Biophys. Acta 1426, 259–273
15. Burda, P., and Aebi, M. (1999) Biochim. Biophys. Acta 1426, 239–257
16. Gentzsch, M., and Tannen, W. (1996) EMBO J. 15, 5752–5759
17. Lussier, M., Silic, A. M., and Bushey, H. (1999) Biochim. Biophys. Acta 1426, 323–334
18. Dean, N. (1999) Biochim. Biophys. Acta 1426, 309–322
19. Munro, C. A., Bates, S., Burman, E. T., Hughes, H. B., MacCallum, D. M., Durr, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G., Atkinson, G,