EUKARYOTIC CELL, Dec. 2007, p. 2184–2193 Vol. 6, No. 12
1535-9778/07/$08.00+0 doi:10.1128/EC.00350-07

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 $Glc_3Man_9GlcNAc_2$ N-glycan is processed by α-glucosidases I and II and α1,2-mannosidase to generate $Man_8GlcNAc_2$. 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 Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ 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 β1,3- and β1,6-glucans and an outer layer that is rich in mannoproteins that 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 $Glc_3Man_9GlcNAc_2$ from $Glc_3Man_9GlcNAc_2$-PP-dolichol to specific asparagine residues of nascent proteins in the endoplasmic reticulum (ER). Subsequently, the $Glc_3Man_9GlcNAc_2$ 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 $Man_8GlcNAc_2$ 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 $Man_8GlcNAc_2$ core. In the *och1*Δ null mutant of *C. albicans*, the $Man_8GlcNAc_2$ 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

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▽ Published ahead of print on 12 October 2007.

TABLE 1. Strains used in this study

Strain	Parent strain	Genotype	Source or reference
CAI4	CAF2-1	*ura3*Δ::*imm434*/*ura3*Δ::*imm434*	22
NGY152	CAI4	Same as CAI4 but *RPS1*/*rps1*Δ::CIp10	6
HMY1	CAI4	Same as CAI4 but *MNS1*/*mns1*Δ::*dp1200-URA3-dp1200*	This study
HMY2	HMY1	Same as CAI4 but *MNS1*/*mns1*Δ::*dp1200*	This study
HMY3	HMY2	Same as CAI4 but *mns1*Δ::*dp1200*/*mns1*Δ::*dp1200-URA3-dp1200*	This study
HMY4	HMY3	Same as CAI4 but *mns1*Δ::*dp1200*/*mns1*Δ::*dp1200*	This study
HMY5	HMY4	Same as CAI4 but *mns1*Δ::*dp1200*/*mns1*Δ::*dp1200*, *RPS1*/*rps1*Δ::CIp10	This study
HMY6	HMY4	Same as CAI4 but *mns1*Δ::*dp1200*/*mns1*Δ::*dp1200*, *RPS1*/*rps1*Δ::CIp10-*MNS1*	This study
HMY7	HMY2	Same as CAI4 but *MNS1*/*mns1*Δ::*dp1200*, *RPS1*/*rps1*Δ::CIp10	This study
HMY8	CAI4	Same as CAI4 but *ROT2*/*rot2*Δ::*dp1200-URA3-dp1200*	This study
HMY9	HMY8	Same as CAI4 but *ROT2*/*rot2*Δ::*dp1200*	This study
HMY10	HMY9	Same as CAI4 but *rot2*Δ::*dp1200*/*rot2*Δ::*dp1200-URA3-dp1200*	This study
HMY11	HMY10	Same as CAI4 but *rot2*Δ::*dp1200*/*rot2*Δ::*dp1200*	This study
HMY12	HMY11	Same as CAI4 but *rot2*Δ::*dp1200*/*rot2*Δ::*dp1200*, *RPS1*/*rps1*Δ::CIp10	This study
HMY13	HMY11	Same as CAI4 but *rot2*Δ::*dp1200*/*rot2*Δ::*dp1200*, *RPS1*/*rps1*Δ::CIp10-*ROT2*	This study
HMY14	HMY9	Same as CAI4 but *ROT2*/*rot2*Δ::*dp1200*, *RPS1*/*rps1*Δ::CIp10	This study
HMY15	CAI4	Same as CAI4 but *CWH41*/*cwh41*Δ::*dp1200-URA3-dp1200*	This study
HMY16	HMY15	Same as CAI4 but *CWH41*/*cwh41*Δ::*dp1200*	This study
HMY17	HMY16	Same as CAI4 but *cwh41*Δ::*dp1200*/*cwh41*Δ::*dp1200-URA3-dp1200*	This study
HMY18	HMY17	Same as CAI4 but *cwh41*Δ::*dp1200*/*cwh41*Δ::*dp1200*	This study
HMY19	HMY18	Same as CAI4 but *cwh41*Δ::*dp1200*/*cwh41*Δ::*dp1200*, *RPS1*/*rps1*Δ::CIp10	This study
HMY20	HMY18	Same as CAI4 but *cwh41*Δ::*dp1200*/*cwh41*Δ::*dp1200*, *RPS1*/*rps1*Δ::CIp10-*CWH41*	This study
HMY21	HMY16	Same as CAI4 but *CWH41*/*cwh41*Δ::*dp1200 RPS1*/*rps1*Δ::CIp10	This study

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*), *ROT2* (*GLS2*), and *MNS1* encode three ER enzymes: α-glucosidase I, α-glucosidase II catalytic subunit, and α1,2-mannosidase, respectively, which are involved in N-glycan core processing (27). Cwh41 removes the outermost α1,2-glucose residue of $Glc_3Man_9GlcNAc_2$ before it is trimmed further by α-glucosidase II, which removes the α1,3-glucose residues from $Glc_2Man_9GlcNAc_2$ (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 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 α1,2-mannosidase that trims the $Man_9GlcNAc_2$ oligosaccharide to $Man_8GlcNAc_2$ 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-glycosidases 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 Ca*VRG4* and Ca*SRB1*, 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-glycosidases 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 (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 (HexNAcase) synthesis, cells were grown in SC+GlcNAc (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] neopeptone, 0.4% [wt/vol] glucose, and 0.1% [wt/vol] yeast extract) at 30°C.

Construction of null mutants and control strains. The *MNS1*, *ROT2*, and *CWH41* genes were disrupted by the "mini-ura-blaster" protocol (81). To make the disruption cassette, long primers containing 70-pb 5′ and 3′ regions of the open reading frames were used to amplify by PCR the *dp1200-URA3-dp1200* cassette contained in plasmid pDDB57 (81): for *MNS1*, primer pair 5′-ATGCT ATTAAAAGGTTTTATGTTGTCTTTAGTATTATATGCTGTGTACCATT TAGCATCAAATGGTGGGCTGTGGAATTGTGAGCGGATA-3′ and 5′-TT ACCCAGCAATTTCTTCAATAATTTCTTTAGCTTCTTGATCAGCTGATT

TATCAACTGGTTGAGCTTCCGTTTTCCCAGTCACGACGTT-3′; for *ROT2*, primer pair 5′-ATGAAATTATTTCTAACAATAATTTTTATAATTGCGTCA GTGAATGCTGTAAAGGAGTACTTGTTCAAATTGTGGAATTGTGAGC GGATA-3′ and 5′-TTATAGTTCGTCATGCTCGATTTTTCTGTGGCTGTC AAAACTAAAGGGGAGACTCCAATCCAGGTTGATGGTTTTCCCAGTC ACGACGTT-3′; and for *CWH41*, primer pair 5′-ATGAGATTGTTATCGTG GTTGCCATTTGTATTTCTTTTGGAAGTGATATTTGCCAGCAATCAAA TAAAATTGTGGAATTGTGAGCGGATA-3′ and 5′-TCATTGCAAATGTT CAGGCATTGTCATCATTATCAAGACCAAAGATGACCAGCCCAAAAA GTTTTTAGCTGTTTTCCCAGTCACGACGTT-3′; the regions complementary to plasmid pDDB57 are underlined. The genes were disrupted by sequential rounds of transformation of strain CAI4 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$^-$ *mns1*Δ, *rot2*Δ, and *cwh41*Δ null strains were transformed with StuI-digested CIp10 plasmid (48); hence, *URA3* was expressed at the *RPS1* locus. To construct reintegrant 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′-GCGGCCGCTAAGATCAACTTTTTTCTAT TC-3′ and 5′-GCGGCCGCAATGATACCAATAAGGA-3′; primer pair for *ROT2*, 5′-GCGGCCGCAATCTTATTAGCTCCAGAC-3′ and 5′-GCGGCCG CATACCGTACCAAGAAA-3′; and primer pair for *CWH41*, 5′-GCGGCCGC TTATCTAGACAAATGTTTAAAAT-3′ and 5′-GCGGCCGCTTAGATACT GAACGTCATT-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 by NotI digestion and subcloned into the NotI site of CIp10. The resulting plasmids were digested with StuI and used to transform the Ura$^-$ null strains. As a further control, strain CAI4 was transformed with StuI-digested CIp10. 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 collected by low-speed centrifugation, washed twice with 50 mM sodium phosphate buffer (pH 6.0) (buffer A), and broken with glass beads using a FastPrep machine (Qbiogene), and the homogenate was centrifuged at 21,500 × *g* for 10 min. The supernatant obtained was subjected to ultracentrifugation at 105,000 × *g* for 1 h, and the high-speed supernatant and the mixed membrane fraction were collected, freeze-dried, and kept at −20°C until use. Fractions (10 to 100 μg of protein) were incubated at 37°C with 40 μM MUαMan or MUαGlc 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 adding 3.3 ml of 50 mM glycine-NaOH buffer (pH 11.0), and the fluorescence of 4-methylumbelliferone (MU) was read in a spectrofluorometer with excitation and emission set at 350 and 440 nm, respectively. Specific activity was expressed as nmoles of MU liberated per minute per milligram of protein (1 U = 1 nmol of MU). Total activity is referred as units of specific activity liberated by the total protein of the preparations.

Sensitivity testing. Strains that were tested for sensitivity to specific cell-wall-perturbing agents were initially grown for 24 h in YPD medium and then washed with water and resuspended at an optical density at 600 nm (OD_{600}) of 1.0. These cells were inoculated into YPD medium at an OD_{600} of 0.01, and 95-μl aliquots were spotted into microdilution plate wells. Test agents in 5-μl volumes were added at a range of doubling dilutions. Duplicate plates were incubated for 16 h at 30°C, and the OD_{600} was determined. The agents tested were Calcofluor White (100 μg/ml), Congo red (100 μg/ml), 0.1% sodium dodecyl sulfate (SDS), hygromycin B (500 μg/ml), salts (NaCl and KCl at 1 M), caffeine (50 mM), and tunicamycin (100 μg/ml).

Alcian Blue binding assays. Alcian Blue affinity assays for phosphomannan content were carried out as described previously (4, 30). Assays were also performed on β-eliminated cells that were stripped of O-glycans, by overnight treatment with 100 mM NaOH at room temperature and subsequent washing of the cells with water.

Analysis of the cell wall composition. Yeast cells were grown in YPD medium at 30°C and broken as described above. The homogenate was centrifuged at 1,000 × *g* for 10 min, and the pellet, containing the cell debris and walls, was washed with 1 M NaCl, resuspended in buffer (500 mM Tris-HCl buffer [pH 7.5], 2% [wt/vol] SDS, 0.3 M β-mercaptoethanol, 1 mM EDTA), boiled for 10 min, and freeze-dried. The β-glucan, mannan, and chitin levels were determined by hydrolysis of those polymers and quantification of glucose, mannose, and glucosamine, respectively. For quantification of glucose and mannose, cell walls were resuspended in 2 M trifluoroacetic acid, boiled for 3 h, washed, and centrifuged at 21,500 × *g* for 10 min. The hydrolysates were analyzed by high-performance anion-exchange chromatography as described previously (45). For the determination of chitin content, the cell walls were hydrolyzed and analyzed as described previously (37). For total protein determination, cell walls were resuspended in 1 N NaOH, boiled for 30 min, neutralized with 1 N HCl, and assayed by using the method described by Bradford (5).

In situ β-*N*-acetylhexosaminidase activity staining. Zymograms of native polyacrylamide gel electrophoresis (PAGE) were assayed as reported previously (4). After the growth for 16 h in SC plus GlcNAc, the cells were washed, resuspended in 10 mM Tris-HCl buffer (pH 8.0) containing protease inhibitor cocktail (Roche, Lewes, United Kingdom), and broken with glass beads in a FastPrep machine, and the homogenate was centrifuged at 21,500 × *g* for 10 min. For deglycosylation treatment, the native sample was incubated with 25 mU of endoglycosidase H (Roche) for 16 h at 37°C in 50 mM sodium acetate buffer (pH 5.2). Samples were mixed with native loading dye (62.5 mM Tris-HCl buffer [pH 6.8], 0.01% [wt/vol] bromophenol blue, and 15% [vol/vol] glycerol) and run on a 3 to 8% Tris-acetate PAGE gel (Invitrogen, Paisley, United Kingdom) for 1 h at 19 V/cm under nondenaturing conditions. The gel was washed 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-GlcNAc [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 integrity 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 Calcofluor White (100 μg/ml) 2 h before collection. Cells were washed, resuspended in extraction buffer (100 mM Tris-HCl buffer [pH 7.5], 0.01% [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 electrotransferred to a polyvinylidene difluoride membrane. The membrane was blocked in phosphate-buffered saline plus 0.1% Tween 20 and 5 mg of bovine serum albumin/ml for 2 h at room temperature. Detection was then carried out with the PhosphoPlus p44/42 mitogen-activated protein kinase (Thr202/Tyr204) antibody kit (Cell Signaling Technology, Hertfordshire, United Kingdom), which cross-reacts with *C. albicans* Mkc1 (Slt2) in both its active (phosphorylated) and inactive (nonphosphorylated) forms.

Stimulation of cytokine production in human monocytes. Isolation of human PBMC was performed as described elsewhere (20). Samples of 5×10^5 PBMC 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 10^6 cells/ml. After 24 h of incubation at 37°C, the PBMC-*C. albicans* 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 radioimmunoassays as described previously (19). Interleukin-6 (IL-6) and IL-10 concentrations were measured by commercial enzyme-linked immunosorbent assay kits (Pelikine Compact; 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 1.8×10^4 CFU/g of mouse body weight in a 100-μl volume. For mutants with attenuated growth in vitro, virulence attenuation in vivo was confirmed in groups of two mice intravenously inoculated and monitored over 21 days. Mice showing signs of illness were humanely terminated, and their deaths recorded as occurring the following day.

Nucleotide sequence accession numbers. The Ca*CWH41* and Ca*MNS1* sequences have been submitted to GenBank and were assigned accession numbers DQ295807 and AY167027, respectively.

RESULTS

Isolation, analysis, and deletion of Ca*CWH41*, Ca*ROT2*, and Ca*MNS1*. The analysis of *C. albicans* genome databank identified two DNA fragments as the 5′ and 3′ ends of Ca*CWH41* (GenBank accession XM_718516 and XM_705221, respec-

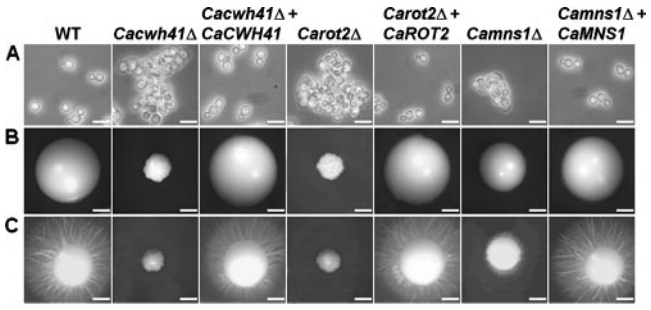

FIG. 1. Cell and colony morphology in the Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ null mutants. (A) Cell morphology after growth at 30°C for 16 h in YPD medium, demonstrating clumping of cells in the Ca*cwh41*Δ (HMY19), Ca*rot2*Δ (HMY12), and Ca*mns1*Δ (HMY5) null mutants. Scale bars, 10 μm. (B and C) Colony morphology after 5 days growth at 30°C on YPD agar plates (B) or solid Spider medium (C). Scale bars, 1 mm.

tively). To identify the complete coding sequence, primers aligning in the 5′ and 3′ of the putative open reading frame were used to amplify Ca*CWH41*, and the fragment was cloned and sequenced. The Ca*CWH41* 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 ^{606}**ELNVDLISW**^{614} sequence similar to substrate binding motifs reported in vertebrate enzymes (58). Residues Ser^{421}, Arg^{467}, and Gly^{717} are homologous to those in human α-glucosidase I, which are necessary for catalytic activity (29, 31, 77).

Ca*ROT2* was identified in the *C. albicans* genome (36) by homology to the *S. cerevisiae* homologue. The Ca*ROT2* 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 (DGXWI**D**MNEXSXF), including a conserved Asp residue that is thought to be involved in catalysis (25, 32, 33, 38, 72). A similar peptide sequence (^{487}IHLWN**D**MNEPSVF^{499}) 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 Ca*MNS1* 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 α1,2-mannosidases are present. These include Cys^{320} and Cys^{363} corresponding to ScMns1 Cys^{340} and Cys^{385}, which are necessary for stabilization of the tertiary structure of the catalytic pocket (42), and Arg^{251}, which would be predicted to be required for the specificity of the ER α1,2-mannosidase reaction (59).

The Ca*CWH41*, Ca*ROT2*, and Ca*MNS1* genes were disrupted in strain CAI4 by sequential gene replacement using the mini-ura-blaster protocol (81). The resulting Ca*cwh41*Δ (HMY-19), Ca*rot2*Δ (HMY-12), and Ca*mns1*Δ (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 Ca*CWH41*, Ca*ROT2*, or Ca*MNS1* were introduced into the null strains under the control of their own promoters at the *RPS1* locus. Strain CAI4 transformed with CIp10 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 Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ 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 Ca*cwh41*Δ and Ca*rot2*Δ yeast cells were swollen and enlarged (Fig. 1A) and small and crenulated (Fig. 1B) colonies. The Ca*mns1*Δ null mutant grew as normal hyphae in 20% (vol/vol) serum, GlcNAc-containing medium, and Lee's medium at pH 6.5, while the Ca*rot2*Δ and Ca*cwh41*Δ 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-glycosidase activities. The α-glucosidase or α-mannosidase activities in the null mutants were determined by using the fluorogenic substrates 4-methylumbellyferyl-α-D-glucopyranoside (MUαGlc) or 4-methylumbellyferyl-α-D-mannopyranoside (MUαMan), respectively. Homogenates of Ca*cwh41*Δ and Ca*rot2*Δ 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-

TABLE 2. α-Glucosidase activity in Ca*cwh41*Δ and Ca*rot2*Δ null mutants and reintegrant strains

Strain genotype	Mean α-glucosidase activity ± SD[a]		
	Total	Soluble (%)[b]	Membrane bound (%)[b]
WT	35 ± 1	55 ± 3	45 ± 3
Ca*cwh41*Δ	17 ± 0.3	99 ± 1	1 ± 1
Ca*cwh41*Δ + Ca*CWH41*	28 ± 0.4	57 ± 3	43 ± 3
Ca*rot2*Δ	18 ± 0.3	2 ± 1	98 ± 1
Ca*rot2*Δ + Ca*ROT2*	28 ± 1	42 ± 3	58 ± 3

[a] Expressed as nmol of MU min^{-1} total protein^{-1} (n = 3).
[b] Expressed as a percentage of the total activity.

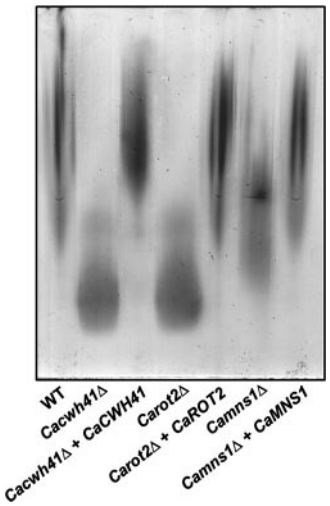

FIG. 2. N-glycosylation defects in Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ null mutants. The electrophoretic mobility of β-*N*-acetylhexosaminidase under nondenaturing conditions was examined. The strains tested include NGY152 (wild type), HMY19 (Ca*cwh41*Δ), HMY20 (Ca*cwh41*Δ + Ca*CWH41*), HMY12 (Ca*rot2*Δ), HMY13 (Ca*rot2*Δ + Ca*ROT2*), HMY5 (Ca*mns1*Δ), and HMY6 (Ca*mns1*Δ + Ca*MNS1*).

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 Ca*cwh41*Δ null mutant had no measurable membrane-associated α-glucosidase activity, whereas Ca*rot2*Δ 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 Ca*mns1*Δ null mutant (data not shown).

Glycosylation defects. We determined the consequences of the alteration in N-glycan structure in Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ 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*HEX1*, 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, Ca*rot2*Δ, and Ca*mns1*Δ 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 Ca*mns1*Δ 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, Ca*rot2*Δ, and Ca*mns1*Δ 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 Ca*rot2*Δ 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*CWH41* and Ca*ROT2* have an important role in N-glycan outer-chain elaboration.

TABLE 3. Alcian Blue binding of Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ null mutants and reintegrant strains

Strain genotype	Mean Alcian Blue binding ± SD[a]	
	No treatment	After β-elimination
WT	111 ± 5	95 ± 6
Ca*cwh41*Δ	20 ± 3	3 ± 2
Ca*cwh41*Δ + Ca*CWH41*	109 ± 5	92 ± 5
Ca*rot2*Δ	21 ± 5	4 ± 3
Ca*rot2*Δ + Ca*ROT2*	108 ± 8	91 ± 4
Ca*mns1*Δ	46 ± 5	29 ± 4
Ca*mns1*Δ + Ca*MNS1*	109 ± 7	92 ± 5

[a] Expressed as μg bound/OD_{600} = 1 cells (n = 3).

Cell wall composition, sensitivity, and cell integrity pathway activation. To determine the effect of the disruption of Ca*CWH41*, Ca*ROT2*, and Ca*MNS1* on the overall cell wall composition, the content of total carbohydrates, and proteins was analyzed. Ca*cwh41*Δ and Ca*rot2*Δ 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 Ca*cwh41*Δ null mutant and of 2.2- and 3.2-fold for the Ca*rot2*Δ null mutant was observed. Ca*mns1*Δ 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-glycosidase 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 Ca*mns1*Δ null mutant was hypersensitive to Congo red, Calcofluor White, and hygromycin B, and Ca*rot2*Δ and Ca*cwh41*Δ null mutants were hypersensitive to Calcofluor White, Congo red, hygromycin B, tunicamycin, and SDS (Fig. 3). Hypersen-

TABLE 4. Cell wall composition of Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ null mutants and reintegrant strains

Strain genotype	Mean amt (μg) of polymer ± SD[a]			
	Glucan	Mannan	Chitin	Protein
WT	546 ± 11	276 ± 15	18 ± 2	140 ± 7
Ca*cwh41*Δ	380 ± 14	97 ± 11	41 ± 2	459 ± 6
Ca*cwh41*Δ + Ca*CWH41*	591 ± 17	246 ± 11	18 ± 1	124 ± 8
Ca*rot2*Δ	384 ± 10	102 ± 13	40 ± 2	444 ± 8
Ca*rot2*Δ + Ca*ROT2*	561 ± 15	277 ± 12	17 ± 1	120 ± 7
Ca*mns1*Δ	602 ± 15	134 ± 13	21 ± 2	215 ± 6
Ca*mns1*Δ + Ca*MNS1*	581 ± 14	258 ± 15	18 ± 4	128 ± 10

[a] That is, per mg of cell wall dry weight (n = 3).

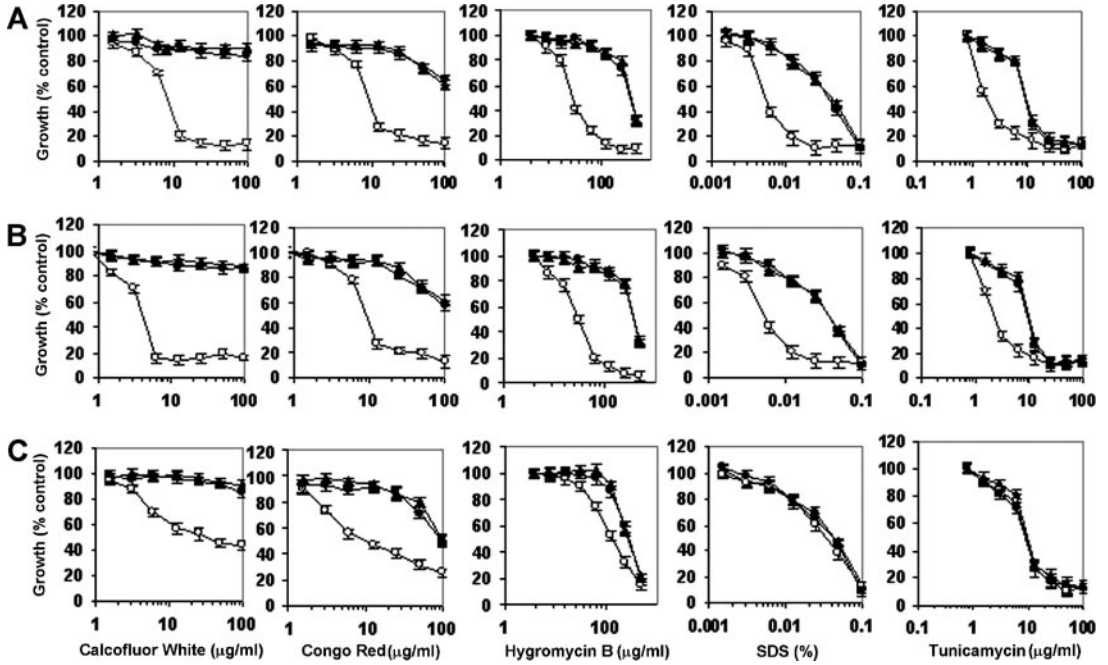

FIG. 3. Sensitivity of Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ null mutants to cell-wall-perturbing agents. Wild type (▲), null mutants (○), and reintegrant controls (●) strains were tested for sensitivity to cell-wall-perturbing agents by using the microdilution method. The strains tested were the Ca*cwh41*Δ (HMY19) (A), Ca*rot2*Δ (HMY12) (B), and Ca*mns1*Δ (HMY5) (C) null mutants. Error bars indicate the means ± the standard deviation ($n = 3$). The results are pooled data from duplicate experiments.

sitivity 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*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ 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*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ mutants. In the Ca*cwh41*Δ 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*rot2*Δ null mutant, with levels of TNF,

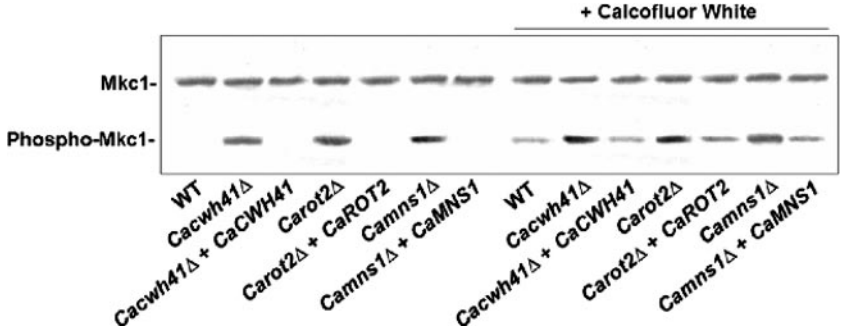

FIG. 4. Activation of the cell integrity pathway in glycosidase null mutants assessed by Western analysis. Protein extracts were prepared from cells in mid-exponential phase. As a positive control for activation of the cell integrity pathway, the strains were treated with Calcofluor White (100 μg/ml) as indicated. Extracts are from the following strains: NGY152 (wild type), HMY19 (Ca*cwh41*Δ), HMY20 (Ca*cwh41*Δ + Ca*CWH41*), HMY12 (Ca*rot2*Δ), HMY13 (Ca*rot2*Δ + Ca*ROT2*), HMY5 (Ca*mns1*Δ), and HMY6 (Ca*mns1*Δ + Ca*MNS1*). Equal loading was confirmed by Ponceau S staining and determining the intensity of nonspecific bands.

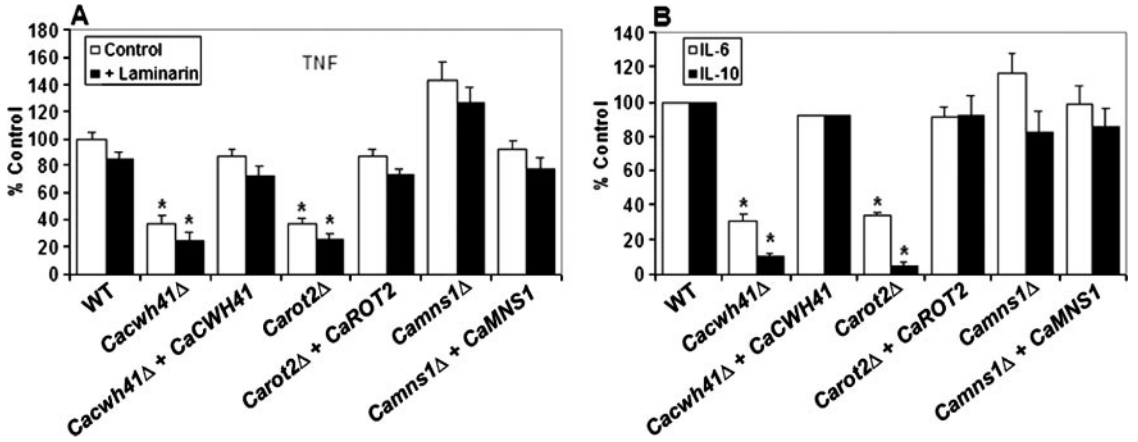

FIG. 5. Cytokine stimulation by Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ null mutants. Human PBMC were stimulated 24 h with 10^6 yeast cells/ml and the TNF (A) and IL-6 and IL-10 (B) concentrations were determined. The experiments for panel A were carried out in the absence (□) or presence (■) of laminarin, a blocking agent of the β-glucan/dectin-1 recognition pathway. The strains tested are NGY152 (wild type), HMY19 (Ca*cwh41*Δ), HMY20 (Ca*cwh41*Δ + Ca*CWH41*), HMY12 (Ca*rot2*Δ), HMY13 (Ca*rot2*Δ + Ca*ROT2*), HMY5 (Ca*mns1*Δ), and HMY6 (Ca*mns1*Δ + Ca*MNS1*). The results are pooled data from four volunteers. Error bars indicate the means ± the standard deviation. *, Significant differences in the mutant compared to the wild type ($P < 0.05$).

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*mns1*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 Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*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-glycosidase loss encoded by Ca*CWH41*, Ca*ROT2*, and Ca*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 Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ 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 reintegrant 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 $Glc_3Man_9GlcNAc_2$ N-oligosaccharide is processed by ER α-glycosydases to generate the $Man_8GlcNAc_2$ 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 Ca*rot2*Δ and Ca*cwh41*Δ 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 Ca*och1*Δ null mutant, which lacks the entire N-glycan outer chain (4). The Ca*mns1*Δ null mutant had a milder phenotype than the Ca*cwh41*Δ and Ca*rot2*Δ 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 MUαMan, 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 Ca*mns1*Δ null mutant indicates that Ca*MNS1* is likely to encode both the soluble and the membrane-bound activities. The absence of soluble α-glucosidase activity in the Ca*rot2*Δ 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 Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ 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 Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ 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 Ca*mnt1*Δ/Ca*mnt2*Δ (46), Ca*pmr1*Δ (3), Ca*och1*Δ (4), Ca*mnn9*Δ (66), and Ca*vrg4*Δ (51) null mutants.

Evidence was found for defects in N glycosylation in the three null mutants generated. This was more severe in the Ca*cwh41*Δ and Ca*rot2*Δ null mutants, as demonstrated by the underglycosylation of HexNAcase. HexNAcase 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 Ca*CWH41* or Ca*ROT2* resulted in defects in N glycosylation demonstrated by the increased mobility of HexNAcase 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 Ca*och1*Δ 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 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 Ca*mns1*Δ null mutant was not as severe as in Ca*rot2*Δ and Ca*cwh41*Δ 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 Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ 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 CaCwh41, 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 Ca*mns1*Δ null mutant was least affected by cell-wall-perturbing agents, correlating with a milder N-glycosylation mutant phenotype. Disruption of Ca*CWH41* and Ca*ROT2* resulted in a 30% reduction in the cell wall β-glucan content, but loss of Ca*MNS1* 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 och1*Δ 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 Ca*cwh41*Δ and Ca*rot2*Δ null mutants may be due to a failure in the glycoprotein quality control/protein refolding pathways that are dependent on glucosylation 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 Ca*cwh41*Δ and Ca*rot2*Δ null mutants is likely to be the result of the cell wall perturbation rather than effects on the plasma membrane.

The Ca*cwh41*Δ, Ca*rot2*Δ, and Ca*mns1*Δ 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 Ca*cwh41*Δ and Ca*rot2*Δ null mutants. This finding is in agreement with the observation that approximately 70% less cytokine production was stimulated in PBMC by the Ca*och1*Δ null mutant (49). The disruption of Ca*MNS1* 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-1 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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