Signaling Domains of Mucin Msb2 in *Candida albicans*

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*Candida albicans* adapts to the human host by environmental sensing using the Msb2 signal mucin, which regulates fungal morphogenesis and resistance characteristics. Msb2 is anchored within the cytoplasmic membrane by a single transmembrane (TM) region dividing it into a large N-terminal exodomain, which is shed, and a small cytoplasmic domain. Analyses of strains carrying deleted Msb2 variants revealed an exodomain segment required for cleavage, shedding, and all functions of Msb2. Phosphorylation of the mitogen-activated protein kinase (MAP kinase) Cek1 was regulated by three distinct regions in Msb2: in unstressed cells, N-terminal sequences repressed phosphorylation, while its induction under cell wall stress required the cytoplasmic tail (C-tail) and sequences N-terminally flanking the TM region, downstream of the proposed cleavage site. Within the latter Msb2 region, overlapping but not identical sequences were also required for hyphal morphogenesis, basal resistance to antifungals, and, in unstressed cells, downregulation of the *PMT1* transcript, encoding protein O-mannosyltransferase-1. Deletion of two-thirds of the exodomain generated a truncated Msb2 variant with a striking ability to induce hyperfilamentous growth, which depended on the presence of the Msb2-interacting protein Sho1, the MAP kinase Cek1, and the Efg1 transcription factor. Under cell wall stress, the cytoplasmic tail relocated partially to the nucleus and contributed to regulation of 117 genes, as revealed by transcriptomic analyses. Genes regulated by the C-tail contained binding sites for the Ace2 and Azf1 transcription factors and included the ALS cell wall genes. We concluded that Msb2 fulfills its numerous functions by employing functional domains that are distributed over its entire length.

Upon proliferation and dissemination in a host tissue or target cell, pathogens encounter different types of host cells, extracellular matrices, and molecules that participate in pathogen defense. Upon contact with fungal pathogens, the human host utilizes an efficient and complex immune response that acts in multiple layers, including pathogen recognition and production of soluble innate effectors (1). To survive and overcome host immunity, pathogenic fungi have evolved sophisticated strategies which include two major steps: immune evasion and tissue invasion (2). Polysaccharides and glycoproteins in the fungal cell wall are the first contact points between the fungus and human immune cells, representing pathogen-associated molecular patterns (PAMPs). PAMPs are detected by human pattern recognition receptors (PRRs) and respond to a variety of innate immune components (3), including antimicrobial peptides (AMPs), which constitute major weapons of innate immunity that can directly kill fungal pathogens (4, 5). In response to the host, modification of PAMPs by cell wall remodeling or by hypha formation alters their availability for PRRs in many fungal species (6). The cellular morphology of the important human fungal pathogen *Candida albicans* is determined by host contact, which regulates morphogenic signaling pathways depending on the type and intensity of environmental cues in body niches (7).

Fungal signaling mucins are large, transmembrane glycoproteins that undergo posttranslational modifications, such as glycosylation and proteolytic processing, which are crucial for their cellular functions (8–13). The *C. albicans* mucin Msb2 triggers responses and rescue pathways required for hyphal morphogenesis and growth in the presence of antifungal compounds (8, 14). Msb2 is cleaved to release and shed its large glycosylated exodomain, which is able to bind AMPs with a high affinity and thereby to protect fungal cells (15). The yeast-to-hypha transition is regulated by several signal transduction cascades containing protein kinase A (PKA), mitogen-activated protein kinases (MAP kinases), and pH-responsive modules (16). Msb2 triggers hyphal morphogenesis via the Cek1 MAP kinase module. Defects in cell wall glycostructures affecting protein glycosylation and 1,3-glucan levels are sensed by Msb2 and transmitted to the Cek1 MAP kinase cascade regulating target genes, e.g., *PMT* genes, encoding protein-O-mannosyltransferases (14, 17). Intact N-glycosylation is detected by Msb2 and represses *PMT1* transcription, while defective N-glycosylation induces Cek1 phosphorylation and derepresses *PMT1* transcription. The responses to defective O-glycosylation also require Msb2 to upregulate *PMT2* and *PMT4* expression (17). Recently, the transcription factor Ace2 was implicated in the regulation of *PMT* genes (17).

The structure of signaling mucins consists of a large, highly glycosylated, rod-shaped extracellular domain that is connected to a small, cytosolic carboxy-terminal domain via a transmembrane (TM) region (9). Previous results indicated that the cytoplasmic tail (C-tail) of Msb2 in *C. albicans* is needed to activate Cek1 in response to cell wall defects (8). In the present work, a systematic deletion analysis of Msb2 identified a mosaic of functional regions in the Msb2 structure, which contribute to various functions.

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TABLE 1 C. albicans strains used for this study

| Strain       | Genotype                                                                 | Reference |
|--------------|---------------------------------------------------------------------------|-----------|
| AS1          | CA4, but tpk2Δ::hisG/tpk2Δ::hisG                                            | 41        |
| CAF2-1       | ura3Δ::imm434/URA3                                                         | 42        |
| CA14         | ura3::imm434/ura3::imm434                                                 | 43        |
| CJK34B-16L   | ura3/ura3 cek1Δ::hisG/cek1Δ::hisG                                          | 44        |
| CJKY157      | ura3/ura3 czf1::hisG/czf1::hisG                                           | 45        |
| HLC67        | CA4, but efg1::hisG/efg1::hisG                                            | 46        |
| JKC18        | CA4, but cph1::hisG/cph1::hisG                                            | 47        |
| IIHH6-4a     | CA4, but tpk1Δ::hisG/tpk1Δ::hisG                                           | 48        |
| FCCa27       | CA4, but msb2Δ::hisG/msb2Δ::hisG                                          | 8         |
| FCCa28       | CA4, but msb2Δ::hisG/msb2Δ::hisG                                          | 8         |
| ESCa3        | CA4, but msb2Δ::hisG/msb2Δ::hisG/LEU2/LEU2::pES11a(ACT1p-MSB2::HA-V5)      | 8         |
| ESCa25       | CA4, but msb2Δ::hisG/msb2Δ::hisG/LEU2/LEU2::pES14(ACT1p-MSB2::ΔN::HA-V5)  | 8         |
| ESCa38       | FCCa28, but LEU2/LEU2::pES16(ACT1p-MSB2-ΔC16)                              | 8         |
| ESCa39       | FCCa28, but LEU2/LEU2::pES17(ACT1p-MSB2-ΔTM-C16)                          | 8         |
| REP22        | ura3Δ::imm434/ura3Δ::imm434 his1Δ::hisG/Δ::hisG/msb2Δ::FRT/msb2Δ::FRT sho1::hisG/sho1::hisG | 14        |
| LvW1000      | CA4, but LEU2/LEU2::pDS1044-1 (empty vector)                              | This study |
| MScCa1       | FCCa28, but LEU2/LEU2::pSM4(ACT1p-MSB2::ΔG::1293~1307)                    | This study |
| MScCa3       | FCCa28, but LEU2/LEU2::pSM4(ACT1p-MSB2::ΔG::1278~1292)                    | This study |
| MScCa8       | FCCa28, but LEU2/LEU2::pSM6(ACT1p-MSB2ΔN114~1285)                         | This study |
| MScCa29      | FCCa28, but LEU2/LEU2::pSM7(ACT1p-MSB2ΔN65~780)                           | This study |
| MScCa30      | FCCa28, but LEU2/LEU2::pSM9(ACT1p-MSB2ΔN700~835)                          | This study |
| MScCa31      | FCCa28, but LEU2/LEU2::pSM10(ACT1p-MSB2ΔN514~579)                         | This study |
| MScCa34      | FCCa28, but LEU2/LEU2::pSM11(ACT1p-MSB2ΔN500~1087)                         | This study |
| MScCa35      | FCCa28, but LEU2/LEU2::pSM12(ACT1p-MSB2ΔN1055~1282)                       | This study |
| MScCa37      | FCCa28, but LEU2/LEU2::pSM14(ACT1p-MSB2ΔN514~1087)                        | This study |
| MScCa38      | CA4, but LEU2/LEU2::pSM14(ACT1p-MSB2-ΔN1294~1307)                         | This study |
| MScCa39      | CK34B-16L, but LEU2/LEU2::pSM14(ACT1p-MSB2ΔN514~1087)                     | This study |
| MScCa40      | CKY157, but LEU2/LEU2::pSM14(ACT1p-MSB2ΔN514~1087)                        | This study |
| MScCa41      | HLC67, but LEU2/LEU2::pSM14(ACT1p-MSB2ΔN514~1087)                        | This study |
| MScCa42      | JKC18, but LEU2/LEU2::pSM14(ACT1p-MSB2ΔN514~1087)                        | This study |
| MScCa43      | IIHH6-4a, but LEU2/LEU2::pSM14(ACT1p-MSB2ΔN514~1087)                     | This study |
| MScCa44      | AS1, but LEU2/LEU2::pSM14(ACT1p-MSB2ΔN514~1087)                          | This study |
| MScCa45      | FCCa28, but LEU2/LEU2::pSM15(ACT1p-MSB2ΔN514~1087)                       | This study |
| MScCa46      | FCCa28, but LEU2/LEU2::pSM16(ACT1p-MSB2ΔN-TMC514~1087)                   | This study |
| MScCa47      | REP22, but LEU2/LEU2::pSM14(ACT1p-MSB2ΔN514~1087)                        | This study |
| MScCa48      | FCCa28, but LEU2/LEU2::pSM17(ACT1p-MSB2ΔN514~1087)                       | This study |

Materials and Methods

Strains and media. C. albicans strains used for this study are listed in Table 1. MSB2 expression vectors digested with EcoRV were integrated ectopically into the LEU2 locus of strain FCCa28, which places MSB2 alleles under transcriptional control of the ACT1 promoter (8). Strains were grown on/in complex yeast extract-peptone-dextrose (YPD) or minimal supplemented SD medium (8). Standard drop dilution tests (10-fold dilutions to 10−5) were used to determine sensitivity to inhibitors on YPD agar. Hypha formation was induced by growth at 37°C on YPM medium (1% yeast extract, 2% peptone, 2% mannitol).

MSB2 expression vectors. Expression vectors encoding Msb2 variants were constructed by primer-directed mutagenesis of plasmid pES11a (8), using a QuikChange kit (Stratagene). Sequences of oligonucleotide primers are listed in Table S1 in the supplemental material. Designations of deletion variants (with the encoding plasmids and mutagenic primers) were as follows: ΔD3 (plasmid pSM3; primer pair Msb2-15ASD61 Fwd/Rev), ΔD4 (pSM4; Msb2-15ASD62 Fwd/Rev), ΔD5 (pSM12; Msb2-15ASD62 Fwd/Rev), ΔD6 (pSM11; Msb2-15ASD61 Fwd/Rev), ΔD7 (pSM10; Msb2-Del10 Fwd/Rev), ΔD8 (pSM9; Msb2-Del9 Fwd/Rev), ΔD9 (pSM17; Msb2-Del6 Rev/Msb2-Del11 Fwd), ΔD11 (pSM14; Msb2-Del6 Rev/Msb2-Del11), ΔD14 (pSM7; Msb2-Del7 Fwd/Rev), and ΔD15 (pSM6; Msb2-Del6 Fwd/Rev). pSM15/16 was based on construct pSM14. pES16/17 (8) and pSM14 were cut with Apal and Bsu36I. Fragments including stop codons upstream or downstream of the TM domain were ligated into pSM14, encoding the ΔD12 and ΔD13 variants. Plasmids were integrated into the LEU2 locus of strain FCCa28. Integration into the LEU2 locus was verified by colony PCR with primers Kolo AMP Fwd and Kolo LEU2 Rev.

RNA methods. RNAs were isolated from all cultures, and transcriptomic analyses were performed essentially as described previously (18). Cy3- and Cy5-labeled cDNAs generated from RNAs of strains ESca3 and ESCa38 were cohybridized to C. albicans genomic arrays (Eurogentec, Belgium). The arrays were read and evaluated using GeneSpring software as described previously. Genes were considered to be significantly regulated (P < 0.05) if the expression ratios for strains ESca3 and ESCa38 were ≥1.5 during caspofungin treatment (50 ng/ml; 30 min).

For quantitative PCR (qPCR) analyses, total RNA was isolated, treated with DNase I (Turbo DNase kit; Ambion), and purified using an RNA isolation kit. g of purified RNA was reverse transcribed into cDNA (Maxima First Strand cDNA synthesis kit; Thermo Scientific), using nuclear-free water (protocols of the suppliers were used for all steps). A parallel sample was not reverse transcribed as a control for...
contaminated DNA. The qPCR assay was done using a model Mx3000P (Stratagene) machine, with 10 µl of cDNA sample (1:10), 4 µl EvaGreen QPCR-mix II (Bio-Budget), and 3 µl each of forward and reverse oligonucleotide primers (400 pmol/µl) in each reaction mixture. AmpliTaq polymerase was activated at 95°C for 10 min, and annealing was performed at 60°C for 20 s. The extension step was performed at 72°C for 30 s, and the denaturation step was performed at 95°C for 30 s; a total of 40 cycles were completed.

**Protein methods.** Strains were grown in 50 ml YPD or SD medium at 30°C to an optical density at 600 nm (OD_{600}) of 0.8 or 6 to 10, respectively, and cells were harvested by centrifugation. Cells were washed with water and resuspended in lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100) containing protease inhibitors (Complete Mini; Roche). Cells were broken by shaking with glass beads at 4°C with a FastPrep homogenizer (MP Biochemicals). Cell debris and glass beads were separated from the crude cell extract by centrifugation. For immunoblotting, proteins were separated by SDS-PAGE (12% SDS, 4 to 20% acrylamide) and blotted to polyvinylidene difluoride (PVDF) membranes. Protein standards used were the PageRuler set (11 to 170 kDa; Fermentas) and the HiMark set (31 to 460 kDa; Invitrogen) of proteins. Membranes were probed using a rat anti-heamatglutinin (anti-β) monoclonal antibody (1:2,000; Roche) and visualized using peroxidase-coupled goat anti-rat or anti-mouse antibody (1:10,000; Thermo Scientific) and the SuperSignal West Dura chemiluminescent substrate (Pierce). The membrane was processed as for immunoblotting, and the resulting signals were recorded using a Fujifilm LAS400 mini-image analyzer and evaluated with the Fujifilm Multi Gauge program. Signals were quantitated using ImageJ software (http://imagej.nih.gov/ij/).

**MAP kinase activation assay.** Strains were grown overnight to stationary phase in YPD medium and diluted in YPD medium to an OD_{600} of 0.1. Cells were grown to an OD_{600} of 0.8 at 37°C and incubated further for 1 h in the presence (+) or absence (−) of tunicamycin (2 µg/ml). Immunoblots were prepared as described previously, with verification of equal loading by Ponceau red staining of the membranes. Blots were probed with anti-phospho-p44/42 MAP kinase (Cell Signaling Technology) to detect phosphorylated Cek1 antibody (8) or with a polyclonal Cek1 antibody to detect total Cek1 (14).

**Fluorescence microscopy.** Strains were grown in YPD at 30°C to an OD_{600} of 1 and were stained with calcofluor white (0.1% calcofluor white solution; Sigma) to visualize chitin. Cells (OD_{600} = 0.8) used for immunofluorescence microscopy were fixed with 4% formaldehyde, and 1 ml of cell suspension was treated with Zymolyase T100 (100 µg; 50 kDa); glucuronidase (30 µl), and 10 mM diithiothreitol (DTT) for 30 min at 30°C. Cells were pelleted and treated with 0.1% Triton X-100 for 5 min at room temperature. Cells (20 µl) were fixed to polylysine-coated glass slides and washed with phosphate-buffered saline (PBS), followed by blocking of unspecific binding sites by use of 2% milk powder in PBS. The blocking solution was removed, and 40 µl of rat anti-V5 antibody (1:100; AbD Serotec) was allowed to react for 90 min at room temperature or overnight at 4°C in a wet chamber. Cells were washed, and fluorescein isothiocyanate (FITC)-coupled anti-mouse antibody (1:100; Sigma–Aldrich) in 0.2% milk powder was added and allowed to react for 90 min at room temperature. For nuclear staining, 20 µl 4',6-diamidino-2-phenylindole (DAPI; 1 µg/ml) was added for 15 min at room temperature. Slides were washed with PBS, and a drop of antifade reagent (Pro-Long; Sigma) was added before covering the specimen with a coverslip, which was sealed with nail polish. Microscopic inspection of FITC and DAPI fluorescence was done using an Axioskop 40 microscope (Carl Zeiss, Goettingen, Germany), and the ImageJ program (http://imagej.nih.gov/ij/) was used for evaluation of fluorescence signals.

**Antimicrobial peptide assays.** Overnight cultures of C. albicans were diluted and grown in YPD at 30°C to an OD_{600} of 0.3. Cells were harvested by centrifugation and washed and resuspended in PBS. Triplicate assay mixtures containing 5 µl cell suspension and 2 µg LL-37 (Sigma) in a total volume of 25 µl were incubated for 1.5 h at 37°C, diluted 500-fold, and plated on YPD. Numbers of CFU were determined after 2 days of growth at 30°C. Sensitivity to long-term exposure to LL-37 was determined by growing strains in the presence or absence of LL-37 (1 µg/200 µl; final concentration, 5 µg/ml). Triplicate assay mixtures were inoculated to an OD_{600} of 0.1 and were incubated for 7 h at 37°C on a rotary shaker at 115 rpm. To assess killing, the OD_{600} was measured and compared to that of untreated cells.

**Statistical analysis.** Statistical significance was determined using the unpaired t test (*, P < 0.05; **, P < 0.01; and ***, P < 0.001).

**RESULTS.** C. albicans strains producing Msb2 deletion variants. The precursor of the membrane sensor protein Msb2 in C. albicans contains a single transmembrane (TM) region that separates a large, N-terminal glycosylated exodomain from a short, C-terminal cytoplasmic tail. The precursor is cleaved and the exodomain released in substantial amounts into the environment during planktonic and surface growth (8, 15, 19). Previous studies suggested that the N-terminal, TM, and cytoplasmic domains of Msb2 convey different cellular functions (8). To further define Msb2 functional domains, strains were constructed to produce additional Msb2 deletions; variants were designated ΔD1 to ΔD15 and included the previously described variants ΔN (ΔD10), ΔC (ΔD1), and ΔTM–C (ΔD2) (Fig. 1). The respective MSB2 alleles were chromosomally integrated into an msb2 mutant strain and expressed under the control of the constitutive ACT1 promoter (8), because preliminary results revealed that MSB2 transcription is autoregulated via the Msb2-Cek1 pathway (L. van Wijlick and J. F. Ernst, unpublished results). Transformants produced all Msb2 variants at similar levels, although amounts of cell-associated/secrete proteins were different for certain variants (see below).

**Hyphal morphogenesis and basal resistance to glycostress require specific Msb2 domains.** As previously described, the msb2 mutant and a strain producing an Msb2 variant lacking both the TM region and the cytoplasmic tail (ΔD2 variant) were not able to form hyphae on YPM agar, while removal solely of the N-terminal glycosylation (ΔD1 variant) had no effect (8). Interestingly, a series of small deletions directly upstream of the TM region (ΔD3, ΔD4, and ΔD5 variants) led to defective hypha formation, indicating that the respective sequences (residues 1084 to 1308) contribute to hyphal development (Fig. 2A). Defective hypha formation was not observed for any of the deletions further upstream, assigning Msb2 requirements for hyphal morphogenesis to a specific internal Msb2 segment.

Previous work indicated that msb2 mutants are supersensitive to caspofungin and to other agents generating glycostress, including Congo red and Zymolase (14). To define functional domains of Msb2 required for basal resistance, we tested the sensitivity of strains producing Msb2 variants to caspofungin and to low concentrations of tunicamycin, which blocks N-glycosylation. As expected, the msb2 mutant was more sensitive than the wild-type strain to tunicamycin. Similarly, the Msb2 deletion series encompassing sequences immediately N-terminal to the TM region (ΔD3, ΔD4, and ΔD5 variants) was also more sensitive, while further upstream deletions (ΔD6 to ΔD10 variants) restored basal resistance (Fig. 1 and 2B). Collectively, the results indicate that basal resistance to glycostress requires specific internal Msb2 sequences, which coincide essentially with sequences required for hypha formation.

**Shedding of the exodomain requires a specific Msb2 segment.** To establish if specific sequences of Msb2 are needed for its
shedding from *C. albicans* cells, we examined the presence of Msb2Δ1 variant via the msb2Δ1 allele (8). The positions of the signal sequence (SS), the transmembrane region (TM), and HA and V5 epitope tags in Msb2 are indicated. The transformants (encoded variants) were strains ESCa3 (Msb2Δ1HA-V5), ESCa38 (ΔD1), ESCa39 (ΔD2), MSCa1 (ΔD3), MSCa3 (ΔD4), MSCa4 (Δ06), MSCa35 (ΔD5), MSCa30 (ΔD8), MSCa31 (ΔD7), MSCa48 (ΔD9), ESCa25 (ΔD10), ESCa37 (Δ11), MSCa45 (Δ12), MSCa46 (ΔD13), MSCa29 (ΔD14), and MSCa8 (ΔD15). Data for strains ESCa25 (ΔD10; Msb2ΔN), ESCa38 (ΔD1; Msb2−ΔC), and ESCa39 (ΔD2; Msb2−ΔTM−C) have been reported previously (8). Listed Msb2 phenotypes include Msb2 shedding, hypha formation on YPM agar at 37°C, tunicamycin (TM) and caspofungin (CaspR) resistances, phosphorylation of Cek1 induced by tunicamycin treatment (TM Cek1-P), and repression of PMT1 expression (PMT1 rep) under normal growth conditions. Hyperfilamentous growth (hyp) and constitutive phosphorylation of Cek1 during growth in YPD at 37°C (const) are listed. n.d., not determined.

**FIG 1** Structures and functions of Msb2 protein variants. Plasmids encoding Msb2 variants were chromosomally integrated into strain FCC2a8, which produces the inactive Msb2−Δ1 variant via the msb2Δ1 allele (8). The positions of the signal sequence (SS), the transmembrane region (TM), and HA and V5 epitope tags in Msb2 are indicated. The transformants (encoded variants) were strains ESCa3 (Msb2Δ1HA-V5), ESCa38 (ΔD1), ESCa39 (ΔD2), MSCa1 (ΔD3), MSCa3 (ΔD4), MSCa4 (Δ06), MSCa35 (ΔD5), MSCa30 (ΔD8), MSCa31 (ΔD7), MSCa48 (ΔD9), ESCa25 (ΔD10), ESCa37 (Δ11), MSCa45 (Δ12), MSCa46 (ΔD13), MSCa29 (ΔD14), and MSCa8 (ΔD15). Data for strains ESCa25 (ΔD10; Msb2ΔN), ESCa38 (ΔD1; Msb2−ΔC), and ESCa39 (ΔD2; Msb2−ΔTM−C) have been reported previously (8). Listed Msb2 phenotypes include Msb2 shedding, hypha formation on YPM agar at 37°C, tunicamycin (TM) and caspofungin (CaspR) resistances, phosphorylation of Cek1 induced by tunicamycin treatment (TM Cek1-P), and repression of PMT1 expression (PMT1 rep) under normal growth conditions. Hyperfilamentous growth (hyp) and constitutive phosphorylation of Cek1 during growth in YPD at 37°C (const) are listed. n.d., not determined.
examined the phosphorylation status of Cek1 by immunoblotting crude extracts of cells grown either without stress or in the presence of tunicamycin. Interestingly, under both conditions, separate Msb2 domains were found to be responsible for Cek1 regulation. First, under tunicamycin stress, the control strain (Msb2) revealed a strong upregulation of phosphorylated Cek1 and total Cek1, which did not occur in the \textit{msb2} mutant and occurred at reduced levels in strains producing the \textit{/H9004 D3}, \textit{/H9004 D4}, and \textit{/H9004 D5} variants (defective in Msb2 shedding) or the \textit{/H9004 D13} variant (Fig. 4). In addition, the C-terminal deletion variants \textit{ΔD1} and \textit{ΔD2} were reported to be defective for upregulation of phosphorylated Cek1 with tunicamycin treatment (8). All other Msb2 variants were able to produce phosphorylated Cek1 as in control cells (Fig. 4; see Fig. S1 in the supplemental material). We concluded that the C-terminal region surrounding the transmembrane region of Msb2 is required to increase levels of phosphorylated Cek1 in response to glycostress by tunicamycin and also by caspofungin (see below).

The second, repressive function of the functional domains in Msb2 was detected by analyzing Cek1 in unstressed cells. Under these conditions, Cek1 and Cek1-P levels were low in both the control strain (Msb2) and the \textit{msb2} mutant but were as high as those with tunicamycin induction in cells with the \textit{ΔD11}, \textit{ΔD12}, \textit{ΔD14}, and \textit{ΔD15} Msb2 variants; no other deleted Msb2 variant showed this characteristic (Fig. 4). Collectively, the results indicate that N-terminal sequences of Msb2 prevent upregulation of Cek1 levels in unstressed cells, while its C-terminal sequences are crucial for increasing the levels of Cek1 and its phosphorylated form under glycostress conditions.

\textbf{Msb2 domains repress transcription of \textit{PMT1} in unstressed cells.} Previous results indicated that the Msb2-Cek1 pathway regulates transcription of \textit{PMT} genes, encoding protein \textit{O}-mannosyltransferases (17). Specifically, the presence of Msb2 and Cek1 proteins was found to downregulate transcript levels of \textit{PMT1}, encoding Pmt isoform 1. When \textit{PMT1} transcript levels were ana-

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**FIG 2** Msb2 variants affect morphogenesis and glycostress resistance. (A) Msb2 variants are defective in formation of hyphae on YPM agar. Colonies of strains were photographed following growth for 2 days at 37°C. Strains FCCa27 (msb2), ESCa38 (\textit{ΔD1}), MSCa1 (\textit{ΔD3}), MSCa3 (\textit{ΔD4}), MSCa34 (\textit{ΔD6}), and MSCa35 (\textit{ΔD5}) were compared to ESCa3 (Msb2 \textit{HA-V5}). (B) Sensitivities of strains to tunicamycin (1 \(\mu\)g/ml) and caspofungin (100 ng/ml) were tested by a drop dilution test.

**FIG 3** Msb2 shedding. Immunoblotting was performed to detect HA-tagged Msb2. Fifty-microgram samples of proteins in cell extracts and 15-\(\mu\)l aliquots of the secretome were separated in 4 to 20\% SDS-PAGE gels, and immunoblots were incubated with rat anti-HA antibody. Strains tested included ESCa3 (Msb2; \textit{MSB2 HA-V5}), FCCa27 (msb2), MSCa1 (\textit{ΔD3}), MSCa3 (\textit{ΔD4}), MSCa34 (\textit{ΔD6}), and MSCa35 (\textit{ΔD5}).
lyzed in tunicamycin-stressed cells, high levels were detected in the control strain, in the msb2 mutant, and in all strains producing Msb2 variants (Fig. 5), suggesting that Msb2 is dispensable for upregulating PMT1 transcripts under tunicamycin stress conditions. In unstressed cells, however, Msb2 appeared to have a repressive function, since the PMT1 transcript level was low in the control strain but was upregulated in the msb2 mutant strain. Furthermore, the ΔD2 to ΔD5 variants upregulated the PMT1 transcript in unstressed cells, suggesting that the encompassed sequences (residues 1084 to 1308) mediate repression of PMT1.

Low levels of phosphorylated Cek1 (Fig. 4) but derepressed high transcript levels of its downstream element PMT1 in the msb2 mutant and the cek1 mutant (17) suggest that transcriptional repression of PMT1 depends on Cek1 in its unphosphorylated form. The identified Msb2 sequences may transmit information on the intactness of glycostructures to Cek1 to permit downregulation of PMT1 transcription.

Msb2 sequences involved in basal resistance to LL-37. In previous studies, it was shown that the basal resistance of C. albicans to the AMP LL-37 depends on the shed Msb2 glycofragment (8, 15). In this study, we tested the ability of Msb2 variants to mediate resistance during short- and long-term exposures to LL-37. In experiments employing high LL-37 concentrations for short exposure times, we found that only the msb2 mutant, not the strains producing Msb2 deletion variants, was supersensitive to LL-37 (Fig. 6A). Physiological concentrations of LL-37 are low but persist at sites of infection and inflammation (20). Therefore, we also incubated strains for 7 h in the presence or absence of low levels of LL-37 (5 μg/ml). In this experimental setting, the elevated sensitivity of the msb2 mutant was confirmed (Fig. 6B). Remarkably, Msb2 variants lacking C-terminal sequences (ΔD1 to ΔD5 variants) were also more sensitive to LL-37. Thus, it appears that Msb2 sequences involved in basal glycostress resistance (Fig. 2B) also contribute to basal LL-37 resistance. In these experiments, we also examined the involvement of Cek1 in LL-37 resistance by including a cek1 mutant. This mutant was supersensitive to LL-37.

FIG 4 Levels of MAP kinase Cek1 in strains producing Msb2 variants. (A) Cells were grown to stationary phase, diluted in YPD medium, grown to an OD₆₀₀ of 0.8 at 37°C, and incubated further for 1 h in the presence (+) or absence (−) of tunicamycin (2 μg/ml). Cell extracts (50 μg) were separated by SDS-PAGE, and immunoblots were incubated with anti-phospho-p44/42 MAP kinase antibody, which detects phosphorylated Cek1 (Cek1-P), or with a polyclonal antibody to detect total Cek1 (Cek1). The actin protein (Act1), detected by anti-Act1 antibody, was used as the loading control. Strains tested included ESCa3 (Msb2; MSB2 HA-V5), FCCa27 (msb2), ESCa38 (ΔD3), ESCa39 (ΔD4), ESCa40 (ΔD6), ESCa41 (ΔD11), ESCa42 (ΔD12), ESCa43 (ΔD13), ESCa44 (ΔD14), and ESCa45 (ΔD15). (B) Relative intensities of Cek1-P and total Cek1 compared to Act1 and of Cek1-P compared to total Cek1. Band intensities were quantified using ImageJ software (http://imagej.nih.gov/ij/).

FIG 5 Regulation of PMT1 transcripts in strains producing Msb2 variants. The level of the PMT1 transcript relative to the ACT1 transcript (RTL) was determined by qPCR for cells grown to an OD₆₀₀ of 0.8 in the presence or absence of tunicamycin (0.5 μg/ml). Strains tested included ESCa3 (Msb2; MSB2 HA-V5), FCCa27 (msb2), ESCa38 (ΔD1), ESCa39 (ΔD2), ESCa40 (ΔD3), ESCa41 (ΔD4), ESCa42 (ΔD6), and ESCa43 (ΔD5). Means and standard deviations of results for two biological replicates (black and white bars) in triplicate assays are shown. Statistical significance was determined using the t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
and /H9004 required for hypha formation, we constructed the partial deletions of the segment missing in the (not observed with the 0.001. (Fig. 6) Hyperfilamentous phenotype generated by Msb2 variants. ESCa3 grown under identical conditions in the presence of caspofungin, the cytoplasmic tail of Msb2 is killing short-term exposure to determine conditions under which caspofungin concentrations, ranging from 10 to 100 ng/ml, during short-term exposure to determine conditions under which <10% of C. albicans cells were killed for both strains (data not shown). As in the case of glycostress by caspofungin, the cytoplasmic tail of Msb2 is partially cleaved and enters the nucleus.

The cytoplasmic tail of Msb2 regulates transcriptional responses to glycostress. To further investigate the role of the Msb2 cytoplasmic tail, we first analyzed the activation of Cek1 under caspofungin stress conditions (Fig. 9A). We first tested different caspofungin concentrations, ranging from 10 to 100 ng/ml, during short-term exposure to determine conditions under which <10% of C. albicans cells were killed for both strains (data not shown). As in the case of glycostress by caspofungin, the cytoplasmic tail of Msb2 is partially cleaved and enters the nucleus.

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genes according to their functional categories (21; http://www.candidagenome.org/cgi-bin/GO/goTermFinder) revealed that deletion of the Msb2 C-tail had a significant effect on ALS genes (ALS1, ALS3, and ALS5), which are responsible for the entry of C. albicans into host cells (see Table S3A). GO analysis (Slim Mapper) of the complete regulated gene set assigned 19 genes to stress responses, 16 to cellular protein modification processes, and 15 to responses to chemicals (see Table S3B). To confirm the transcriptional profiling data, we performed qPCR on two selected Msb2 target genes. Transcript levels for the ALS1 and ALS3 genes, encoding cell wall proteins, were increased by a factor of 4 or 6 (Fig. 9B). The results confirmed the increased levels of ALS gene transcripts in cells producing the /H9004D1 variant under caspofungin stress conditions.

The promoter regions of the regulated genes were analyzed using dyad analysis and YeTFaSCo (22, 23), which revealed the following two highly significant transcription factor motifs: GA2GA5 (101 sites) and CAC2AC2 (46 sites) (Fig. 9C). The identified GA2GA5 motif matches the motif for binding of the glucose-induced transcriptional regulator Azf1 in Saccharomyces cerevisiae (24). The identified CAC2AC2 motif matches the Ace2 binding site in S. cerevisiae (23) and in C. albicans (L. van Wijlick and J. F. Ernst, unpublished results). Collectively, the transcriptomic results support the notion that the cytoplasmic portion of Msb2 is functional in the nucleus to regulate transcriptional circuits alleviating glycostress. Ace2 was previously identified as contributing to regulation of PMT genes (17).

**DISCUSSION**

The fungal pathogen *C. albicans* is able to adapt to and proliferate in various complex environments of the human host (25). Fungal survival requires stress response pathways to restore cell wall integrity during attack of host immune effectors (26). The signaling mucin Msb2 has dual functions: sensing environmental stress signals and blocking an important aspect of immune defenses by inactivating AMPs (5, 14). Here we showed that different se-
sequences of Msb2 provide different functions in its proteolytic maturation and in hyphal morphogenesis, basal antifungal resistance, and gene regulation. A scheme depicting the established functional domains of Msb2 is shown in Fig. 10.

Mammalian signaling mucins are known to become proteolytically processed to generate a large extracellular glycodomain as well as a cytoplasmic domain conveying regulatory functions (9). Autoproteolytic cleavage of the MUC1 mucin depends on its internal SEA module, comprising about 120 residues, which cleaves the precursor protein at a G↑SVVV motif; the VVV sequence is replaced by other bulky hydrophobic residues in homologs (27).

FIG 9 Phosphorylation of Cek1 and regulation of ALS1 and MSB2 gene expression by caspofungin treatment. (A) Msb2 C-tail is required for Cek1 phosphorylation during caspofungin treatment. Strains tested include ESCa3 (Msb2; MSB2<sup>WT-V5</sup>) and mutant strain ESCa38, producing Msb2 lacking the cytoplasmic tail (ΔD1). Cells were grown to stationary phase, diluted in fresh YPD medium, grown to an OD<sub>500</sub> of 0.8 at 37°C, and incubated further for 30 min in the presence (+) or absence (−) of caspofungin (50 ng/ml). Immunoblot detection of Cek1 and Act1 proteins was performed as described in the legend to Fig. 4. (B) ALS1 and ALS3 expression. Transcript levels relative to the 18S rRNA transcript levels in wild-type and ESCa38 mutant cells left untreated or treated with caspofungin (50 ng/ml; 30 min) were determined by qPCR. Means and standard deviations of results for two biological replicates (black and white bars) in triplicate assays are shown. Statistical significance was determined using the t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (C) Consensus sequences for transcription factors in promoters of genes regulated by the C-tail of Msb2. Genome-wide expression analysis revealed 117 genes regulated by the presence of the Msb2 C-tail (see Table S2 in the supplemental material). Promoter regions were analyzed using dyad analysis (22) and YeTFaSCo (23) to define consensus sequences and revealed binding sites for the Azf1 and Ace2 transcription factors.

The Msb2 sensor regulates the activity of the MAP kinase Cek1, which is required for hypha formation on semisolid surfaces (8). Here we showed that glycosylation defects upregulate Cek1 levels, in agreement with the finding that the CEK1 transcript level is upregulated by tunicamycin (L. van Wijlick and J. F. Ernst, unpublished results). We identified two modes of Msb2 function on Cek1 phosphorylation, which are conveyed by three separate functional Msb2 domains. In unstressed cells, N-terminal sequences (encompassed by the ΔD10 variant) repressed the appearance of phosphorylated Cek1, while under tunicamycin stress, C-terminal sequences flanking the TM region (encompassed by the ΔD1 and ΔD3 variants) mediated induction of Cek1 and its phosphorylated form. Previously, the Mkc1 MAP kinase was described to become activated by surface growth, oxidative stress, and cell wall defects, including caspofungin glycostress (29–32); thus, Msb2 may mediate basal caspofungin resistance by activating both Mkc1 and Cek1. In contrast, increased Mkc1-P levels were not consistently reported for tunicamycin treatment conditions (14, 33), suggesting that defective protein N-glycosylation triggers upregulation of Mkc1-P only under as yet undefined conditions (possibly upon prolonged tunicamycin treatment, when cell wall proteins become significantly underglycosylated). The ability to generate hyphae was not correlated with phosphorylated
Cek1, since the ΔD1 variant showed low levels of phosphorylated Cek1 but formed hyphae, while the ΔD4 variant produced high levels of phosphorylated Cek1 but did not undergo filamentation. This result suggests that Msb2 triggers hypha formation, at least partially, independently of Cek1 and/or its state of phosphorylation. Surprisingly, a remarkable hyperfilamentation phenotype was observed when about two-thirds of the N-terminal sequences of Msb2 were deleted (ΔH9004 D11 and ΔH9004 D12 variants). Strong filamentation occurred in liquid media and led to large hyphal aggregates. Hyperfilamentation was still observed when the cytoplasmic tail of Msb2 was removed but did not occur when the TM region was deleted (ΔH9004 D13 variant). Thus, the presence of the TM region and/or its close flanking sequences appears to be sufficient to strongly induce hyphal morphogenesis. Interestingly, the Msb2-generated abnormal hypha formation also occurred in the wild-type genetic background, suggesting the dominance of the Msb2 fragment over full-length Msb2. On the other hand, hyphal formation was not induced in cells carrying the sho1, cek1, or efg1 mutation. These results indicate that hyperfilamentation induced by Msb2 sequences in the ΔH9004 D11 variant requires a known Msb2 interactor (Sho1) (14) and its downstream kinase (Cek1), but also a transcription factor essential for hypha formation (Efg1) (34). However, the contribution of Msb2 to regular hyphal induction under physiological conditions requires further analysis.

Previous results have implicated the Msb2-Cek1 pathway in fungal basal resistance and cell wall integrity (8, 14, 17). Shed Msb2 binds human AMPs and a peptide antibiotic with high affinity, thereby generating a protective cloud surrounding cells (15). Here we showed that Msb2 sequences that probably are not shed (C-terminal to the proposed cleavage region) also contribute to AMP resistance. C-terminal sequences extending from the cytoplasmic tail to residue 1085 are necessary to increase basal resistance not only to the AMP LL-37 but also to the cell wall-damaging compounds caspofungin and tunicamycin. Although sequences comprising residues 1278 to 1308 are not essential for either Msb2 shedding or Cek1 phosphorylation, they are relevant for basal resistance. The underlying mechanisms are not yet clear, but it appears that extracellular Msb2 sequences that remain after cleavage of the exodomain regulate nuclear activities, as observed in the case of mammalian N-CAM and β-dystroglycan proteins (35, 36).

The transcriptional output of Msb2-Cek1 signaling consists of altered gene expression that restores cell wall integrity. We previ-
ously found that PMT1 expression is repressed by Msb2-Cek1 proteins during normal growth, while the induction of PMT2 and PMT4 genes by inhibition of Pmt1 requires both proteins (17). Since no significant phosphorylation of Cek1 occurs in unstressed cells, it appears that PMT1 repression requires the presence of Cek1, but not in its phosphorylated form. Msb2 sequences upstream of the TM region, which are also needed to provide basal resistance to caspofungin and tunicamycin, were found to be required for PMT1 repression, although the latter process was unaffected by absence of the cytoplasmic tail (ΔD1 variant). With regard to the mechanism by which Msb2-Cek1 signaling alters gene expression, we obtained evidence by immunofluorescence microscopy that the C terminus of Msb2 (possibly the cytoplasmic domain, including the TM region) partially relocates to the cytoplasm and the nucleus if cells are stressed by caspofungin. Thus, the carboxy end of Msb2 may enter the nucleus either by itself or as a passenger of the MAP kinase modules and be involved in target gene regulation. Cleavage of type I transmembrane proteins in the membrane, releasing an intracellular fragment capable of transducing nuclear signals, has been described for the Notch-1 protein, ErbB-4, and CN14 (producing nuclear signals, has been described for the Notch-1 pro-
gene regulation. Cleavage of type I transmembrane proteins in the plasm and the nucleus if cells are stressed by caspofungin. Thus, microscopy that the C terminus of Msb2 (possibly the cytoplasmic
gene expression, we obtained evidence by immunofluorescence
of strains containing Msb2 with and without its cytoplasmic tail indeed revealed 117 differentially regu-
lated genes. Intriguingly, the list of genes includes five cell wall genes (ALSI, ALS3, ALS5, CHT2, and PGA14). Previously, it was shown that caspofungin-induced cell aggregation activates an Efg1-dependent signaling pathway to regulate ALS1 expression (41). We suggest that Msb2 is an upstream signaling molecule for Efg1-mediated hyphal morphogenesis and for upregulation of ALS1. Promoter comparisons of all regulated genes revealed binding motifs for two transcription factors (Ace2 and Azf1) known to activate transcription of genes involved in the maintenance of cell wall integrity. Interestingly, previous results identified Ace2 as an essential protein for regulation of PMT genes and for basal anti-
fungal resistance (17). Future research should be directed at clari-
fying the details of Msb2 processing and its influence in directing environment-regulated gene expression.

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