Outer Chain N-Glycans Are Required for Cell Wall Integrity and Virulence of *Candida albicans*  

Received for publication, September 21, 2005, and in revised form, November 1, 2005. Published, IBC Papers in Press, November 1, 2005, DOI 10.1074/jbc.M510360200

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The outer layer of the *Candida albicans* cell wall is enriched in highly glycosylated mannoproteins that are the immediate point of contact with the host and strongly influence the host-fungal interaction. *N*-Glycans are the major form of mannoprotein modification and consist of a core structure, common to all eukaryotes, that is further elaborated in the Golgi to form the highly branched outer chain that is characteristic of fungi. In yeasts, outer chain branching is initiated by the action of the α1,6-mannosyltransferase Och1p; therefore, we disrupted the *C. albicans* OCH1 homolog to determine the importance of outer chain *N*-glycans on the host-fungal interaction. Loss of CaOCH1 resulted in a temperature-sensitive growth defect and cellular aggregation. Outer chain elongation of *N*-glycans was absent in the null mutant, demonstrated by the lack of the α1,6-linked mannobiose backbone and the underglycosylation of *N*-acetylglucosaminidase. A null mutant lacking OCH1 was hypersensitive to a range of cell wall perturbing agents and had a constitutively activated cell wall integrity pathway. These mutants had normal growth rates in vitro but were attenuated in virulence in a murine model of systemic infection. However, tissue burdens for the Caoch1Δ null mutant were similar to control strains with normal *N*-glycosylation, suggesting the host-fungal interaction was altered such that high burdens were tolerated. This demonstrates the importance of *N*-glycan outer chain epitopes to the host-fungal interaction and virulence.

*Candida albicans* is a commensal organism carried by a significant proportion of healthy individuals. It is the most common opportunistic fungal pathogen of humans causing superficial infections of the mucosa and in the immunocompromised host life-threatening systemic infections (1–4). The cell wall is the immediate point of contact between fungus and host and plays an important role in adherence, antigenicity, and in the immunocompromised host life-threatening systemic infections (1–4). The cell wall is enriched in highly glycosylated mannoproteins (10), and the modulation of the host immune response (5–9). The outer layer of the fungus and host plays an important role in adherence, antigenicity, and in the immunocompromised host life-threatening systemic infections (12, 28–31). Phosphomannan has been implicated in the interaction with phagocytic leukocytes; however, deletion of *MNN4*, required for phosphomannan production, demonstrated that it was not required for macrophage interaction or virulence (32).

In *S. cerevisiae* OCH1 encodes the specific α1,6-mannosyltransferase that initiates outer chain branching through its action on the Man*α*6GlcNAc2 core (33–35). Scoch1p is unusual among mannosyltransferases because it displays a narrow acceptor specificity requiring the whole Man*α*6GlcNAc2 core for efficient recognition (34, 36). Mutants lacking the OCH1 gene in *S. cerevisiae* are viable but display temperature-sensitive growth (33). The Scoch1Δ mutant has a severe *N*-glycosylation defect, with a complete loss of the α1,6-linked mannobiose backbone. *N*-Glycans isolated from the Scoch1Δ null mutant consist of only Man*α*6GlcNAc2, where the Man*α*6GlcNAc2 core is modified by the addition of α1,3-linked mannose residues to antennae of the core (35). *N*-Glycosylation *och1Δ* mutants have also been characterized for *Schizosaccharomyces pombe* and *Pichia pastoris* (37–39).

To assess the importance of outer chain *N*-glycosylation in virulence and host-fungal interactions, we disrupted the *C. albicans* OCH1 homolog. The Caoch1Δ null mutant had a severe defect in *N*-glycosylation, displaying loss of the α1,6-linked mannobiose backbone, although the core was further modified by the addition of α1,2-mannose residues. The Caoch1Δ null mutant displayed cell wall defects and was attenuated...
in virulence in a murine model of systemic infection despite being able to colonize organs to almost wild type levels. This work demonstrates the importance of epitopes carried on branched outer chain N-glycans for the host-fungal interaction and for virulence.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Culture Conditions**—The strains constructed and used in this work are listed in Table 1. Strains were grown at 30 °C in YEPD (2% (w/v) mycelial peptone, 1% yeast extract (w/v), 2% glucose (w/v)) or SD (0.67% (w/v) yeast nitrogen base with ammonium sulfate without amino acids, 2% glucose) with uridine (50 μg/ml) as required. To repress expression of the tetracycline-regulatable promoter, doxyxycycline was added to the medium at a final concentration of 20 μg/ml. To induce β-N-acetylhexosaminidase (HexNCase), activity strains were grown in SC + GlcNac (0.67% (w/v)) yeast nitrogen base, 0.077% complete supplement mixture, 25 mM acetate. Hyphae were induced in 20% fetal calf serum, RPMI 1640, and Lee’s medium, pH 6.5 (40), at 37 °C or Spider medium (41) at 30 °C. Strains were grown in NGY medium (0.1% neopentone, 0.4% glucose, 0.1% yeast extract) at 30 °C prior to virulence testing. To disperse cellular aggregates, strains were grown in NGY medium containing 2 units/ml chitinase.

**Construction of Caoch1 Null Mutant, Re- integrating, and Blastic Strains**—The Caoch1 gene was disrupted by the ura-blaster method (42). The 5′ and 3′ regions of homology were amplified by PCR (5′ primer pair 5′-CTCAAGCTGCTGCTCAATAGGAAAGCC- C-3′ and 5′-GATGATGAAGATGCTGAGAGCTTTAGGATGTTTATGAGC-3′; the SacI and BglII restriction sites are underlined, respectively; 3′ primer pair 5′-CTCAAGCTGCTGCTCAATAGGAAAGCC- C-3′ and 5′-GATGATGAAGATGCTGAGAGCTTTAGGATGTTTATGAGC-3′; the SacI and BglII restriction sites are underlined, respectively) and cloned into the relevant sites in pMB-7. The ura-blaster cassette was released by digestion with SacI and BglII and was flanked by 505-bp upstream and 521-bp downstream sequences complementary to Caoch1. Caoch1 was disrupted in strain CAI-4 by sequential gene replacement and recycling of the URA3 marker by selection on SD plus 5-ﬂuoroorotic acid (1 mg/ml) and uridine (50 μg/ml). The Ura− Caoch1Δ null mutant was constructed with the Sul1-digested Clp10 plasmid so that URA3 was expressed from the neutral RPS1 locus (43, 44). As a control, a re-integrant strain was also constructed in which a wild type copy of Caoch1 was transformed into the null mutant. The Caoch1 open reading frame plus 969 bp of its own promoter and 425 bp of its terminator sequences were amplified by PCR (primer pair 5′-CAAGCGGGCCGATTTGAGTGTTTCTTC3′ and 3′-TAATTTGTTTAGGTCTGC-3′), and the product was cloned into pGEM-T Easy (Promega Ltd., Southampton, UK). The plasmid insert was subcloned into the NotI site of Clp10. The resulting plasmid was then linearized with Stul before transformation into the Caoch1Δ null mutant.

A conditional mutant was also constructed in which CaOCH1 was placed under the control of the tetracycline-regulated promoter (45) in strain THE1 that expresses the tetracycline transactivator (a fusion protein of Escherichia coli TetR and the activation domain of S. cerevisiae HAP4). The first allele of CaOCH1 was disrupted by means of the URA3 recycleable PCR-directed gene disruption system (46). The recycleable URA3 cassette was amplified from pDBB57 (primer pair 5′-GAAACCA GACACACCTTCTACTATAACTGATACTTTTGTTTCTT- TCTTATTCCATATTGGTGAATATTTTCAAATGAAAATATAGCTGATT AAAATAGGAAAGAAAATGCGTATTCGTTAAAATATGATGGGTGT G-3′ and 5′-GATGATTTAGGTAAACAAATCGGTTTATGTTATTGCCTA ACAGGAGGTCT3′; the region of homology to pDBB57 is underlined) and transformed into THE1 to generate a heterozygous strain. The URA3 marker was then recycled by selection on SD plus 5-fluoroorotic acid (1 mg/ml) and uridine (50 μg/ml). The promoter replacement cassette was produced by a modification of the published system in order to utilize PCR-directed targeting. The URA3-TR promoter cassette was amplified from p99-CAU1 (primer pair 5′-GAAACCA GACACACCTTCTACTATAACTGATACTTTTGTTTCTT- TCTTATTCCATATTGGTGAATATTTTCAAATGAAAATATAGCTGATT AAAATAGGAAAGAAAATGCGTATTCGTTAAAATATGATGGGTGT G-3′ and 5′-GATGATTTAGGTAAACAAATCGGTTTATGTTATTGCCTA ACAGGAGGTCT3′; the region of homology to p99CAU1 is underlined) and transformed into the heterozygous mutant in the THE1 strain background.

**Mannosyltransferase Activity Assay**—Mixed membrane fractions were prepared from exponentially growing cells as described previously (47). For mannosyltransferase activity assays, 200 μg of mixed membrane protein preparation was incubated for 1 h at 30 °C in 50 μl of 50 mM Tris-HCl, pH 7.5, 10 mM MnCl2, 0.6% Triton X-100, 0.5 mM 1-deoxyxamnopoiojirimycin, GDP-[3H]mannose (95 Bq, specific activity 296 mCi/mmol; Amersham Biosciences), and 0.06 mM ManGlcNAc2 (Oligomannose-BS, D. D. D., Dextra Laboratories, Reading, UK) as the acceptor. Assays were carried out in triplicate, and unincorporated GDP-mannose was removed by passage through 0.6 ml of QAE-Sephadex. Neutral products were eluted with water, and radioactivity was counted. Specific activities were calculated and expressed as nanomoles transferred/mg/min.

**Labeling of Glycans and TLC**—For the analysis of acid-labile and O-linked glycans, the strains were labeled with d-[2-3H]mannose. Cells

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**ACCEPTED MANUSCRIPT**

**TABLE 1**

| Strain | Parent strain | Genotype | Source |
|--------|---------------|----------|--------|
| CAI-4  | ura3Δ:imr434/ura3Δ:imr434 | [42] |
| NGY52  | CAI-4 | As CAI-4 but RPS1::Clp10 | 44 |
| NGY202 | CAI-4 | As CAI-4 but OCH1::hisG-URA3-hisG | This study |
| NGY203 | NGY202 | As CAI-4 but OCH1::hisG-URA3-hisG | This study |
| NGY204 | NGY203 | As CAI-4 but och1::hisG-och1::hisG-URA3-hisG | This study |
| NGY357 | NGY205 | As CAI-4 but och1::hisG-och1::hisG-URA3-hisG | This study |
| NGY358 | NGY205 | As CAI-4 but och1::hisG-och1::hisG-URA3-hisG | This study |
| THE1   | ura3Δ:imr434/ura3Δ:imr434 ade2::hisG ade2Δ::hisG ENO1::Eno1-eno1-tetR-Sch4PAD-3xHA-ADE2 | [45] |
| NGY359 | THE1 | As THE1 but och1::clp100/99-URA3 | This study |
| NGY360 | NGY359 | As THE1 but och1::clp100/99-URA3 | This study |
| NGY361 | NGY360 | As THE1 but och1::clp100/99-URA3 | This study |
| NGY355 | As CAI-4 but pmr1Δ::hisG/pmrrΔ::hisG, RPS1::Clp10 | 27 |

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3 The abbreviations used are: HexNCase, β-N-acetylhexosaminidase; ES-MS, electrospray-mass spectrometry; MS/MS, tandem mass spectrometry; cfu, colony-forming units; Endo H, endoglycosidase H; MAPK, mitogen-activated protein kinase.
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growing in 2 ml of SC + GlcNAc were incubated with 1.85 MBq of \(^{3}H\)mannose (555 GBq mmol\(^{-1}\); PerkinElmer Life Sciences) for 16 h at 30 °C. Cells were then collected and washed twice with water before O-linked glycans were released by β-elimination with 100 mM NaOH for 24 h at room temperature. Cells were then pelleted, and the supernatant containing O-glycans was retained for TLC analysis. The cells were washed twice in water and then boiled in 10 mM HCl for 1 h to release acid-labile glycans. The remaining cellular material was pelleted, and the supernatant was retained for TLC analysis.

For TLC the samples were spotted and dried onto Silica Gel 60 TLC plates (Whatman). The plates were eluted twice in the solvent (3:4:2.5:4 ethyl acetate/butan-1-ol/acetonic acid/water). For detection of labeled carbohydrates, the plates were sprayed with En3Hance (PerkinElmer Life Sciences) and visualized by autofluorography (Kodak BioMax XLS).

In Situ N-Acetylgalosaminidase Activity Staining—Strains were grown for 16 h in SC + GlcNAc to induce HexNacase expression. Cells were washed and resuspended in 10 mM Tris-HCl, pH 8, containing protease inhibitor mixture (Roche Applied Science) and then disrupted with glass beads in a FastPrep machine (Qiobiogene, Cambridge, UK). The lysate was clarified by centrifugation at 21,500 \( \times g \) for 10 min. For endoglycosidase H (Endo H) treatment, the native sample was treated with 25 unit of cell suspension. The remaining cellular material was pelleted, and the supernatant containing N-glycans was retained for TLC analysis. The cells were washed twice in water and then boiled in 10 mM HCl for 1 h to release acid-labile glycans. The remaining cellular material was pelleted, and the supernatant was retained for TLC analysis.

Preparation of Total N-Glycan—Total N-linked glycans were released from cell walls by enzymatic digest with peptide-N-glycosidase F. Cell walls were prepared by a method modified from de Groot et al. (50). Cells from a stationary phase culture (100 ml) were washed three times in 50 mM Tris-HCl, pH 6.8, resuspended in 50 mM Tris-HCl, pH 6.8, containing protease inhibitor mixture (Roche Applied Science) to a total volume of 5 ml, and disrupted by three passes through a French press at 15.5 MPa. Cell walls were washed extensively in 1 mM NaCl followed by water and extracted twice with 2% (w/v) SDS, 100 mM EDTA, 40 mM β-mercaptoethanol, 50 mM Tris-Cl, pH 7.8, at 100 °C for 5 min to remove noncovalently bound proteins. SDS-extracted cell walls were then washed extensively in water.

Phosphodiester-linked glycans were removed from the cell wall preparations by mild hydrolysis with 40 mM trifluoroacetic acid at 100 °C for 10 min. The cell walls were washed three times with water, resuspended in 250 μl of 1% (w/v) SDS, 50 mM Tris-HCl, pH 6.8, and incubated at 100 °C for 10 min to denature cell wall proteins. The suspension was adjusted to 2.5 ml of 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 50 mM Tris-Cl, pH 6.8, and N-glycans were released by treatment with 3750 units of peptide-N-glycosidase F (New England Biolabs, Hitchin, UK) at 37 °C and shaken for 16 h (51). Cell wall material was pelleted, and the supernatants containing N-glycans were retained and adjusted to pH 8.8 with 1 M NaOH. Phosphate groups present on the N-glycans were removed by treatment with 400 units of bovine intestinal alkaline phosphatase (Sigma) at 37 °C for 16 h. SDS was removed by precipitation with potassium acetate (final concentration 20 mM) overnight on ice, and Triton X-100 was removed by extensive extraction with toluene (52). The N-glycans were desalted on columns of 2.5 ml of AG-50-H+ over 1 ml AG-4 OH\(^{-}\), eluted fully in water, and lyophilized. The N-glycans were further desalted by gel filtration on a Bio-Gel P2 column (25 × 1.5 cm) and eluted with water at 12 ml h\(^{-1}\). Fractions were collected and glycans detected by spotting onto Silica Gel 60 TLC plates and staining with orcinol. Fractions containing glycans were pooled, lyophilized, and dissolved in water.

Methylation Linkage Analysis—Methylation linkage analysis was performed on N-glycans isolated from the wild type and \( \textit{Caocl} \Delta \) null mutant. Glycans were converted to their component monosaccharides in the form of partially methylated alditol acetates and analyzed by gas chromatography-mass spectrometry on a Supelco SP2380 column as described previously (53).

Mass Spectrometric Analysis of Permethylated N-Glycans—Samples of permethylated glycans in 80% acetonitrile containing 0.5 mM sodium acetate were loaded into nanospray tips (Micromass type F) for ES-MS and ES-MS/MS. Samples were analyzed in positive ion mode with captur argon as the collision gas and with collision voltages of 45–70 V. All spectra were collected and processed using MassLynx software.

Protein Extracts and Western Analysis—Activation of the cell integrity pathway was assayed by Western blot analysis utilizing the PhosphoPlus p44/42 MAPK antibody kit (New England Biolabs) that cross-reacts with \( \text{CaMyc} \) in its phosphorylated form. Strains were grown in YEPD at 30 °C to mid-exponential phase, as positive control strains were also treated with Calcofluor White (100 μg/ml) 2 h before collection. Protein extracts were prepared in 100 mM Tris-HCl, pH 7.5, 0.01% SDS, 1 mM dithiothreitol, 10% glycerol containing protease inhibitor mixture (Roche Applied Science) by means of glass bead disruption in a FastPrep machine (Qiobiogene, Cambridge, UK). The resulting lysate was clarified by centrifugation at 21,500 \( \times g \) for 10 min. Protein samples (50 μg) were separated on a 4–14% NuPAGE gel (Invitrogen) and blotted onto a polyvinylidene difluoride membrane. The membranes were blocked in phosphate-buffered saline plus 0.1% Tween 20 and 5% bovine serum albumin for 2 h at room temperature. The PhosphoPlus p44/42
MAPK antibody kit (New England Biolabs) was then used to develop Western blots according to the manufacturer’s instructions.

**Virulence Tests**—Female, immunocompetent BALB/c mice (Harlan Sera-Lab Ltd., Loughborough, UK) were challenged intravenously with yeasts grown for 18–24 h in NGY medium at 30 °C. The cells were collected, washed twice with water, and resuspended in physiological saline to give a challenge inoculum of 1.8 × 10⁴ cfu/g mouse body weight in a 100-μl volume. The challenge inoculum concentration was confirmed by the determination of cellular ATP content, hemocytometer counting, and viable counting. Groups of five or six mice were intravenously inoculated and monitored over 28 days. Mice showing signs of illness were humanely terminated and their deaths recorded as occurring the following day. Mice surviving the course of the experiment were humanely terminated on day 28. Kidneys and brain were removed aseptically postmortem, homogenized in 0.5 ml of water, and tissue burdens determined by viable counting.

**RESULTS**

**Isolation and Deletion of CaOCH1—**CaOCH1 was initially isolated by screening a cDNA library with PCR primers based on an unpublished sequence displaying homology to ScOCH1. The mannan polymerase II complex in *S. cerevisiae* contains ScHoc1p that displays homology to ScOch1p. Completion of the *C. albicans* genome-sequencing project confirmed the CaOCH1 open reading frame (GenBank™ accession number AF900420; orf19.7391) and identified a CaHOC1 homolog (orf19.3445). The degree of overall sequence identity confirms that CaOch1p reported here is the true homolog of ScOch1p. CaOch1p displays 37.5% identity to ScOch1p and 33.8% identity to ScHoc1p, whereas CaHoc1p displays 25% identity to ScOch1p but 41.4% identity to ScHoc1p. A multiple sequence alignment (ClustalW) also supports the direct sequence comparisons with the *C. albicans* and *S. cerevisiae* Och1p homologs and the Hoc1p homologs clustering together (not shown). It also identified regions that were only present in either the Och1p or Hoc1p homologs. The CaOCH1 open reading frame of 1158 bp is predicted to encode a 385-amino acid type II membrane protein with a 17-amino acid cytosolic tail, followed by an 18-amino acid membrane region before the catalytic domain. It also displays a classic DXD motif (residues 165–214) known to be required for binding the donor nucleotide sugar.

The CaOCH1 open reading frame was disrupted in strain CAI-4 by sequential gene replacement following the ura-blaster method (42). The resulting Caoch1Δ null mutant had *URA3* introduced into the neutral *RSP1* locus to avoid problems with the level of *URA3* expression (43, 44). *CaOCH1* was reintroduced into the null mutant under the control of its own promoter at the *RSP1* locus as a re-integrant control. The CaOCH1 open reading frame was also placed under the control of a tetracycline-regulatable promoter in the *THE1* strain that expresses the tetracycline transactivator (45). The first allele of CaOCH1 was disrupted in THE1, and the second allele was regulated by promoter replacement. In the resulting strain, TET-OC1H1, expression of CaOCH1 was repressed by the addition of doxycycline to the medium. Under inducing conditions, the strain is referred to as TET-OC1H1 ON and in repressing conditions, through the addition of doxycycline, as TET-OC1H1 OFF.

**Growth and Morphology of the Caoch1Δ Null Mutant**—The Caoch1 null mutant had a specific growth rate of 75% of the wild type control in YEPD at 30 and 37 °C. The mutant also displayed an altered cellular morphology with cells forming tight aggregates and the presence of swollen cells (Fig. 1A). After growth in medium containing chitinase, cellular aggregates were dispersed, suggesting they were the result of a failure in cell separation. The clumping of mutant cells resulted in a change in colony morphology from the smooth white appearance of wild type to a highly crenulated appearance in the Caoch1Δ null mutant (Fig. 1B). The Caoch1Δ null mutant colonies invaded the agar surface more than the wild type (Fig. 1C), and the invading cells were predominantly hyphal. The mutant also displayed a temperature-sensitive defect with a failure to grow at 42 °C (Fig. 2). In terms of hyphal development, the Caoch1Δ null mutant responded normally to the presence of 20% (v/v) serum, although germ tubes were shorter, indicating either a decrease in extension rate or a structural defect limiting cell extension. In response to the weaker hypha-inducing signal of pH and temperature shift, in Lee’s medium or RPMI 1640, the null mutant generated mixed morphologies consisting of true hyphal and pseudohyphal forms (data not shown). The null mutant also failed to induce filamentous growth on solid Spider medium (data not shown). In all cases the growth defects were restored to wild type in the re-integrant strain and the tetracycline-regulated strain displayed the same phenotype as the Caoch1Δ null mutant after growth in repressing conditions.

**Reduced Mannosyltransferase Activity toward N-Linked Core Glycer in the Caoch1Δ Mutant**—In *S. cerevisiae* Och1p initiates outer chain elongation of N-linked core oligosaccharides. The α1,6-mannosyltransferase activity of ScOch1p is novel in that it displays a tight substrate specificity requiring Man9GlcNAc2 for efficient acceptor recognition (36). We carried out *in vitro* mannosyltransferase activity assays on mixed membrane preparations of the Caoch1Δ null mutant and the TET-OC1H1 strain under inducing and repressing conditions utilizing Man9GlcNAc2 as the acceptor (Table 2). The Caoch1Δ null mutant had 59% of the wild type activity. The remaining mannosyltransferase activity suggests that other mannosyltransferases can act on the complex Man9GlcNAc2 acceptor structure *in vitro*. The TET-OC1H1 strain also displayed a significant drop in transferase activity when grown under repressing conditions (73% of wild type). When growing exponentially under inducing conditions, the TET-OC1H1 strain appeared to overexpress Och1p with detected mannosyltransferase activity rising 2-fold (20% of wild type).

**FIGURE 1.** Cell and colony morphology in the Caoch1Δ null mutant. *A*, cell morphology after growth at 30 °C for 16 h in YEPD medium, demonstrating clumping of cells in the Caoch1Δ null mutant and in the TET-OC1H1 strain under repressing conditions. Scale bars = 10 μm. *B* and *C*, colony morphology and agar invasion after 5 days of growth at 30 °C on YEPD agar plates. Colonies were photographed before (*B*) and after (*C*) agar plates were washed. Scale bars = 1 mm.

**FIGURE 2.** Temperature sensitivity of Caoch1Δ null mutant. Growth was scored after 3 days on YEPD agar plates at the temperatures shown.
**TABLE 2**

Mannosyltransferase activity and Alcian blue binding of Caoch1Δ null mutant and control strains

| Strain          | Mannosyltransferase activity | Alcian blue binding |
|-----------------|------------------------------|--------------------|
|                 | nmol transferred/ng/min ± S.D. | µg bound/A600 cell ± S.D. |
| CaOCH1          | 9.8 ± 1.1                    | 46.8 ± 0.4         |
| Caoch1Δ         | 5.8 ± 0.1                    | 7.8 ± 2.5          |
| Caoch1Δ + OCH1  | ND*                         | 44.1 ± 1.6         |
| TET- OCH1 ON    | 20.3 ± 1.1                   | 43.8 ± 5.6         |
| TET- OCH1 OFF   | 7.1 ± 0.1                    | 11.5 ± 0.5         |

* ND indicates not determined.

**FIGURE 3.** N-Glycosylation defects in the Caoch1Δ null mutant. The extent of N-glycosylation was determined by activity staining for βN-acetylhexosaminidase after protein samples were separated by nondenaturing electrophoresis. A, samples are as follows: lane 1, wild type; lane 2, Caoch1Δ null mutant; lane 3, Caoch1Δ + OCH1 re-integrant control; lane 4, TET- OCH1 ON; lane 5, TET- OCH1 OFF. Samples were also treated with Endo H as indicated to remove N-glycans. B, the N-glycosylation defect was compared with that seen in the general glycosylation mutant Capmr1Δ. Samples are as follows: lane 1, wild type; lane 2, Caoch1Δ null mutant; lane 3, Capmr1Δ null mutant.

Glycosylation Defects in the Caoch1Δ Null Mutant—Previously, the electrophoretic mobility of secreted acid phosphatase on native gels has been used as a maker of N-glycosylation status (13, 25, 27). However, no acid phosphatase activity was detected in zymograms of the Caoch1Δ null mutant. Additionally, in *in vitro* assays, only low levels of acid phosphatase activity were detected for the Caoch1Δ null mutant, in cell-associated assays, and in secreted fractions (data not shown). We therefore developed an *in situ* activity assay for the well characterized hydrolytic enzyme HexNCase as an alternative marker for N-glycosylation status in *C. albicans*. HexNCase, encoded by CaHEX1, is induced after growth in medium containing GlcNAc as the sole carbon source. It has seven potential N-glycosylation sites and has been demonstrated to be highly N-glycosylated (54–56). The *in situ* activity assay used a naphthyl derivative of GlcNAc as the substrate and the tetrazolium salt Fast Blue for visualization after the separation of protein extracts by native gel electrophoresis. The HexNCase of the Caoch1Δ null mutant had an increased electrophoretic mobility, indicating an N-glycosylation defect (Fig. 3A). The TET- OCH1 strain also showed a similar increase in the electrophoretic mobility of HexNCase after growth under repressing conditions. The electrophoretic mobility of HexNCase of the re-integrant control and TET- OCH1 strain grown under inducing conditions was similar to wild type. After Endo H treatment to remove N-glycans, the HexNCase migrated faster through the gel as two distinct bands. It has been reported previously that HexNCase contains three N-glycosylation sites that receive only the mature core N-glycan and lack outer chain elongation. These sites were partially resistant to Endo H cleavage when HexNCase was in its native nondegenerated form (55). The N-glycosylation defect in the Caoch1Δ null mutant was more severe than in the Capmr1Δ null mutant (27) that has a general glycosylation defect (Fig. 3B), indicating that CaoCH1 acts at an early step in N-glycan outer chain elaboration.

The outer chain N-glycans contain the majority of the acid-labile phosphomannan fraction consisting of β1,2-linked mannose residues attached via a phosphodiester linkage. The extent of mannosephosphorylation can be detected by the extent of binding of the cationic dye Alcian blue. The Caoch1Δ null mutant and TET- OCH1 OFF strains had reduced Alcian blue binding, with 16.7 and 24.6% of wild type levels, respectively (Table 2). The re-integrant control and TET- OCH1 ON had wild type levels of Alcian blue binding. The acid-labile phosphomannan fraction was also directly resolved by TLC analysis. This had greatly reduced levels of mannosephosphate with only small amounts of Man3 and Man4 β1,2-linked mannose residues present in the Caoch1Δ null mutant and TET- OCH1 OFF strains (Fig. 4A). The wild type, re-integrant control, and TET- OCH1 ON had a normal phosphomannan structure. The structure of O-mannan was also assessed by TLC analysis and displayed no change resulting from the loss of CaOCH1 (Fig. 4B).

**Analysis of N-Linked Glycans**—Cell walls were treated with SDS/β-mercaptoethanol followed by mild acid hydrolysis to remove noncovalently linked mannanproteins and phosphomannan, respectively. The purified cell walls were then treated with peptide-N-glycosidase F to release the N-linked glycans. These were further treated with alkaline phosphatase to render the glycans neutral by removing the 6-linked phosphate groups that remain after removal of phosphomannan. Desalted N-linked glycans were permethylated and analyzed by ES-MS and gas chromatography-mass spectrometry methylation linkage analysis.

The methylation analysis of the wild type glycans (Table 3) was in accord with the detailed analysis of Shibata et al. (57). The material contained substantial amounts of nonreducing terminal Man, 2-O-substituted Man, 6-O-substituted Man, and 2,6-di-O-substituted Man, as well as the 3,6-di-O-substituted Man of the core glucan and occasional outer arm branch points (Fig. 5). Methylation analysis of the Caoch1Δ null mutant glycans (Table 3) showed an almost complete loss of 6-O-substituted Man and 2,6-di-O-substituted Man and a significant decrease in nonreducing terminal Man residues. This result is consistent with the loss of the 1,6-linked arm of the wild type N-glycan structures. Methylation analysis of the Caoch1Δ null mutant glycans was consistent with the residual N-linked glycans being based on the conventional Manα1GlcNAcβ core glucan because they contained almost exclusively terminal Man, 2-O-substituted Man and 3,6-di-O-substituted Man. However, ES-MS analysis revealed that they were larger than the conventional core ranging from Hex1HexNAc2 to Hex12HexNAc3 (Fig. 6). The methylation analysis suggests these additional hexoses were in the form of 1,2-linked Man. The original doubly charged [M + 2Na]+ ions that were deconvoluted (Fig. 6) were analyzed...
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TABLE 3
Gas chromatography-mass spectrometry methylation linkage analysis of wild-type and Caoch1Δ mutant N-glycans

| PMAA derivative                                      | Origin          | Peak area ratio |
|------------------------------------------------------|-----------------|----------------|
| 2,3,4,6-Tetra-O-methyl-1,5-di-O-acetyl-1-[1H]mannitol | Terminal mannose| 14.0           |
| 3,4,6-Tri-O-methyl-1,2,5-tri-O-acetyl-1-[1H]mannitol  | 2-O-Substituted Man | 22.4           |
| 2,3,4-Tri-O-methyl-1,2,5,6-tetra-O-acetyl-1-[1H]mannitol | 6-O-Substituted Man | 4.6           |
| 3,4-Di-O-methyl-1,2,5,6-tetra-O-acetyl-1-[1H]mannitol  | 2,6-Di-O-substituted Man | 8.2           |
| 2,4-Di-O-methyl-1,3,5,6-tetra-O-acetyl-1-[1H]mannitol  | 3,6-Di-O-substituted Man | 2.0           |

FIGURE 5. Model of N-linked glycans in wild type and Caoch1Δ null mutant.

FIGURE 6. ES-MS analysis of the Caoch1Δ null mutant permethylated glycans. Mild acid-treated N-linked glycans from the wild type and Caoch1Δ null mutant strains were permethylated and analyzed by positive ion ES-MS. No ions were recorded for the wild type structures, presumably because of their extreme size, but doubly charged [M + 2Na]2+ ions were recorded from Hex1HexNAc2 to HexnHexNAc2 species for the null mutant. The figure shows the masses (M + 2Na) of glycan disodium adducts after deconvolution of the spectrum using the MassLynx maximum entropy 3 algorithm.

The additional 1,2-linked Man residues can be attached to one or more antennae of the conventional Man3GlcNAc2 core (Fig. 5).

Cell Wall Composition, Sensitivity, and Cell Integrity Pathway Activation—Total cell wall carbohydrate composition was analyzed by Dionex HPAE-PAD. The chitin/glucan/mannan ratio was 1.0:59.5:39.4 for the wild type control and 1.8:88.3:10.0 for the Caoch1Δ null mutant. The decrease in the proportion of mannan was reciprocated with a rise in both glucan and chitin.

To determine the effect of the Caoch1Δ mutation on the integrity of the cell wall, we tested the null mutant for sensitivity to a range of cell wall perturbing agents and other compounds associated with glycosylation defects. The Caoch1Δ null mutant was hypersensitive to the cell wall perturbing agents Calcofluor White and Congo Red and to SDS that would affect both cell wall proteins and the plasma membrane (Fig. 7). The null mutant was also hypersensitive to hygromycin B and tunicamycin (Fig. 7) and slightly more resistant to vanadate (data not shown), characteristics that are common to N-glycosylation mutants in other fungi (59, 60). There was no change in sensitivity toward other agents and stress such as caffeine, NaCl, or KCl (data not shown). A similar sensitivity profile was seen with the TET-OCH1 strain when the initial inoculum was pre-grown under repressing conditions. Antifungal susceptibility testing demonstrated no change in sensitivity to itraconazole, fluconosine, amphotericin B, or caspofungin (data not shown).

Because the Caoch1Δ null mutant had a cell wall defect, we carried out Western blotting to test whether the protein kinase C cell integrity pathway was activated by using an antibody that recognizes the phospho-specific form of the MAPK Mkc1p. Mkc1p was activated in the Caoch1Δ null mutant and TET-OCH1 strain under repressing conditions but was not activated in the wild type and TET-OCH1 strain under inducing conditions (Fig. 8). As a positive control, the strains were also treated with Calcofluor White, which is known to activate the pathway. An additional band was also detected only in the Caoch1Δ null mutant and TET-OCH1 strain under repressing conditions. This band migrated at 49 kDa and most likely corresponds to the phosphorylated form of CaCek1p. It has been shown recently that this phospho-specific antibody can recognize activated CaCek1p (61). Therefore, CaCek1p may act in an alternative cell integrity pathway activated by glycosylation defects. A similar role has been reported for Kss1p in S. cerevisiae (62, 63). The increased sensitivity to cell wall perturbing agents and constitutive activation of the cell integrity pathway confirm a cell wall defect resulting from the loss of CaOCH1.

Attenuation of Virulence in Caoch1Δ—The effect of the loss of CaOCH1 on virulence was assessed in a mouse model of systemic infection. The Caoch1Δ null mutant was significantly attenuated in virulence with a mean survival time of 22 days, compared with 6 days for the wild type control (Fig. 9, log rank test; p < 0.01). The re-integrant control was completely restored in virulence with a mean survival time of 7 days. Mice infected with the wild type, re-integrant control, or Caoch1Δ null mutant all had similar tissue burdens in both brain and kidney (Table 4). Therefore, the host-fungal interaction is altered in the Caoch1Δ null mutant that lacks N-glycan outer chains, such that significant tissue by ES-MS/MS, and the product ion spectra confirmed that these contained a GlcNAc2 core and were of the triantennary oligomannose type (data not shown). Analysis of cross-ring cleavage ions (58) suggests that the additional 1,2-linked Man residues can be attached to one or more antennae of the conventional Man3GlcNAc2 core (Fig. 5).
Och1p Required for Outer Chain N-Glycosylation and Virulence

![Image](365x26 to 393x38)

**FIGURE 7.** Sensitivity of Caoch1Δ null mutant to cell wall perturbing agents. The wild type (closed squares), Caoch1Δ null mutant (open squares), and Caoch1Δ = OCH1 re-integrant (open triangles) strains were quantitatively tested for sensitivity to cell wall perturbing agents using the microdilution method. The agents to which the Caoch1Δ null mutant displays hypersensitivity are shown (Calcofluor White, Congo Red, SDS, hygromycin B, and tunicamycin). Error bars are means ± S.D.

![Image](49x30 to 300x457)

**FIGURE 8.** Activation of the cell wall integrity pathway through the loss of CaOCH1. Activation of the cell integrity pathway was determined by Western blotting using the PhosphoPlus p44/42 MAPK antibody that detects CaMkk1p (59 kDa) in its phosphorylated form. This antibody also detects CaCek1p (49 kDa) in its phosphorylated form. Protein extracts were prepared from cells in mid-exponential phase, and as a positive control for activation of the cell integrity pathway, the strains were also treated with 100 μg/ml Calcofluor White as indicated. Samples are as follows: lane 1, wild type; lane 2, Caoch1Δ null mutant; lane 3, TET-OCH1 ON; lane 4, TET-OCH1 OFF. Equal loading was confirmed with Ponceau S staining and the intensity of nonspecific bands.

![Image](49x54 to 300x600)

**FIGURE 9.** Attenuation of virulence in the Caoch1Δ null mutant. Virulence was assayed in a mouse model of systemic infection. Mice (n = 5 for wild type (closed squares), n = 6 for null mutant (open squares), and re-integrator (open triangles)) were infected intravenously with the strains at 1.8 × 10^5 cfu/g of body weight.

| Strain                  | Mean survival | Kidney burden | Brain burden |
|-------------------------|---------------|---------------|--------------|
| CaOCH1                  | 6 ± 2.3       | 6.6 ± 0.5     | 5.0 ± 1.0    |
| Caoch1Δ                 | 22 ± 9.1      | 6.5 ± 1.2     | 4.9 ± 1.0    |
| Caoch1Δ = OCH1          | 7 ± 2.3       | 6.6 ± 0.5     | 4.0 ± 1.1    |

**TABLE 4**

Mean survival times and organ burden for BALB/c mice infected with Caoch1Δ null mutant and control strains.

Burdens are generated but evidently cause less damage resulting in an increased survival time. This work demonstrates the importance of N-glycan outer chain epitopes in the host-fungal interaction and virulence.

**DISCUSSION**

Here we describe the importance of outer chain elongation of N-glycans in the structure of the C. albicans cell wall and the interaction of these epitopes with the host. The addition of N-glycans is the major form of modification of mannoproteins. The carbohydrate structure consists of the Man, GlcNAc, and core that is extensively elaborated in the Golgi by the addition of the highly branched outer chain. Previous studies have demonstrated the overall importance of glycosylation to the cell wall structure and virulence (25–27). Loss of O-mannan has been shown to result in reduced adherence and the attenuation of virulence, suggesting that O-mannan may act as a ligand in the interaction with host surfaces (12, 14, 28–31). Conversely, phosphomannan has been shown not to be required for normal host-fungal interactions or virulence (32). We have extended these studies by analyzing the importance of the N-glycan outer chain epitopes. This was achieved by disrupting the C. albicans homolog of OCH1, which initiates outer chain elongation. The Caoch1Δ null mutant had a clear defect in the addition of the N-glycan outer chain and correspondingly displayed a weakened cell wall. The N-glycan outer chain was shown to be necessary for normal host-fungal interactions and virulence.

The Caoch1Δ null mutant had slightly reduced growth rate and formed cellular aggregates. These aggregates could be dispersed by treatment with chitinase suggesting that they are the result of a cell separation defect. After chitinase treatment, it was clear that the null mutant was growing in chains of short pseudohyphae. These aggregates of short pseudohyphae resulted in the formation of a crenulated colony morphology. Most notably, the null mutant cells also invaded the agar surface, and the invading cells were predominantly hyphal in morphology under conditions that normally support growth by budding. Western analysis has demonstrated that both the Cek1p and Mkc1p MAPKs are constitutively activated in the null mutant. Both Cek1p and Mkc1p have been shown to be important in invasive growth, signaling through nutrient limiting conditions or colonial growth and physical contact, respectively (64, 65). Therefore, the constitutive activation of these pathways in the Caoch1Δ null mutant may explain why it so readily invades the agar surface under nonhyphal inducing conditions.

Previously we have measured the electrophoretic mobility of secreted acid phosphatase as a marker for changes in N-glycosylation. However, we were not able to detect acid phosphatase in soluble protein extracts of the Caoch1Δ null mutant, and only low levels of cell-associated or secreted activity were seen. In crude cellular extracts, low activity was detected after native PAGE, but it failed to migrate into the polyacrylamide gel. This would suggest that the glycosylation defect in Caoch1Δ resulted in acid phosphatase becoming partially insoluble with low activity. As an alternative we developed an in-gel activity assay for HexNCase. HexNCase is exclusively N-glycosylated and is readily detected in nondenaturing polyacrylamide gels. Therefore, it can be used as a more sensitive marker of N-glycosylation defects.

Loss of CaOCH1 resulted in clear defects in N-glycosylation demonstrated by the increased mobility of HexNCase and a reduction in phosphomannan. The remaining phosphomannan present in the null mutant, low levels of Manα and Manβ could be attached to the N-linked core structure or potentially O-mannan. As expected, the O-mannan in the Caoch1Δ null had a normal structure. The null mutant also displayed a significant reduction in the in vitro mannosyltransferase activ-
ity toward the Man₉GlcNAc₂ core structure. However, activity toward this acceptor was not completely abolished in the null mutant. The Man₉GlcNAc₂ core is a complex structure and could be modified in vivo by additions to the terminal antennae of the core by other mannosyltransferases. Mannosylphosphate is also added to the N-glycan core, although in this case the resulting charged product would not be detected in the assay. Additionally, in vitro assays of activity many mannosyltransferases display low substrate specificity. Indeed, heterologously expressed CaMnt1p has been shown to display activity toward a Man₉GlcNAc₂ core-like structure in vitro (66), although in vivo it specifically acts in the extension of O-linked glycans (14). Therefore, background activity toward the Man₉GlcNAc₂ core in the Caoch1Δ null mutant is not unexpected.

Purified N-glycans from the wild type and Caoch1Δ null mutant were analyzed by methylation linkage analysis and ES-MS. Wild type N-glycans had no ions detectable by ES-MS because of their extreme size. Methylation linkage analysis revealed substantial amounts of terminal mannose and 2-O-substituted, 6-O-substituted, and 2,6-di-O-substituted mannose, consistent with the current structural model for the outer chain (57) (Fig. 5). The two unique 3,6-di-O-substituted mannose linkages in the N-glycan core were used to determine the stoichiometry of the various linkage groups in the outer chain. However, the outer chain has also been reported to contain some branched side chains (57), and these structures would also contribute to the level of 3,6-di-O-substituted mannose. Hence, the proportions of linkages in the wild type N-glycans may be a minimum estimate of the extent of outer chain elongation.

Methylation linkage analysis of the N-glycans purified from the Caoch1Δ null mutant demonstrated an almost complete loss of 6-O-substituted and 2,6-di-O-substituted mannose. This confirms the absence of the outer chain glycan in the Caoch1Δ null mutant. Therefore, CaoCH1 functions in initiating outer chain elongation in C. albicans. Consistent with the loss of the outer chain, there was a reduction in the proportion of terminal and 2-O-substituted mannose residues. However, 2-O-substituted mannose residues were not reduced to the level expected for a simple Man₉GlcNAc₂ core. Indeed, the core appears to undergo further modification in the Caoch1Δ null mutant, with elaborations from Man₁₀GlcNAc₂ to Man₁₆GlcNAc₂. This is in contrast to the situation in S. cerevisiae where N-glycans of the Scoch1Δ null mutant consist of only Man₁₀GlcNAc₂ (35). These further modifications to the core in the Caoch1Δ null mutant appear to consist of chains of 1,2-linked mannose residues added to one or more of the antenna residues of the core (Fig. 5). It is not yet clear whether such modifications to the core also occur in wild type N-glycans or are the result of the lack of normal acceptor substrates for mannosyltransferases in the absence of the outer chain. The presence of these modifications is in accord with the mannosyltransferase activity detected toward the Man₉GlcNAc₂ core in the null mutant. The migration pattern of HexNAcase from the core in the null mutant appears to consist of chains of Man₈–₁₀GlcNAc₂ (35). These further modifications to the core also occur in wild type S. cerevisiae or are the result of the lack of normal acceptor substrates for mannosyltransferases in the absence of the outer chain glycan in the Caoch1Δ null mutant. The balance of O-linked glycans from the wild type and Caoch1Δ null mutant were used to determine the stoichiometry of these structures and these would also contribute to the level of 3,6-di-O-substituted mannose. Hence, the proportions of linkages in the wild type N-glycans may be a minimum estimate of the extent of outer chain elongation.

ACKNOWLEDGMENTS—We thank Dr. Jean Marie François and Blanca Aguilar-Usconga (Institut National des Sciences Appliquées de Lyon) for their help with the cell wall composition analysis.

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