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

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Received 11 July 2007/Accepted 27 September 2007

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 GlcMan9GlcNAc2 N-glycan is processed by α-glucosidases I and II and α1,2-mannosidase to generate Man9GlcNAc2. This N-oligosaccharide is then elaborated in the Golgi to form N-glycans with highly branched outer chains rich in mannose. In Saccharomyces cerevisiae, CWI41, ROT2, and MNS1 encode for α-glucosidase I, α-glucosidase II catalytic subunit, and α1,2-mannosidase, respectively. We disrupted the C. albicans CWI41, 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 C. albicans CWI41Δ, Carot2Δ, and Cunns1Δ 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-acetylmuramidase, 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 proteins (10, 52, 53, 61). The cell wall of this fungus and other Candida species is heterogenous, composed of β-glucans and α-glucans, and proteins (10, 52, 53, 61). The β-glucans are predominantly mannoproteins, and the α-glucans are complex branched 1,3- and 1,6-linked α-D-mannosides as well as 1,3- and 1,6-linked α-D-glucans (10, 52, 53, 61). The terminal mannose residues of O-glycans are attached by 1,3- and 1,6-linkages rather than 1,2-linkages as in S. cerevisiae (46), and β1,2-linked mannose residues are attached by α1,2-linkages rather than α1,3-mannose residues as in S. cerevisiae (46), and β1,2-linked mannose residues are attached by α1,2-linkages rather than α1,3-mannose residues as in S. cerevisiae (46).

Saccharomyces cerevisiae. N glycosylation occurs by a stepwise process involving the transfer of GlcMan9GlcNAc2 from GlcMan9GlcNAc2-PP-dolichol to specific asparagine residues of nascent proteins in the endoplasmic reticulum (ER). Subsequently, the GlcMan9GlcNAc2 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 attached by α1,2-linkages rather than α1,3-mannose residues as in S. cerevisiae (46).
In S. cerevisiae, CWH41 (GLS1), ROT2 (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 Glc₃Man₃GlcNAc₂ before it is trimmed further by α-glucosidase II, which removes the α,1,3-glucose residues from Glc₃Man₃GlcNAc₂ (23, 62, 76). In S. cerevisiae, the α-glucosidase II is a heterodimer with the catalytic α-subunit encoded by ROT2, a member of the glycosyl hydrolyase 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 Man₃GlcNAc₂ 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 processing (27, 8). 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

| Strain | Parent strain | Genotype | Source or reference |
|--------|---------------|----------|---------------------|
| CAI4   | CAF2-1        | ura3Δ::imm434/ura3Δ::imm434 | 22 |
| NGY152 | CAI4          | Same as CAI4 but RPS1/ps1Δ::Clp10 | 6 |
| HMY1   | CAI4          | Same as CAI4 but MNS1/mns1::dp1200-URA3-dp1200 | This study |
| HMY2   | HMY1          | Same as CAI4 but MNS1/mns1::dp1200 | This study |
| HMY3   | HMY2          | Same as CAI4 but mns1::dp1200/mns1::dp1200-URA3-dp1200 | This study |
| HMY4   | HMY3          | Same as CAI4 but mns1::dp1200/mns1::dp1200 | This study |
| HMY5   | HMY4          | Same as CAI4 but mns1::dp1200/mns1::dp1200 | This study |
| HMY6   | HMY5          | Same as CAI4 but mns1::dp1200/mns1::dp1200 | This study |
| HMY7   | HMY6          | Same as CAI4 but mns1::dp1200 | This study |
| HMY8   | CAI4          | Same as CAI4 but ROT2/rot2Δ::dp1200-UR3-dp1200 | This study |
| HMY9   | HMY8          | Same as CAI4 but RPS1/ps1Δ::dp1200 | This study |
| HMY10  | HMY9          | Same as CAI4 but RPS1/ps1Δ::dp1200 | This study |
| HMY11  | HMY10         | Same as CAI4 but RPS1/ps1Δ::dp1200 | This study |
| HMY12  | HMY11         | Same as CAI4 but RPS1/ps1Δ::dp1200-UR3-dp1200 | This study |
| HMY13  | HMY12         | Same as CAI4 but RPS1/ps1Δ::dp1200-UR3-dp1200 | This study |
| HMY14  | HMY13         | Same as CAI4 but RPS1/ps1Δ::dp1200 | This study |
| HMY15  | CAI4          | Same as CAI4 but CWH41/cwh41::dp1200-URA3-dp1200 | This study |
| HMY16  | HMY15         | Same as CAI4 but CWH41/cwh41::dp1200-URA3-dp1200 | This study |
| HMY17  | HMY16         | Same as CAI4 but CWH41/cwh41::dp1200-URA3-dp1200 | This study |
| HMY18  | HMY17         | Same as CAI4 but CWH41/cwh41::dp1200-URA3-dp1200 | This study |
| HMY19  | HMY18         | Same as CAI4 but CWH41/cwh41::dp1200-URA3-dp1200 | This study |
| HMY20  | HMY19         | Same as CAI4 but CWH41/cwh41::dp1200-URA3-dp1200 | This study |
| HMY21  | HMY20         | Same as CAI4 but CWH41/cwh41::dp1200-URA3-dp1200 | This study |

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 Ca⁺²/Mn⁺²-ATPase involved in the transport of Ca⁺² and Mn⁺² 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, ROT2, 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.
erone (MU) was read in a spectrofluorometer with excitation and emission set at 360 nm (M SERVICES glove box). Control samples, with water and resuspended at OD600 of 1.0. These preparations.

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First, the lysate was centrifuged at 21,500 x g at 20°C until complete. Fraction (10 to 100 µl of protein) were incubated at 37°C with 40 µg MUCan-M or MUCan-G and buffer A for assay of α-mannosidase or α-glucosidase, respectively, in a final volume of 200 µl. After 30 min, the reaction was stopped by addition of 3.0 ml of 50 mM glycine-NaOH buffer (pH 11.0), and the fluorescence of 4-methylumbelliferone (MU) was measured by using fluorogenic substrates as described previously (45). In situ β-N-acetylhexosaminidase activity staining. Zymograms of native poly-
Mns1 from fungi and mammals, respectively, and also had a characteristic type II membrane protein structure with one cysteolic 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,1,2-mannosidases are present. These include Cys^{320} and Cys^{390} corresponding to ScMns1 Cys^{340} and Cys^{380}, which are necessary for stabilization of the tertiary structure of the catalytic pocket (42), and Arg^{351}, which would be predicted to be required for the specificity of the ER α,1,2-mannosidase reaction (59).

The CaCW41, CaROT2, and CaMNS1 genes were disrupted in strain CA14 by sequential gene replacement using the mini-ura-blaster protocol (81). The resulting CaCW41Δ (HMY-19), CaRot2Δ (HMY-12), and CaMns1Δ (HMY-5) null mutants (Table 1) had URA3 reintroduced at the neutral RSP1 locus to avoid problems due to ectopic expression of URA3 (6, 48). Reintegrant control strains were also constructed in which CaCW41, 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 CaCW41Δ, CaRot2Δ, and CaMns1Δ 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 reintegant controls had specific growth rates identical to that of the parent strain. Yeast cells of the three null mutants tended to form small and crenulated (Fig. 1B) colonies. The CaMns1Δ null mutant grew as normal hyphal in 20% (vol/vol) serum, GlcNAc-containing medium, and Lee's medium at pH 6.5, while the CaRot2Δ and CaCW41Δ 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 reintegration 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 (MUGl) or 4-methylumbelliferyl-α-D-mannopyranoside (MUMaMan), respectively. Homogenates of CaCW41Δ 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 CaCW41Δ, CaRot2Δ, and CaMns1Δ null mutants. (A) Cell morphology after growth at 30°C for 16 h in YPD medium, demonstrating clumping of cells in the CaCW41Δ (HMY19), CaRot2Δ (HMY12), and CaMns1Δ (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)
plemented 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 Cačwh41Δ 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 Cačwh41Δ, 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 CaHEXI, 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 Cačwh41Δ, 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 Cačwh41Δ, 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 Cačwh41Δ 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. These results indicate that CaCWΗ41 and Carot2Δ have an important role in N-glycan outer-chain elaboration.

Table 3: Alcian Blue binding of Cačwh41Δ, Carot2Δ, and Camns1Δ null mutants and reintegrant strains

| Strain genotype | Mean Alcian Blue binding ± SD* |
|-----------------|---------------------------------|
| WT              | 111 ± 5                         |
| Cačwh41Δ        | 20 ± 3                          |
| Cačwh41Δ + CaCWΗ41 | 109 ± 5                       |
| Carot2Δ         | 21 ± 5                          |
| Carot2Δ + Carot2Δ | 108 ± 8                       |
| Camns1Δ         | 46 ± 5                          |
| Camns1Δ + Camns1Δ | 109 ± 7                       |

*Expressed as µg bound/OD600 - 1 cells (n = 3).

Cell wall composition, sensitivity, and cell integrity pathway activation. To determine the effect of the disruption of CaCWΗ41, Carot2Δ, and Camns1Δ on the overall cell wall composition, the content of total carbohydrates, and proteins was analyzed. Cačwh41Δ 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 the Cačwh41Δ 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 Cačwh41Δ null mutants were hypersensitive to Calcofluor White, Congo red, hygromycin B, tunicamycin, and SDS (Fig. 3). Hypersen-

Table 4: Cell wall composition of Cačwh41Δ, Carot2Δ, and Camns1Δ null mutants and reintegrant strains

| Strain genotype | Glucan (µg) | Mannan (µg) | Chitin (µg) | Protein (µg) |
|-----------------|-------------|-------------|-------------|--------------|
| WT              | 546 ± 11    | 276 ± 15    | 18 ± 2      | 140 ± 7      |
| Cačwh41Δ        | 380 ± 14    | 97 ± 11     | 41 ± 2      | 459 ± 6      |
| Cačwh41Δ + CaCWΗ41 | 591 ± 17    | 246 ± 11    | 18 ± 1      | 124 ± 8      |
| Carot2Δ         | 384 ± 10    | 102 ± 13    | 40 ± 2      | 444 ± 8      |
| Carot2Δ + Carot2Δ | 561 ± 15    | 277 ± 12    | 17 ± 1      | 120 ± 7      |
| Camns1Δ         | 602 ± 18    | 134 ± 13    | 21 ± 2      | 215 ± 6      |
| Camns1Δ + Camns1Δ | 581 ± 14    | 258 ± 15    | 18 ± 4      | 129 ± 10     |

*That is, per mg of cell wall dry weight (n = 3).
sensitivity to these agents is shared by other N-glycosylation mutants 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 integrity 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>ccwh41</sup>/H9004, Ca<sup>cror2</sup>/H9004, and Ca<sup>cmns1</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 pathway. 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>ccwh41</sup>, Ca<sup>cror2</sup>, and Ca<sup>cmns1</sup> mutants. In the Ca<sup>ccwh41</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>cror2</sup> null mutant, with levels of TNF,

![Figure 3](image1.png)

**FIG. 3.** Sensitivity of Ca<sup>ccwh41</sup>, Ca<sup>cror2</sup>, and Ca<sup>cmns1</sup> null mutants to cell-wall-perturbing agents. Wild type (▲), null mutants (○), and reintegrant controls (●) strains were tested for sensitivity to cell-wall-perturbing agents by using the microdilution method. The strains tested were the Ca<sup>ccwh41</sup> (HMY19) (A), Ca<sup>cror2</sup> (HMY12) (B), and Ca<sup>cmns1</sup> (HMY5) (C) null mutants. Error bars indicate the means ± the standard deviation (n = 3). The results are pooled data from duplicate experiments.

![Figure 4](image2.png)

**FIG. 4.** Activation of the cell integrity pathway in glycosidase null mutants assessed by Western analysis. Protein extracts were prepared from cells in mid-exponential phase. As a positive control for activation of the cell integrity pathway, the strains were treated with Calcofluor White (100 µg/ml) as indicated. Extracts are from the following strains: NGY152 (wild type), HMY19 (Ca<sup>ccwh41</sup>), HMY20 (Ca<sup>ccwh41</sup> + Ca<sup>WH41</sup>), HMY12 (Ca<sup>cror2</sup>), HMY13 (Ca<sup>cror2</sup> + Ca<sup>ROT2</sup>), HMY5 (Ca<sup>cmns1</sup>), and HMY6 (Ca<sup>cmns1</sup> + Ca<sup>MNS1</sup>). Equal loading was confirmed by Ponceau S staining and determining the intensity of nonspecific bands.
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 the Ca\textsuperscript{mns1}\textsuperscript{Δ} null mutant were not statistically different (P = 0.1001, P > 0.5, and P > 0.5, respectively) from those stimulated by wild-type cells (Fig. 5). In order to determine whether the changes in the cell wall of the null mutants led to exposure of elements present in the inner layers such as the β-glucans, human PBMC 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 Ca\textsuperscript{mns1}\textsuperscript{Δ} 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 Ca\textsuperscript{CWH41}, Ca\textsuperscript{ROT2}, and Ca\textsuperscript{MNS1} 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 Ca\textsuperscript{mns1}\textsuperscript{Δ} null mutants were highly attenuated in virulence and had an altered cytokine induction profile. 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 Cacwh41Δ null mutant, which lacks the entire N-glycan outer chain (4). The Ca\textsuperscript{mns1}\textsuperscript{Δ} 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 \textit{C. albicans}, but alterations in core-mannan production at different steps resulted in different phenotypes.

We previously demonstrated, using the fluorogenic substrate MUA\textsubscript{Man}, that \textit{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 Ca\textsuperscript{mns1}\textsuperscript{Δ} null mutant indicates that Ca\textsuperscript{MNS1} 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 \textit{C. albicans}.
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 Cacb41Δ, Carot2Δ, and Camms1Δ 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 Cacb41Δ, Carot2Δ, and Camms1Δ null mutants tended to clump as small aggregates. Aggregation may be the result of changes in the cell wall hydrophobicity due to decreased charged phosphomannan content. Alternatively, 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), Capmt1Δ (3), Cloch1Δ (4), Cammr9Δ (66), and Cavg4Δ (51) null mutants.

Evidence was found for defects in N glycosylation in the three null mutants generated. This was more severe in the Cacb41Δ and Carot2Δ null mutants, as demonstrated by the underglycosylation of HenNAcase. HenNAcase is exclusively N glycosylated, is readily detected in non-denaturing PAGE gels, and has been used as a sensitive marker of N-glycosylation defects (4). Loss of CaCWH41 or CaROT2 resulted in defects in N glycosylation demonstrated by the increased mobility of HenNAcase 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 Cloch1Δ 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 ability of the Caoch1 α,1,6-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 α,1,3-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 Camms1Δ null mutant was not as severe as in Carot2Δ and Cacb41Δ 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 Cacb41Δ, Carot2Δ, and Camms1Δ 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 Camms1Δ 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 Cacb41Δ and Carot2Δ null mutants may be due to a failure in the glycoprotein 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 Cacb41Δ and Carot2Δ null mutants is likely to be the result of the cell wall perturbation rather than effects on the plasma membrane.

The Cacb41Δ, Carot2Δ, and Camms1Δ 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 Cacb41Δ and Carot2Δ null mutants. This finding is in agreement with the observation that approximately 70% less cytokine production was stimulated in PBMC by the Cloch1Δ 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.

ACKNOWLEDGMENTS

This study was supported by grant CONACyT-2002-CO1-39528/A from the Consejo Nacional de Ciencia y Tecnología, México, and the Dirección de Investigación y Posgrado, Universidad de Guanajuato, and by Wellcome Trust Programme grants 063204 and 080088 to N.A.R.G., A.J.P.B., and F.C.O.

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GLYCOSIDASES OF C. ALBICANS

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