Endoplasmic Reticulum α-Glycosidases of Candida albicans Are Required for N Glycosylation, Cell Wall Integrity, and Normal Host-Fungus Interaction

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The cell surface of Candida albicans is enriched in highly glycosylated mannoproteins that are involved in the interaction with the host tissues. N glycosylation is a posttranslational modification that is initiated in the endoplasmic reticulum (ER), where the Glc₃Man₉GlcNAc₂ N-glycan is processed by α-glucosidases I and II and α₁,2-mannosidase to generate Man₈GlcNAc₂. This N-oligosaccharide is then elaborated in the Golgi to form N-glycans with highly branched outer chains rich in mannose. In Saccharomyces cerevisiae, CWH41, ROT2, and MNS1 encode for α-glucosidase I, α-glucosidase II catalytic subunit, and α₁,2-mannosidase, respectively. We disrupted the C. albicans CWH41, ROT2, and MNS1 homologs to determine the importance of N-oligosaccharide processing on the N-glycan outer-chain elongation and the host-fungus interaction. Yeast cells of Cacwh41Δ, Carot2Δ, and Cammns1Δ null mutants tended to aggregate, displayed reduced growth rates, had a lower content of cell wall phosphomannan and other changes in cell wall composition, underglycosylated β-N-acetylhexosaminidase, and had a constitutively activated PKC-Mkc1 cell wall integrity pathway. They were also attenuated in virulence in a murine model of systemic infection and stimulated an altered pro- and anti-inflammatory cytokine profile from human monocytes. Therefore, N-oligosaccharide processing by ER glycosidases is required for cell wall integrity and for host-fungus interactions.

Candida albicans is an opportunistic fungal pathogen of humans that can cause superficial infections of the mucosa and, in the immunocompromised host, life-threatening systemic infections (10, 52, 53, 61). The cell wall of C. albicans is the immediate point of contact between the fungus and host and therefore plays a key role in the host-fungus interaction. The cell wall is composed of an inner layer of chitin and an outer layer that is rich in mannoproteins which accounts for 40% of the yeast form cell wall mass (39). The mannoproteins have important roles in adhesion, antigenicity, modulation of the host immune response, and recognition of this fungus by innate immune cells (4, 9, 11, 49, 56, 67, 78). Therefore, studies of cell wall glycosylation can provide insights into the molecular basis of the pathogenic and commensal interactions between C. albicans and the human host.

The N-glycosylation pathway has been studied extensively in Saccharomyces cerevisiae. N glycosylation occurs by a stepwise process involving the transfer of Glc₃Man₉GlcNAc₂ from Glc₃Man₉GlcNAc₂-PP-dolichol to specific asparagine residues of nascent proteins in the endoplasmic reticulum (ER). Subsequently, the Glc₃Man₉GlcNAc₂ core oligosaccharide is processed by α-glucosidases I and II, which sequentially remove the terminal α₁,2-linked and two remaining α₁,3-linked glucose residues, respectively. Further processing by ER α₁,2-mannosidase I generates Man₉GlcNAc₂ isomer B (27, 28). In the Golgi compartment, Och1 initiates the outer-chain branching of N-glycans by the addition of a single α₁,6-linked mannose residue to Man₉GlcNAc₂ core. In the och1Δ null mutant of C. albicans, the Man₉GlcNAc₂ N-glycan core is subjected to further modification with three to eight mannose residues added to one or more of the antenna residues of the core (4). The α₁,6-mannose backbone of the N-mannan outer chain is extended by the enzyme complexes mannan polymerase I and II, and branched side chains are attached by a range of Golgi mannosyltransferases to yield a high-mannose N-glycan that may represent 95% of the glycoprotein mass (15, 39, 47). Studies of glycosylation pathways in C. albicans are important because key differences exist between the O- and N-glycan structures of this fungus and S. cerevisiae. For example, in C. albicans, the terminal mannose residues of O-glycans are attached by α₁,2-linkages rather than α₁,3-mannose residues as in S. cerevisiae (46), and β₁,2-linked mannose residues are...
present in both the acid-labile and acid-stable N-glycans (64, 73). Such changes are likely reflected in surface-to-surface interactions of these organisms in their natural environments and during human infection. In S. cerevisiae, CWH41 (GLS1), ROUT2 (GLS2), and MNS1 encode three ER enzymes: α-glucosidase I, α-glucosidase II catalytic subunit, and α,1,2-mannosidase, respectively, which are involved in N-glycan core processing (27). CWH41 removes the outermost α,1,2-glucose residue of GlcMan GlcNAc2 before it is trimmed further by α-glucosidase II, which removes the α,1,3-glucose residues from GlcMan GlcNAc2 (23, 62, 76). In S. cerevisiae, the α-glucosidase II is a heterodimer with the catalytic α-subunit encoded by ROUT2, a member of the glycosyl hydrolase family 31. In higher eukaryotes the β-subunit normally contains a KDEL-type ER retention motif (74). In lower eukaryotes such as fungi, no gene with significant homology to the β-subunit has been found. MNS1 is an α,1,2-mannosidase that trims the Man₄GlcNAc2 oligosaccharide to Man₄GlcNAc2 isomer B, the last product of the N-glycan processing carried out in the ER (8, 34). It has been suggested that removal of this unique mannose residue induces a conformational reorganization in the N-glycan core that is required for the outer-chain synthesis (8). The core-processing α-glucosidases are also important for glycoprotein folding and for the ER quality control during glycoprotein biosynthesis (24, 29).

Recent studies in C. albicans have shown that protein glycosylation is essential for fungal pathogenesis and immune recognition. Mannosyltransferases involved in N- and O-linked glycosylation have been shown to be required for virulence (4, 7, 46, 54, 66, 69, 70). Golgi proteins involved in the provision of GDP-mannose for glycosylation, which are encoded by CaVRG4 and CaSRB1, are essential for this fungus, indicating the overall importance of glycosylation to cell viability (26, 51, 79, 80). Also, CaPmr1, a Golgi P-type Ca²⁺/Mn²⁺-ATPase involved in the transport of Ca²⁺ and Mn²⁺ ions into the Golgi compartment, is necessary for normal O- and N-linked glycosylation and virulence (3). However, cell wall phosphomannan synthesis is apparently not required for full virulence (30).

To assess the importance of N-oligosaccharide processing and N-mannan structure on the host-fungus interaction, we disrupted the C. albicans CWH41, ROUT2, and MNS1 homologs. The null mutants displayed a number of cell wall defects, were attenuated in virulence in a murine model of systemic infection, and stimulated an altered cytokine profile by human peripheral blood mononuclear cells (PBMC). Therefore, N-oligosaccharide processing by ER α-glucosidases to generate high-mannose N-glycans is vital for the host-fungus interaction and for virulence.

**MATERIALS AND METHODS**

Strains, media, and culture conditions. All of the strains used and constructed in the present study are listed in Table 1. Strains were grown at 30°C in YPD medium (1% [wt/vol] yeast extract, 2% [wt/vol] mycological peptone, 2% [wt/vol] glucose) or in SD medium (0.67% [wt/vol] yeast nitrogen base with ammonium sulfate without amino acids, 2% [wt/vol] glucose, 0.077% [wt/vol] complete supplement mixture minus uracil [Qbiogene, Cambridge, United Kingdom]) with uridine (50 μg/ml) as required. Hyphal cells were grown in YPD medium plus 20% (vol/vol) newborn calf serum, Lee’s medium (pH 6.5) (41), or salt base containing 20% (vol/vol) newborn calf serum, Lee’s medium (pH 6.5) (41), or salt base (0.45% [wt/vol] NaCl, 0.335% [wt/vol] yeast nitrogen base with ammonium sulfate without amino acids, 2.5 mM GlcNAC) (16) at 37°C or on solid Spider medium (43) at 30°C. To induce β-N-acetylhexosaminidase (HexNAc) synthesis, cells were grown in SC+GlcN (0.67% [wt/vol] yeast nitrogen base with ammonium sulfate without amino acids, 0.077% [wt/vol] complete supplement mixture minus uracil, 25 mM GlcNAC) at 30°C. For virulence assays, the inoculum of yeast cells was grown in NGY medium (0.1% [wt/vol] glucose, and 0.1% [wt/vol] yeast extract) at 30°C.

**Construction of null mutants and control strains.** The MNS1, ROUT2, and CWH41 genes were disrupted by the “mini-ura-blaster” protocol (81). To make the disruption cassette, long primers containing 70-pb 5’ end of the open reading frames were used to amplify by PCR the dp1200-URA3-dp1200 cassette contained in plasmid pDDBS7 (81) for MNS1, primer pair 5’-ATGCATTATTTAAGGTATTATGTTGCTTTATATATATATGCTGTGACCATT-3’ and 5’-TTACCCAGAAATTTCTCTTCAATTTTTTGCTTTATGCACTGGATT-3’.

**TABLE 1. Strains used in this study**

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TATCACTGTTGAGTCTGCGTTTCCGACAGCATTTG-3' for ROT2, primer pair 5'-ATGAGAATTTCTCTAAAACTTTTATTAATGCTCA GTGACCTGTTAAGGAATGTTGCTAATGTTGGGA GGGATG-3' and 5'-TTATAGTGCTGATCGTATTTTTGCTTGTGGCTGC TAAAACAGGGGGAAGATCCACCTTCATTGAGTTTTCCACGT AGCCAGTT-3' and for CWH41, primer pair 5'-ATGAGAATTTCTCTAAAACTTTTATTAATGCTCA GTGACCTGTTAAGGAATGTTGCTAATGTTGGGA GGGATG-3'; and for CWH41, primer pair 5'-ATGAGAATTTCTCTAAAACTTTTATTAATGCTCA GTGACCTGTTAAGGAATGTTGCTAATGTTGGGA GGGATG-3' and 5’-TTATTTTGTATTGCTGTCTCAGACCACAT CAAAATTTTTGATTTGGTGATTGGCATTTG-3'; the regions complementarily to plasmid pDB57 are underlined. The genes were disrupted by sequential rounds of transformation of strain CAH4 and the recycling of the URA3 marker by selection on SD medium plus 5-fluoroorotic acid (1 mg/ml) and uridine. To avoid the problems associated with the ectopic expression of URA3 (6), the Ura Rps1/α, rot2Δ, and cwh41Δ null strains were transformed with Stu-digested Clp10 plasmid (48); hence, URA3 was expressed at the RPS1 locus. To construct reintegrent control strains, the MNS1 open reading frame plus 999 bp of its promoter and 597 bp of its terminator sequences (total of 3.3 kb) and ROT2 (979 bp of promoter and 693 bp of terminator [total of 4.3 kb]) and CWH41 (946 bp of promoter and 593 bp of terminator [total of 4.0 kb]) genes were amplified by PCR (primer pair for MNS1, 5'-GGGCTGCTAGATACCTTCTTCTTCA TCC-3’ and 5’-GGGCGGCAATGATACCAATAAGGA-3’; primer pair for ROT2, 5’-GGGCGGCCGCAATCCTATTAGCTCCAGAC-3’ and 5’-GGGCGGCC TATACCTGAAAAAGTTAATT-3’; and primer pair for CWH41, 5’-GGGCGGCGC TATACCTGAAAAAGTTAATT-3’ and 5’-GGGCGGCCGTATACCT-3’), with the bases to generate a NotI site underlined, and the products were cloned into pGEM-T Easy vector (Promega, Ltd., Southampton, United Kingdom). The inserts were released from NotI digestion and ligated into the NotI site of Clp10. The resulting plasmids were digested with StuI and used to transform the Ura– null strains. As a further control, strain CAH4 was transformed with Stu-digested Clp10. Therefore, all strains analyzed had the URA3 marker expressed at the RPS1 locus.

Assay of α-glycosidase activity. The α-mannosidase and α-glucosidase activities were measured by using fluorogenic substrates as described previously (45, 71). Cells were washed twice with water and resuspended in 2 M trifluoroacetic acid, boiled for 3 h, washed, and centrifuged at 30°C and broken as described above. The homogenate was centrifuged at 21,500g for 10 min. Then, 50 μl of buffer (100 mM Tris-HCl buffer [pH 7.5], 0.01% [wt/vol] bromophenol blue, and 15% [vol/vol] glycerol) and run on a 10% SDS-PAGE gel (Invitrogen, Paisley, United Kingdom) in 0.1 M citrate-KOH buffer (pH 4.0) for 10 min at room temperature and then incubated in the substrate solution (0.18 mM naphthyl-β-D-glucoside [Glycosynth, Ltd., Warrington, United Kingdom]) in 0.1 M citrate-KOH buffer (pH 4.0) for 30 min at 37°C. The reaction product was visualized by incubation in the substrate solution plus 0.7 mM Fast Blue at 60°C until color developed.

Protein extracts and Western blotting. To test for activation of the cell integrit y pathway, cells were grown in YPD medium at 30°C and collected in mid-exponential growth phase. As positive controls, strains were stressed by the addition of Congo Blue (0.1 g/ml), 0.1% sodium dodecyl sulfate (SDS), 0.3 M NaCl, 0.05% [wt/vol] SDS, 1 mM dithiothreitol, 10% [wt/vol] glycerol, protease inhibitor mixture [Roche], and broken with glass beads in a FastPrep machine, and the lysate was centrifuged at 21,500 × g for 10 min. Then, 50 μg of protein was separated on a 4 to 12% NuPAGE Bis-Tris gel (Invitrogen) and electrophoreted to a polyvinylidene difluoride membrane. The membrane was blocked in phosphate-buffered saline plus 0.1% Tween 20 and 5 μg of bovine serum albumin/min/ml for 2 h at room temperature. Detection was then carried out with the PhosphoPlus p44/p42 mitogen-activated protein kinase (Thr202/Tyr204) antibody kit (Cell Signaling Technology, Hertfordshire, United Kingdom), which cross-reacts with C. albicans Mkk1 (Slt2) in both its active (phosphorylated) and inactive (nonphosphorylated) forms.

Stimulation of cytokine production in human monocytes. Isolation of human PBM C was performed as described elsewhere (20). Samples of 5 × 10^9 PBM C in a 100-μl volume were added to round-bottom 96-well plates (Greiner, Alphen a/d Rijn, The Netherlands) and incubated with 100 μl of various strains of heat-killed (30 min at 56°C) C. albicans yeast cells at a concentration 10^5 cells/ml. After 24 h of incubation at 37°C, the PBM C-cell suspensions were centrifuged; supernatants were collected and stored at −70°C until assayed. Human tumor necrosis factor alpha (TNF-α) concentrations were determined by specific sandwich cytokine immunoassay [R&D Systems, Minneapolis, MN (catalog numbers: 80 TM59 and 80 TM10)]. Interleukin-6 (IL-6) and IL-10 concentrations were measured by commercial enzyme-linked immunosorbent assay kits (PeliPak Constant; Sanquin, Amsterdam, The Netherlands). The experiments were performed in duplicate with samples from four volunteers. The differences between strains were analyzed by using the Student t test, and the level of significance was set at P < 0.05.

Virulence tests. Female, immunocompetent BALB/c mice (Harlan Sera-Lab, Ltd., Loughborough, United Kingdom) were challenged intravenously with yeasts grown for 18 to 24 h in NGY medium at 30°C. The cells were washed twice with water and resuspended in physiological saline to give a challenge inoculum of 10^8 CFU/g of mouse body weight in a 100-μl volume. After 24 h of incubation at 37°C, the PBM C-Myc C cell suspensions were centrifuged; supernatants were collected and stored at −70°C until assayed. Virulence tests were performed as described previously (19). Interleukin-6 (IL-6) and IL-10 concentrations were measured by commercial enzyme-linked immunosorbent assay kits (PeliPak Constant; Sanquin, Amsterdam, The Netherlands). The experiments were performed in duplicate with samples from four volunteers. The differences between strains were analyzed by using the Student t test, and the level of significance was set at P < 0.05.

RESULTS

Isolation, analysis, and deletion of CcaW41 and CcaMNS1. The analysis of C. albicans genome database identified two DNA fragments as the 5' and 3' ends of CcaW41 (GenBank accession XM_718516 and XM_705221, respec-
tively). To identify the complete coding sequence, primers aligning in the 5’ and 3’ of the putative open reading frame were used to amplify CaCWH41, and the fragment was cloned and sequenced. The CaCWH41 open reading frame of 2,493 bp (GenBank/EBI accession no. DQ295807) is predicted to encode a protein of 830 amino acids showing homology to other α-glucosidase I enzymes of the glycosyl hydrolase family 63 (60% homology to CwH41 from S. cerevisiae). The predicted protein had a characteristic type II membrane protein domain structure with a single 20-amino-acid transmembrane region at the N terminus and a 2-amino-acid cytosolic tail. CaCwH41 includes a 606EELNVDIL164 sequence similar to substrate binding motifs reported in vertebrate enzymes (58). Residues Ser421, Arg467, and Gly717 are homologous to those in human α-glucosidase I, which are necessary for catalytic activity (29, 31, 77).

CaROT2 was identified in the C. albicans genome (36) by homology to the S. cerevisiae homologue. The CaROT2 open reading frame of 2,616 bp (GenBank accession no. XM_711779) is predicted to encode a protein of 871 amino acids of the glycosyl hydrolase family 31 that is between 57 and 38% identical to other fungal and mammalian Rot21 proteins (61% homology to Rot2 from S. cerevisiae). Again, this sequence was typical of a type II membrane protein, having a predicted 2-amino-acid cytosolic tail and a 20-amino-acid transmembrane region. The glycosyl hydrolase family 31 members contain a short peptide segment of conserved amino acids (DQXWIDMNEXSF), including a conserved Asp residue that is thought to be involved in catalysis (25, 32, 33, 38, 72). A similar peptide sequence (487HLWHDNNEPVSF599) is present in CaRot2, indicating that this protein also belongs to the same protein family. Consistent with its putative role in N-glycan processing, CaRot2 contains the motif HDEL in the C-terminal region responsible for the receptor-mediated retrieval of a number of ER proteins from the Golgi compartment (68).

The CaMNS1 open reading frame of 1,698 bp (GenBank/EBI accession no. AY167027) is predicted to encode a protein of 565 amino acids with significant homology to other ER glycosyl hydrolase family 47 members (65% homology to Mns1 from S. cerevisiae). This protein is 71 and 42% identical to Mns1 from fungi and mammals, respectively, and also had a characteristic type II membrane protein structure with one cytosolic amino acid and 22 amino acids in the membrane region of the N-terminal domain. Conserved catalytic and metal ion coordinating amino acid residues typical of ER α,2-mannosidases are present. These include Cys220 and Cys286 corresponding to ScMns1 Cys340 and Cys385, which are necessary for stabilization of the tertiary structure of the catalytic pocket (42), and Arg651, which would be predicted to be required for the specificity of the ER α,2-mannosidase reaction (59).

The CaCWH41, CaROT2, and CaMNS1 genes were disrupted in strain CA14 by sequential gene replacement using the mini-ura-blastor protocol (81). The resulting CaCWH41Δ (HMY-19), Carot2Δ (HMY-12), and Canberra1Δ (HMY-5) null mutants (Table 1) had URA3 reintroduced at the neutral RPS1 locus to avoid problems due to ectopic expression of URA3 (6, 48). Reintegrant control strains were also constructed in which CaCWH41, CaROT2, or CaMNS1 were introduced into the null strains under the control of their own promoters at the RPS1 locus. Strain CA14 transformed with Clp10 was used as a control in all experiments and is referred as the parent strain, equivalent to the wild type.

**Growth and morphology of the null mutants.** The CaCWH41Δ, Carot2Δ, and Canberra1Δ null mutants had reduced specific growth rates in YPD medium at 30°C of 0.44, 0.47, and 0.56 h⁻¹, respectively, compared to the parent strain (0.69 h⁻¹). The reintegrant controls had specific growth rates identical to that of the parent strain. Yeast cells of the three null mutants tended to form small aggregates, and CaCWH41Δ and Carot2Δ yeast cells were swollen and enlarged (Fig. 1A) and small and crenulated (Fig. 1B) colonies. The Canberra1Δ null mutant grew as normal hyphae in 20% (vol/vol) serum, GlcNAc-containing medium, and Lee’s medium containing 6.5% glucose, while the Carot2Δ and CaCWH41Δ null mutants had delayed filamentation and formed shorter and swollen germ tubes with decreased extension rates (data not shown). All three null mutants failed to induce filaments on solid Spider medium (Fig. 1C). In all cases the mutant phenotypes were fully complemented by reintroduction of a wild-type copy of the respective gene.

**The null mutants have altered α-glucosidase activities.** The α-glucosidase or α-mannosidase activities in the null mutants were determined by using the fluorogenic substrates 4-methylumbelliferyl-α-d-glucopyranoside (MUGluc) or 4-methylumbelliferyl-α-d-mannopyranoside (MUMann), respectively. Homogenates of CaCWH41Δ and Carot2Δ null mutants had total α-glucosidase activities of 48 and 51%, respectively, compared to the total activity present in wild-type cells (Table 2). Com-

![FIG. 1. Cell and colony morphology in the Cacwh41Δ, Carot2Δ, and Canberra1Δ null mutants. (A) Cell morphology after growth at 30°C for 16 h in YPD medium, demonstrating clumping of cells in the Cacwh41Δ (HMY19), Carot2Δ (HMY12), and Canberra1Δ (HMY5) null mutants. Scale bars, 10 μm. (B and C) Colony morphology after 5 days growth at 30°C on YPD agar plates (B) or solid Spider medium (C). Scale bars, 1 mm.](image-url)
implemented reintegrant controls recovered the wild-type activity. In wild-type cells, the α-glucosidase activity was distributed equally between the soluble and mixed membrane fractions. It was demonstrated previously that the soluble activity corresponds to α-glucosidase II (71). As predicted, the Ca\(cwh41\)Δ null mutant had no measurable membrane-associated α-glucosidase activity, whereas Carot2Δ null mutant lacked soluble α-glucosidase activity (Table 2). No measurable α-mannosidase activity was found in soluble fraction or mixed membrane preparations of the Camns1Δ null mutant (data not shown).

Glycosylation defects. We determined the consequences of the alteration in N-glycan structure in Ca\(cwh41\)Δ, Carot2Δ, and Camns1Δ null mutants by measuring changes in the electrophoretic mobility of secreted HexNAcase in native gels using an in situ activity assay (4). HexNAcase, encoded by Ca\(HMX1\), is induced in media containing GlcNAc as the sole carbon source and has been demonstrated to be highly N-glycosylated (12, 44, 50). The HexNAcase from Ca\(cwh41\)Δ, Carot2Δ, and Camns1Δ null mutants had an increased electrophoretic mobility. Mutants lacking α-glucosidase I and II had the greater mobility, indicating a more severe N-glycosylation defect than in Camns1Δ null mutant (Fig. 2). The electrophoretic mobility of HexNAcase of the reintegrant controls under inducing conditions was similar to wild type. After endoglycosidase H treatment to remove N-glycans, the HexNAcase of all mutant and parent strains migrated faster and with the same mobility (data not shown).

In C.\(albicans\) most of the acid-labile phosphomannan fraction is attached to N-linked mannan (30). Phosphomannan accounts for the negative charge of the cell wall, and this binds the cationic dye Alcian Blue. The Ca\(cwh41\)Δ, Carot2Δ, and Camns1Δ null mutants bound 17.7, 18.5, and 41.1% of the Alcian Blue bound by the parent strain, respectively (Table 3). The reintegrant controls showed the wild-type levels of Alcian Blue bound. When the Alcian Blue binding assay was carried out after elimination of O-glycans by β-elimination, the phosphomannan levels in the Carot2Δ and Ca\(cwh41\)Δ null mutants decreased from 17.7 and 18.5% to 3.2 and 3.9%, respectively (Table 3), indicating that the N-glycan phosphomannan residues were almost completely absent in these β-eliminated null mutants strains. These results indicate that Ca\(CWHI\) and Ca\(ROT2\) have an important role in N-glycan outer-chain elaboration.

Cell wall composition, sensitivity, and cell integrity pathway activation. To determine the effect of the disruption of Ca\(CWHI\), Ca\(ROT2\), and Camns1 on the overall cell wall composition, the content of total carbohydrates, and proteins was analyzed. Ca\(cwh41\)Δ and Carot2Δ null mutants showed decreases of 30.4 and 29.7% and of 65.0 and 62.9% in the contents of glucan and mannan, respectively (Table 4). Also, an increase in the chitin and protein levels of 2.2- and 3.3-fold for Ca\(cwh41\)Δ null mutant and of 2.2- and 3.2-fold for the Carot2Δ null mutant was observed. Camns1Δ null mutant had an increase of 10.2, 16.9, and 53.0% in the content of glucan, chitin, and proteins, respectively (Table 4). These changes were reflected in an overall decrease of 51.4% in the mannan levels. The reintegrant controls had cell wall compositions similar to that of wild-type yeast cells.

To investigate the effect of the ER α-glucosidase loss on the integrity of the cell wall, we tested the null mutants for their sensitivity to a range of cell-wall-perturbing agents and other compounds associated with glycosylation defects. The Camns1Δ null mutant was hypersensitive to Congo red, Calcofluor White, and hygromycin B, and Carot2Δ and Ca\(cwh41\)Δ null mutants were hypersensitive to Calcofluor White, Congo red, hygromycin B, tunicamycin, and SDS (Fig. 3). Hypersensitivity of the null mutants and reintegrant strains to Congo red and Calcofluor White was illustrated by the decreased blue binding of Alcian Blue. Hypersensitivity of the null mutants to hygromycin B was illustrated by the increased resistance to the growth of the null mutants on YPD medium supplemented with hygromycin B.

### Table 3. Alcian Blue binding of Ca\(cwh41\)Δ, Carot2Δ, and Camns1Δ null mutants and reintegrant strains

<table>
<thead>
<tr>
<th>Strain genotype</th>
<th>Mean Alcian Blue binding ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No treatment</td>
</tr>
<tr>
<td>WT</td>
<td>111 ± 5</td>
</tr>
<tr>
<td>Ca(cwh41)Δ</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Ca(cwh41)Δ + Ca(CWHI)</td>
<td>109 ± 5</td>
</tr>
<tr>
<td>Carot2Δ</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>Carot2Δ + Ca(ROT2)</td>
<td>108 ± 8</td>
</tr>
<tr>
<td>Camns1Δ</td>
<td>46 ± 5</td>
</tr>
<tr>
<td>Camns1Δ + Ca(MNS1)</td>
<td>109 ± 7</td>
</tr>
</tbody>
</table>

*Expressed as μg bound/OD\(_{600}\) = 1 cells (\(n = 3\)).

### Table 4. Cell wall composition of Ca\(cwh41\)Δ, Carot2Δ, and Camns1Δ null mutants and reintegrant strains

<table>
<thead>
<tr>
<th>Strain genotype</th>
<th>Mean amt (μg) of polymer ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucan</td>
</tr>
<tr>
<td>WT</td>
<td>546 ± 11</td>
</tr>
<tr>
<td>Ca(cwh41)Δ</td>
<td>380 ± 14</td>
</tr>
<tr>
<td>Ca(cwh41)Δ + Ca(CWHI)</td>
<td>591 ± 17</td>
</tr>
<tr>
<td>Carot2Δ</td>
<td>384 ± 10</td>
</tr>
<tr>
<td>Carot2Δ + Ca(ROT2)</td>
<td>561 ± 15</td>
</tr>
<tr>
<td>Camns1Δ</td>
<td>602 ± 15</td>
</tr>
<tr>
<td>Camns1Δ + Ca(MNS1)</td>
<td>581 ± 14</td>
</tr>
</tbody>
</table>

*That is, per mg of cell wall dry weight (\(n = 3\)).
sitivity to these agents is shared by other N-glycosylation mu-
tants of *S. cerevisiae* and *C. albicans* (2–4, 14). There were no
changes in the sensitivity to other stress-inducing agents such
as caffeine, NaCl, or KCl (data not shown). The walls of the
null mutants were therefore sensitive to cell wall stress but not
to osmotic stress. We tested whether the PKC-Mkc1 cell in-
tegrity pathway was activated in the null mutants by Western
analysis with an antibody that recognizes the phosphorylated
form of the Mkc1 mitogen-activated protein kinase (17). Mkc1
was activated in the Ca<sup>cwh41</sup>/H9004, Ca<sup>rot2</sup>/H9004,
and Ca<sup>mns1</sup>/H9004 null
mutants but not in the wild type and reintegrant controls (Fig.
4). As a positive control, the strains were stressed with 100 μg
of Calcofluor White/ml, which is known to activate the path-
way. These results reinforce the conclusion that N-mannan
processing glycosidases are required for the assembly of a
normal robust cell wall.

N-mannan processing is required for a pathogenic host-
fungus interaction. It has been demonstrated previously that
the N-linked mannosyl residues of the *C. albicans* cell wall are
involved in its recognition by monocyte and macrophages and
in the induction of proinflammatory and anti-inflammatory
cytokines by these mononuclear cells of the innate immune
system (49). Therefore, cytokine production by human PBMC
was investigated after stimulation by yeast cells of the
Ca<sup>cwh41</sup>/H9004, Ca<sup>rot2</sup>/H9004, and Ca<sup>mns1</sup>/H9004 mutants. In the Ca<sup>cwh41</sup>
null mutant stimulation of TNF, IL-6, and IL-10 was reduced
by 62, 70, and 90%, respectively (Fig. 5). Similar results were
obtained with the Ca<sup>rot2</sup> null mutant, with levels of TNF,
IL-6, and IL-10 reduced by 63, 66, and 95%, respectively (Fig. 5). Normal cytokine release was recovered in the respective reintegrant controls. TNF, IL-6, and IL-10 levels stimulated by wild-type cells were treated with laminarin before the challenge with the yeast cells to block signaling via the β-glucan/dectin-1 receptor system (49). For the wild-type cells, there was a small but statistically insignificant decrease in the stimulation of TNF. A similar reduction was observed in the null mutants and reintegrants control tested (Fig. 5A). The results indicate that recognition of the Cacwh41Δ, Carot2Δ, and Cams1Δ null mutants by the dectin-1 receptor was not a significant factor in the recognition of these stains under these conditions.

The effect of the N-mannan processing α-glucosidase loss encoded by Cacwh41, Carot2, and Cams1 on virulence was assessed in a mouse model of systemic infection. Because mutants with reduced growth rates are usually attenuated in virulence, we simply confirmed the virulence loss in a systemic mouse model using only duplicate mice. The Cacwh41Δ, Carot2Δ, and Cams1Δ null mutants were highly attenuated in virulence with a mean survival time of 21 days, compared to 5.5 days for the wild-type control (data not shown). Virulence was restored in the reintegrants controls, which displayed mean survival times similar to the wild-type cells. Therefore, normal N-glycan processing is essential for pathogenesis and a normal host-fungus interaction and virulence.

DISCUSSION

In this study, we describe the importance of N-glycan processing for normal N-glycosylation, structure, and integrity of the C. albicans cell wall and for interaction with the host. The GlcManβGlcNAcβ N-oligosaccharide is processed by ER α-glycosydases to generate the ManαGlcNAcβ core, which is a substrate for Golgi mannosyltransferases in charge of the highly branched outer-chain elaboration (27). Previous studies have demonstrated the importance of glycosylation for the cell wall structure, adherence, and virulence (3, 4, 7, 30, 46, 51, 54, 60, 69, 70, 80). For N-mannans, the N-glycan outer-chain elongation is necessary for the assembly of a normal cell wall structure and for virulence (4). We have extended these studies by analyzing the importance of N-glycan processing. Accordingly, we disrupted the C. albicans homologs of CWH41, ROT2, and MNS1 genes of S. cerevisiae, which encode the ER α-glucosidase I, the catalytic subunit of α-glucosidase II and α-mannosidase I, respectively. The Carot2Δ and Cacwh41Δ null mutants had defects in the N-glycan outer-chain elongation, evidenced by the loss of cell wall phosphomannan and increased mobility of HexNAcase, and had a weakened cell wall. Null mutants were strongly affected in virulence and in their ability to induce cytokine production by PBMC. These defects were similar but less dramatic than those displayed by Cacwh1Δ null mutant, which lacks the entire N-glycan outer chain (4). The Cams1Δ null mutant had a milder phenotype than the Cacwh41Δ and Carot2Δ null mutants, in terms of specific growth rate, phosphomannan content, HexNAcase mobility, and cell wall integrity, but was still significantly affected in virulence and had an altered cytokine induction profile. Therefore, N-mannan processing is important for the host-fungus interaction of C. albicans, but alterations in core-mannan production at different steps resulted in different phenotypes.

We previously demonstrated, using the fluorogenic substrate MUA-Man, that C. albicans α1,2-mannosidases belong to the glycosyl hydrolase family 47 and are present in both soluble and membrane-bound forms (45; H. M. Mora-Montes et al., unpublished data). The total absence of α-mannosidase activity in the Cams1Δ null mutant indicates that Cams1 is likely to encode both the soluble and the membrane-bound activities. The absence of soluble α-glucosidase activity in the Carot2Δ null mutant agrees with previous studies indicating that in C.
albicans the α-glucosidase II activity is associated with a soluble 47-kDa polypeptide (71). Because the molecular mass of this soluble protein is lower than that predicted for CaRot2, it is possible that the α-glucosidase II activity is processed by a protease to generate a soluble catalytic domain.

The Cawh41Δ, Carot2Δ, and Cams1Δ null mutants had 36, 32, and 19% reductions, respectively, in the growth rate of the yeast form. This contrasts with previous reports in S. cerevisiae, wherein no defects on the growth rates were observed in mutants lacking α-glucosidase I or II (21, 74). Yeast cells of the Cawh41Δ, Carot2Δ, and Cams1Δ null mutants tended to clump as small aggregates. Aggregation may be the result of changes in the cell wall hydropathy due to decreased charged phosphomannan content. Alternately, clumping may be the result of a cell separation defect due to alterations in the activity of glycosylated cell wall hydrolases that participate in cytokinesis. A similar clumping phenotype has been reported for other C. albicans glycosylation mutants such as the Camnt1Δ/Camnt2Δ (46), Capmr1Δ (3), Coaeh1Δ (4), Cammn9Δ (66), and Cavrg4Δ (51) null mutants.

Evidence was found for defects in N-glycosylation in the three null mutants generated. This was more severe in the Cawh41Δ and Carot2Δ null mutants, as demonstrated by the underglycosylation of HextNacase. HextNacase is exclusively N glycosylated, is readily detected in nondenaturing PAGE gels, and has been used as a sensitive marker of N-glycosylation defects (4). Loss of CaCWH41 and CaROT2 resulted in defects in N-glycosylation demonstrated by the increased mobility of HextNacase and a reduction in N-glycan-linked phosphomannan content of the cell wall. The remaining phosphomannan present in the null mutants was attached to O-mannan and could be removed by β-elimination. A similar result was observed in the Coaeh1Δ null mutant, where the N-glycan outer-chain elongation is blocked (4). Therefore, the presence of glucose residues on the N-glycan core may inhibit the activity of the CaOeh1 α1,6-mannosyltransferase to initiate the outer-N-chain elongation. The absence of α-glucosidase I activity in S. cerevisiae did not prevent outer-chain formation or the addition of α1,3-mannose residues to the core oligosaccharides (75), suggesting that the importance of the N-glycan core glucose residues for subsequent outer-chain elongation may be different in C. albicans and S. cerevisiae. The N-glycosylation defect in Cams1Δ null mutant was not as severe as in Carot2Δ and Cawh41Δ null mutants, indicating that partial elongation of the N-glycan core occurred. This indicates that the removal of mannose from the N-glycan core is not required for outer-chain synthesis, as found in S. cerevisiae (55).

The Cawh41Δ, Carot2Δ, and Cams1Δ null mutants had an altered and weakened cell wall, as demonstrated by changes in cell wall composition and hypersensitivity to a range of cell-wall-perturbing agents and other agents whose action is indicative of glycosylation defects. The consequences of CaCwb41, CaRot2, or CaMns1 loss resulted in the constitutive activation of the PKC-Mkc1 cell integrity pathway. A similar result was observed in other C. albicans null mutants with defects in glycosylation (3, 4, 14). Similar observations have been made for S. cerevisiae, where the lack α-glucosidase I or II leads to alterations in the cell wall composition (35, 63, 65). The Cams1Δ null mutant was least affected by cell-wall-perturbing agents, correlating with a milder N-glycosylation mutant phenotype. Disruption of CaCWH41 and CaROT2 resulted in a 30% reduction in the cell wall β-glucan content, but loss of CaMNS1 did not affect the levels of this polymer. This suggests that proteins involved in the biosynthesis of β-glucans are sensitive to changes in the N-glycosylation pathway. However, C. albicans och1Δ mutants that lack N-glycan outer chain do not have reduced β-glucan levels (4). This suggests that the β-glucan reduction in the walls of Cawh41Δ and Carot2Δ null mutants may be due to a failure in the glycopolypeptide quality control/protein refolding pathways that are dependent on glycosylation of the N-mannan core rather than the shortening of the N-glycan outer chain. Indeed, it has been demonstrated in S. cerevisiae that the presence of glucose residues on the N-mannan core led to an instability of Kre6, a protein required for β1,6-glucan biosynthesis, resulting in decreased levels of β1,6-glucan in the cell wall (1). There was also no change in sensitivity to high salt conditions in the three null mutants, indicating that the mutants were not osmotically fragile and that the SDS sensitivity of the Cawh41Δ and Carot2Δ null mutants is likely to be the result of the cell wall perturbation rather than effects on the plasma membrane.

The Cawh41Δ, Carot2Δ, and Cams1Δ mutants were significantly attenuated in virulence in a mouse model of systemic infection, as has been demonstrated for other C. albicans N-glycosylation null mutants (4, 66).

The balance of pro- and anti-inflammatory response is known to be important for the outcome of a number of fungal infections (13, 18, 40, 57). Decreased levels of TNF, IL-6, and IL-10 were observed with human PBMC challenged with Cawh41Δ and Carot2Δ null mutants. This finding is in agreement with the observation that approximately 70% less cytokine production was stimulated in PBMC by the CaOeh1Δ null mutant (49). The disruption of CaMNS1 did not affect the release of IL-6 and IL-10 but did result in increased release of TNF. This suggests that IL-6 and IL-10 may be stimulated by a different cell wall epitope than that which activates PBMC to release TNF. These effects on cytokine production are not likely to be mediated via the dectin-1 receptor since laminarin-treated PBMC gave similar responses to untreated PBMC in all cases. Therefore, N-glycan core processing is vital to the cell wall architecture of C. albicans, to its pathogenesis, and for host-fungus interactions.

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