The Putative α-1,2-Mannosyltransferase AfMnt1 of the Opportunistic Fungal Pathogen *Aspergillus fumigatus* Is Required for Cell Wall Stability and Full Virulence

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Proteins entering the eukaryotic secretory pathway commonly are glycosylated. Important steps in this posttranslational modification are carried out by mannosyltransferases. In this study, we investigated the putative α-1,2-mannosyltransferase AfMnt1 of the human pathogenic mold *Aspergillus fumigatus*. AfMnt1 belongs to a family of enzymes that comprises nine members in *Saccharomyces cerevisiae* but only three in *A. fumigatus*. A Δafmnt1 mutant is viable and grows normally at 37°C, but its hyphal cell wall appears to be thinner than that of the parental strain. The lack of AfMnt1 leads to a higher sensitivity to calcofluor white and Congo red but not to sodium dodecyl sulfate. The growth of the mutant is abrogated at 48°C but can be restored by osmotic stabilization. The resulting colonies remain white due to a defect in the formation of conidia. Electron and immunofluorescence microscopy further revealed that the observed growth defect of the mutant at 48°C can be attributed to cell wall instability resulting in leakage at the hyphal tips. Using a red fluorescence fusion protein, we localized AfMnt1 in compact, brefeldin A-sensitive organelles that most likely represent fungal Golgi equivalents. The tumor necrosis factor alpha response of murine macrophages to hyphae was not affected by the lack of the *afmnt1* gene, but the corresponding mutant was attenuated in a mouse model of infection. This and the increased sensitivity of the Δafmnt1 mutant to azoles, antifungal agents that currently are used to treat *Aspergillus* infections, suggest that α-1,2-mannosyltransferases are interesting targets for novel antifungal drugs.

The pathogenic mold *Aspergillus fumigatus* is currently the major cause of airborne fungal infections that menace immunocompromised patients. Its conidia are distributed through the air, and it has been estimated that humans inhale several hundred a day. In the immunocompetent host, invading fungal conidia are eliminated by innate immune cells in the lung, but in cases of severe immunosuppression some *A. fumigatus* conidia may escape the impaired immune response. After germination, fungal hyphae can infiltrate the surrounding tissue and, in cases of invasive aspergillosis, subsequently spread to different organs (15). Invasive aspergillosis is associated with high mortality rates due to suboptimal diagnostic and therapeutic options. Therefore, there is an urgent need to develop new antifungal agents and to optimize existing therapeutic strategies.

The fungal cell wall represents an excellent drug target, since it is structurally unique, its integrity is a precondition for the survival of the fungus, and it represents an essential barrier for protection against host defense mechanisms (1, 8, 9, 16). The fungal envelope consists of a framework of glycostructures, e.g., chitin and glucans, and a set of cell wall-associated proteins (1). Although at first sight the cell wall appears as a robust and static structure, it is in fact a highly dynamic entity. Infections by fungi always are associated with morphogenetic changes and growth processes, both requiring profound cell wall dynamics. The underlying reorganization of the cell wall is a delicate process in which the fungus has to find the balance between stability and plasticity, an attempt that requires the concerted action of a variety of cell wall proteins.

The addition of N-linked and/or O-linked oligosaccharides is a frequent modification of cell wall proteins. Mannosyltransferases play a crucial role in this process and most likely also are engaged in the generation of other glycoconjugates. Mannosyltransferases are localized in intracellular compartments of the secretory pathway, e.g., the Golgi apparatus or the endoplasmic reticulum (ER). Distinct or multiple disruptions of members of the *KTR* (for killer toxin resistance) family of mannosyltransferase genes in *S. cerevisiae* resulted in impaired protein glycosylation (10, 17), and the deletion of the homologous *CaMNT1* gene in *Candida albicans* additionally affected fungal adhesion and virulence (22). At least in *C. albicans*, mannosyltransferases have been identified as crucial components assuring the maintenance and the robustness of the fungal cell wall (22).

The *ScKre2/ScMnt1 α-1,2-mannosyltransferase of S. cerevisiae* and the homologous enzymes *CaMnt1* and *CaMnt2* of *C. albicans* have been shown to be required for the addition of the second and third mannosyl residue of *O*-linked carbohydrates (10, 22), and they play a pivotal role in the elaboration of the outer chains of *N*-linked glycanas (17, 27). The importance of

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TABLE 1. Oligonucleotides used in this study

| Name                        | Sequence                                      |
|-----------------------------|-----------------------------------------------|
| mnt1-Afu5g10760-5-fwd       | TTTCGCCGCGCTTTCGGCTGGGAG                      |
| mnt1-Afu5g10760-5-rev       | TGGGCCTAATGGCCAGAAAGAT                     |
| mnt1-Afu5g10760-3-fwd       | TGGGCAATGAGCTCCATTGTCG                      |
| mnt1-Afu5g10760-3-rev       | TTGGCCTCATTTGCGGTTCCTCC                    |
| mnt1-5                      | ATGCTTCCGGAGCAAATAT                        |
| mnt1-3                      | ATGCTTCCGGAGCAAATAT                        |
| mnt1-Afu5g10760-5-cast      | GTTGGGATATCTAAAGACGCC                      |
| hph-3-Sma                   | TCCCCGGCTATTCGTCCTCC                      |
| trpCt-fwd                   | CAGAATGCAAGTAACCTCTCTG                      |
| mnt1-3-UTR-rev              | ATGGATGATAGACCCCTCTACTG                     |
| mnt1-mRFP1-fu-rev           | GGCCTGACAGTCTGCTGCTCCTC                    |
| mRFP1-5-Pme                  | AAAATGGCGCTTCCCGACACGAC                    |
| mRFP1-3-Smal                | TCCCGGGGTACCGGTCCCGCG                      |

α,1,2-mannosyltransferases for the synthesis of O- and N-linked carbohydrates and their possible role in the generation of other glycoconjugates, as well as the fact that humans do not possess any homologous enzymes, make α,1,2-mannosyltransferases promising targets for novel antifungal therapies.

MATERIALS AND METHODS

Strains and culture conditions. The A. fumigatus wild-type strain D141 and its Δafmnt1 derivative A535 have been described before (14, 31). The procedure used for the isolation of conidia and the composition of the yeast glucose medium (YG) and the Aspergillus minimal medium (AMM) have been described previously (20). The human lung epithelial cell line A549 (ATCC CCL-185) was grown in RPMI 1640 medium supplemented with 5% fetal calf serum.

Sequence analysis and database searches. Database searches were performed using BlastP and the following databases: GenBank/EMBL/DDBJ, the Central Aspergillus Data Repository (http://www.cadre-genomes.org.uk/Aspergillus_fumigatus/, the Saccharomyces Genome Database (http://www.yeastgenome.org), and the Candida Genome Database (http://www.candidagename.org/). Alignments, phylogenetic trees, and bootstrap analyses were generated using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Accession numbers of the protein sequences used are as follows: Aflmnt1 (AFUA_5G10760, AFUA_SG12160, and AFUA5G02740), ScKtr1 (P27810/YOR099W), ScKtr2 (P35350/YKR061W), ScKtr3 (P35183/YBR205W), ScKtr4 (P35183/YBR199W), ScKtr5 (P35966/YNL029C), ScKtr6 (P40504/YIL005C), ScKtr7 (P40405/YIL085C), ScKtr2 (P27809/YDR463W), ScKtr1 (P26725/YEL139C), CaMnt1 (orf19.1665), CaMnt2 (orf19.1663), CaMnt3 (orf19.1010), CaKtr4 (orf19.4475), and CaKtr2 (orf19.4494).

Construction of the Δafmnt1 mutant and the complemented strain. The generation of A. fumigatus CDNA has been described previously (28). The Δafmnt1 (Afua5g10760) gene was amplified from cDNA using oligonucleotides mnt1-5 and mnt1-3. The oligonucleotides used in this study are summarized in Table 1.

The PCR product was cloned, sequenced (Eurofin, Medigenomix, Munich, Germany), and compared to the predicted mRNA sequence derived from the A. fumigatus genome project using the BlastN algorithm. To construct a suitable replacement cassette, the protocol of Kamper (13) was followed. For this purpose, a 3.5-kb hygromycin resistance cassette was excised from pSK379 (14) using the SfiI restriction enzyme. The flanking regions of the Δafmnt1 gene (approximately 1.1 kb each) were amplified by PCR from chromosomal DNA using the oligonucleotides pairs mnt1-Afu5g10760-5-fwd/mnt1-Afu5g10760-5-rev and mnt1-Afu5g10760-3-fwd/mnt1-Afu5g10760-3-rev. These oligonucleotides harbored incompatible SfiI sites. After digestion with SfiI, a ligation of the three fragments (the resistance cassette and flanking regions) yielded a 5.6-kb deletion cassette that was purified using the PrepEase gel extraction kit (USB, Cleveland, OH). The fragment was cloned into the pBlueScript KS vector (Stratagene, La Jolla, CA) using oligonucleotide-derived NotI sites. Purified NotI fragments from the resulting plasmid were used for transformation.

For complementation, the afmnt1 gene was amplified using oligonucleotides mnt1-5’ and mnt1-3’. The PCR product was purified and cloned into pSK379 to drive expression from the gpdA promoter. The resulting plasmid was isolated using the PureYield Plasmid Miniprep System (Promega, Mannheim, Germany). A. fumigatus protoplasts were generated, and transformation was performed essentially as described previously (26). The resulting protoplasts were transferred to AMM plates containing 1.2 M sorbitol and either 200 μg/ml hygromycin (Roche Applied Science, Mannheim, Germany) or 0.1 μg/ml pyrithiamine (Sigma, Deisenhofen, Germany).

Genomic DNA analysis. A. fumigatus clones that showed the expected resistance on selective plates were analyzed further by PCR. In the first PCR, one oligonucleotide (mnt1-Afu5g10760-5-cast) that hybridized upstream of the 1.1-bp 5′-flanking region of the afmnt1 gene was combined with a second primer (mnt1-3′) localized at the 3′ end of the afmnt1 gene (see Fig. 2). This reaction (designated PCR1) was used to detect the afmnt1 gene in its wild-type context. The core afmnt1 gene was amplified using oligonucleotides mnt1-5’ and mnt1-3’ (PCR2). The correct integration of the hygromycin resistance cassette was analyzed at the 5′ end using oligonucleotides mnt1-Afu5g10760-5-cast and pJW101-3′ (PCR3) and at the 3′ end using oligonucleotides trpCt-fwd and mnt1-3-UTR-rev (PCR4) (see Fig. 2).

Genomic DNA was isolated from mycelia grown in liquid culture (AMM supplemented with 1% glucose) using the MasterPure yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI) and digested using HaeIII. This enzyme cuts solely outside of the afmnt1 region including the 1.1-bp flanking regions and is not present in the hygromycin resistance cassette. Complementation of the markers associated with the agaroase, and the DNA restriction fragments were transferred to nylon membranes (Hybond-N; GE Healthcare, Freiburg, Germany). A 1.1-kb probe corresponding to the upstream flanking region of the afmnt1 gene was amplified by PCR using oligonucleotides Afua5g10760-5-fwd and mnt1-Afu5g10760-5-rev and digoxigenin-labeled nucleotides (Roche Applied Science, Mannheim, Germany). The separation of the samples and the hybridization and detection of the probe were performed according to standard procedures and the instructions of the vendor.

Construction of a strain expressing Aflmnt1-mRFP1. The sequence of monomeric red fluorescence protein 1 (mRFP1) was amplified using oligonucleotides mRFP1-5-Pme1 and mRFP1-3-Smal and the template vector pMT-mRFP1 (32). The resulting 695-bp fragment was cloned into pSK379 by retaining a PmeI restriction site at the 5′ end of the integrated fragment, resulting in pJW101. The genomic sequence of afmnt1 without the stop codon was amplified using oligonucleotides mnt1-5’ and mnt1-mRFP1-fu-rev. The resulting 1,378-bp fragment was cloned into pJW101 using the PmeI restriction site. The resulting plasmid pJW102 was transformed into A. fumigatus protoplasts as described above. To test whether the Aflmnt1-mRFP-containing organelles are sensitive to brefeldin A, hyphae grown in minimal medium (AMM) at 37°C were further incubated for 6 h in the presence of 20 μg/ml brefeldin A (Sigma, Deisenhofen, Germany). For microscopic analysis, samples were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 5 min. Samples were embedded using Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA) and analyzed using an SP-5 confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany).

Transmission electron microscopy. For transmission electron microscopy, samples grown at 37°C were fixed in a fixation solution containing 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 0.01 M CaCl2, 0.09 M sucrose, pH 6.9) and washed with cacodylate buffer. Pellets were embedded in 2% water agar and cut into small cubes. Dehydration was achieved with a graded series of acetone (10, 20, and 50%) for 30 min on ice, followed by contrasting with 2% uranyl acetate in 70% acetone overnight at 4°C and further dehydrated with 90 and 100% acetone. Samples in 100% acetone were allowed to reach room temperature and were infiltrated with the epoxy resin according to Spurr’s formula for a medium resin (30): 1 part 100% acetone/1 part resin overnight, 1 part 100% acetone/2 parts resin for 8 h, pure resin overnight, and several changes the following 2 days. Samples then were transferred to resin-filled gelatin capsules and polymerized for 8 h at 75°C. Ultrathin sections were cut with a diamond knife, picked up with formvar-coated copper grids (300 mesh), and counterstained with 4% aqueous uranyl acetate. After being washed, samples were examined in a Zeiss transmission electron microscope TEM910 at an acceleration voltage of 80 kV. Images were recorded digitally with a slow-scan charge-coupled display camera (1,024 by 1,024 pixels; ProScan, Scheuring, Germany) with ITEM software (Olympus Soft Imaging Solutions, Münster, Germany), and cell wall thickness...
used was measured by applying the measurement function of the software at the calibrated \( \times 20,000 \) magnification step.

**Field emission scanning electron microscopy.** For scanning electron microscopy, *A. fumigatus* strains were grown on glass coverslips in AMM at 48°C. Samples were fixed in 5% formaldehyde–50 mM Tris, pH 7.0, for 10 min, and then incubated in 5% glutaraldehyde–50 mM Tris, pH 7.0, for 4 h. Samples were washed several times with cacodylate buffer and subsequently washed with TE buffer (20 mM Tris, 1 mM EDTA, pH 8.9) before being dehydrated in a graded series of acetone (10, 30, 50, 70, 90, and 100%) on ice for 15 min for each step. Samples in the 100% acetone step were allowed to reach room temperature before another change in 100% acetone. Samples then were subjected to critical-point drying with liquid CO\(_2\) (CPD 30; Balzers Union, Liechtenstein). Dried samples were mounted onto conductive carbon adhesive tabs on an aluminum stub and sputter coated with a thin gold film (SCD 40; Balzers Union, Liechtenstein). Samples then were examined in a field emission scanning electron microscope (Zeiss DSM 982 Gemini) using the Everhart Thornley SE detector and the in-lens SE detector at a 50:50 ratio at an acceleration voltage of 5 kV and at calibrated magnifications.

**Immunofluorescence microscopy.** Conidia of the respective *A. fumigatus* strain were transferred to a well of a 24-well plate containing a glass coverslip and 1 ml of AMM. Plates were incubated in sealed chambers to prevent excessive evaporation. After incubation for 15 h at 37°C, the cultures were shifted to 48°C, cultured for another 6 h, and fixed using 3.7% formaldehyde–PBS for 10 min. Samples then were blocked using 2% normal goat serum in PBS at 37°C for 30 min. To detect the acidic ribosomal proteins, the coverslips were incubated in a moistening chamber. In the first step, the samples were covered with culture supernatant of the hydridoma B8-C4 (29) and incubated for 30 min at 37°C. After three washing steps using PBS, bound antibodies were visualized with a Cy3-conjugated secondary antibody (Dianova, Hamburg, Germany). Samples were examined using a Leica SP5 confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany).

**Phenotypic characterization of the mutant.** Isolated conidia were counted using a Neubauer chamber. For plate assays, defined numbers of conidia were spotted onto agar plates. These plates were supplemented with the indicated agents and incubated at the indicated temperatures. For the quantification of the radial growth assays, plates were run in triplicate.

Adhesion to epithelial cells was examined using the human lung epithelial cell line A549. Infection was performed in 24-well plates. Semiconfluent monolayers of 2 \( \times 10^5 \) cells grown on glass coverslips were infected with 2 \( \times 10^6 \) conidia. After 2 and 4 h at 37°C, cells were fixed using 3.7% formaldehyde–PBS. After permeabilization (0.2% Triton X-100–PBS for 1 min), the F-actin cytoskeleton of the epithelial cells was visualized using fluorescein isothiocyanate-labeled phalloidin (Sigma, Deisenhofen, Germany). Epithelial cells and bound conidia were counted using a Leica DMLB microscope (Leica, Wetzlar, Germany) equipped with epifluorescence.

**Susceptibility testing.** Eset strips of voriconazole, posaconazole, caspofungin, and amphotericin B were obtained from Inverness Medical (Cologne, Germany). A total of 25,000 conidia were plated onto YG (pH 6.0) plates and incubated for 8 days. Conidia of the respective strains were seeded in 96-well plates at 2.5 \( \times 10^5 \) conidia per well. The plates were further incubated in DMEM without serum and supplemented with 10% L929 cell-conditioned medium and 10% fetal calf serum. Differentiated to macrophages in Dulbecco’s modified Eagle’s medium (DMEM) were transferred to a well of a 24-well plate containing a glass coverslip and 1 ml of AMM. Plates were incubated in sealed chambers to prevent excessive evaporation. The survival of infected animals was monitored once a day. Kaplan-Meier survival curves were compared using the log-rank test (SPSS 15.0 software). Values of \( P \geq 0.05 \) were considered statistically significant.

**RESULTS**

The genome of *A. fumigatus* harbors only three putative \( \alpha\)-1,2-mannosyltransferase genes. In an attempt to analyze the functional importance of mannosyltransferases in *A. fumigatus*, we used sequences of the known members of the Ktr family of \( \alpha\)-1,2-mannosyltransferases from *S. cerevisiae* and the BlastP algorithm and analyzed the *A. fumigatus* genome database at the Central Aspergillus Data Repository for homologous sequences. We identified three predicted open reading frames with significant homology. All of them were already annotated as putative \( \alpha\)-1,2-mannosyltransferases (AFUA_5G10760, AFUA_5G12160, and AFUA_5G02740). The size of the Ktr family differs significantly between different yeast and fungal species, being nine in *S. cerevisiae* (ScKre2/ScMnt1, ScKtr1, ScKtr2, ScKtr3, ScKtr4, ScKtr5, ScKtr6, ScKtr7, ScKre2, and ScYur1), and *C. albicans* (CaMnt1, CaMnt2, CaMnt3, CaKtr2, and CaKtr4). Percentages are bootstrap values (relative to 2,000). Only bootstrap values of at least 50% are shown. A branch length standard is indicated.
The ferase domain (COG5020; E value, 2e-150) and the H9251-1,2-mannosyltransferase domain (Pfam 01793; E value, 2e-141). Interestingly, no protein with significant homology was found in the human genome database. Members of the Ktr family are type II membrane proteins consisting of a highly variable N-terminal part that comprises a short cytoplasmic tail and a transmembrane domain, a conserved central domain characteristic for glycolipid H9251-1,2-mannosyltransferases (Pfam 01793), and a variable C-terminal tail (19). Since both the N- and the C-terminal sequence stretches are highly variable, we generated a phylogenetic tree based on the sequences of the conserved central domains. The partial sequences used for the phylogenetic analysis were defined according to a previous study in which a phylogenetic tree of the Ktr proteins of S. cerevisiae was generated (19) (e.g., the central domain of AFUA_5G10760 comprises residues 80 to 354) (Fig. 1A). The phylogenetic tree comprising the conserved domains of the three putative H9251-1,2-mannosyltransferases of A. fumigatus, AFUA_5G12160 and AFUA_5G02740, were localized in two separate gene clusters. AFUA_5G12160 turned out to be closely related to this cluster, but the bootstrap values already indicate a significant evolutionary distance. ScKtr1, ScKtr3, ScKre2, CaMnt1, and CaMnt2 are proven H9251-1,2-mannosyltransferases with overlapping functions. Experimental evidence has been published that demonstrates that these enzymes collectively add the second and third mannosyl residue to O-linked carbohydrates and also are jointly involved in N-linked glycosylation (10, 11, 17, 20, 22, 27).

The two other putative A. fumigatus mannosyltransferases, AFUA_5G12160 and AFUA_5G02740, were localized in two separate gene clusters. AFUA_5G12160 turned out to be closely related to the putative mannosyltransferases ScKtr5 and ScKtr7, whereas AFUA_5G02740 shared the highest homologies with the putative mannosyltransferases CaKtr4 and ScKtr4 (Fig. 1).

AFUA_5G10760 tentatively has been named Kre2 in the A. fumigatus genome database. The KRE2 gene of S. cerevisiae originally was identified in a screen of mutants resistant to the K1 killer toxin (20). It has not been shown so far that A. fumigatus is sensitive to the K1 killer toxin, and differences in the cell walls of yeasts and filamentous fungi argue against such
sensitivity. We therefore propose to use the alternative designation AfMnt1, for *A. fumigatus* mannosyltransferase 1. This nomenclature is used for the homologous *C. albicans* proteins (3), and MNT1 is an alternative name for the KRE2 gene in *S. cerevisiae* (10).

**Disruption of the afmnt1 gene by homologous recombination.** We amplified the afmnt1 mRNA from cDNA using oligonucleotides mnt1-5/H11032 and mnt1-3/H11032 (Table 1). A sequence analysis of the cloned PCR product confirmed the predicted structure of the afmnt1 gene with four exons and three introns. The obtained nucleotide sequence was identical to the predicted mRNA sequence of the *A. fumigatus* reference strain 293 (24).

A deletion construct was generated containing 1.1-kb regions upstream and downstream of the afmnt1 gene and a hygromycin resistance cassette (Fig. 2A). This linear deletion construct was used for the transformation of protoplasts of strain AfS35. AfS35 is a derivative of the wild-type strain D141 that lacks *akuA*, an essential gene of the nonhomologous end-joining apparatus, and therefore enables high frequencies of homologous integrations (14). Several hygromycin-resistant clones were obtained and tested for correct integration by PCR (Fig. 2C). To complement the deletion mutant, the afmnt1 gene was cloned into the plasmid pSK379, which harbors a pyrithiamine resistance gene (*ptrA*), and the plasmid was introduced into the afmnt1 mutant. Several resistant clones were obtained and analyzed by PCR (Fig. 2C). The Δafmnt1 mutant and the complemented strain then were verified by Southern blot analysis (Fig. 2B). The data revealed the expected difference in the length of the hybridizing fragments of the parental strain and the Δafmnt1 mutant and thereby confirmed a correct and unique integration of the deletion construct.

**Deletion of the afmnt1 gene results in thinner hyphal cell walls.** Under standard growth conditions, the Δafmnt1 mutant and the complemented mutant showed a comparable radial growth on AMM plates. We observed neither a difference in the sporulation of the tested strains at 37°C nor a delayed conidial germination (data not shown), as has been reported very recently for an *A. fumigatus* mutant in the protein O-mannosyltransferase 1 (36). Immunofluorescence studies revealed no differences between the Δafmnt1 mutant and the corresponding control strains with respect to the surface exposure of β1-3 glucan or galactomannan, or in the staining pattern obtained with calcofluor white (data not shown). To further analyze a potential influence of the afmnt1 gene on the *A. fumigatus* cell wall, we used transmission electron microscopy. Images of cross-sections obtained from hyphae of the different strains grown at 37°C were used to determine the thickness of the cell walls. Representative images of the parental strain AfS35 and the Δafmnt1 mutant are shown in Fig. 3A. The data obtained for the thickness of the cell walls are summarized in Fig. 3B. They strongly suggest that the cell wall of the Δafmnt1 mutant is significantly thinner than that of the parental and the complemented strains. This defect was largely restored in the complemented strain. The observed difference between the parental and the complemented strain might be due to a position effect.

A *C. albicans* mutant in the CaMnt1 mannosyltransferase showed a reduced binding to epithelial cells (22). We therefore analyzed the binding of conidia to the human lung epithelial...
increased sensitivity to calcofluor white (60 g/ml) and Congo red (30 μg/ml), whereas no difference was found between the Δafmnt1 mutant and the parental AfS35 strain in the presence of 0.01% sodium dodecyl sulfate (SDS) (Fig. 4) or H₂O₂ (data not shown). The observed sensitivity of the Δafmnt1 mutant to cell wall stress could be completely restored by complementation, demonstrating that this phenotype is associated specifically with the lack of the afmnt1 gene (Fig. 4).

The cell wall of the Δafmnt1 mutant is unstable at elevated temperatures. Aspergillus fumigatus is thermotolerant and grows at temperatures of up to 50°C. To analyze whether an increased temperature affects the growth of the mutant, we measured growth at elevated temperatures. As mentioned above, we observed no obvious difference at 37°C. In contrast, plating series of tenfold dilutions on plates that subsequently were incubated at 48°C revealed a striking growth defect of the Δafmnt1 strain that was abolished in the complemented mutant (Fig. 5A). Osmotic stabilization due to the presence of 1.2 M sorbitol in the plates restored the growth of the Δafmnt1 mutant, but the resulting colonies remained white, suggesting a dramatically reduced sporulation (Fig. 5B). A closer inspection of these colonies by scanning electron microscopy revealed that the mutant still was able to form vesicles and phialides, whereas only very few conidia were found (data not shown).

The quantification of the radial growth of the different strains at temperatures between 30 and 48°C on plates revealed a marginal delay in the growth of the Δafmnt1 mutant between 37 and 44°C, whereas a dramatic growth defect was apparent at 48°C (Fig. 5C). Although we observed only a slight reduction in the growth of the Δafmnt1 mutant at 44°C, the resulting colonies were white, indicating a defect in sporulation already at this temperature. Defects in growth and sporulation were completely restored in the complemented mutant (Fig. 5). A microscopic inspection of hyphae grown for 12 h at 37°C and then for an additional 6 h at 48°C revealed bulky structures at the tips of the Δafmnt1 mutant that were hardly observed for the parental or complemented strains (Fig. 6A). Quantification analysis revealed that after 6 h at 48°C, approximately 38% of the hyphal tips of the Δafmnt1 mutant had an abnormal morphology (Fig. 6B).

The scanning electron microscopy of fungal cells grown at 48°C revealed that the Δafmnt1 mutant is able to germinate at this temperature, but the resulting germ tubes were short and characterized by amorphous structures at their terminal tips (Fig. 7C and E). In contrast, the parental strain and the complemented mutant showed normal growth and intact hyphal tips (Fig. 7A, B, and D). To determine whether the amorphous material at the hyphal tips is released cytoplasm, we stained Δafmnt1 samples using the monoclonal antibody B8-C4, which specifically recognizes an epitope in the conserved domain of the acid ribosomal proteins P0, P1, and P2 (29). Immunofluorescence analysis revealed a strong labeling of the amorphous material at the hyphal tips of the Δafmnt1 mutant (Fig. 8A) but no staining with intact hyphae and hyphal tips of strains D141, AfS35, and the complemented strain (Fig. 8B and data not shown) and in a control experiment with mutant hyphae and the secondary antibody alone (data not shown). These data strongly suggest that the cell wall at the growing hyphal tip of the Δafmnt1 mutant is unstable at 48°C, resulting in a localized disruption of the cell wall and a subsequent cellular leakage.
**Intracellular localization of the AfMnt1 protein.** The high homologies of AfMnt1 to the conserved domains of the members of the Ktr family strongly suggest that AfMnt1 is an α-1,2-mannosyltransferase. These enzymes are engaged in protein glycosylation and are supposed to reside within the Golgi equivalents of fungi. For the *S. cerevisiae* Kre2/Mnt1 protein, this localization has been proven by immunofluorescence (18). To analyze the localization of AfMnt1, we generated a pSK379 derivative in which the *afmnt1* gene was fused to the 5′ end of the *mrfp1* reporter gene. After transformation into the *afmnt1* mutant, we obtained pyrithiamine-resistant clones that showed a striking localization of the RFP fusion protein within distinct intracellular structures in hyphae grown at 37°C (Fig. 9A) and 48°C (data not shown), whereas a control construct containing the *mrfp1* gene alone led to a diffuse cytoplasmic distribution of the red fluorescence (data not shown). Interestingly, the RFP fusion protein was able to fully complement the temperature-sensitive phenotype of the *Δafmnt1* mutant, thereby providing evidence for a correct targeting of the fusion protein. An analysis of swollen conidia and germlings revealed a localization of the fusion protein in small spot-like structures (Fig. 9B and C, respectively), suggesting that Golgi equivalents already exist in swollen conidia.

Brefeldin A is known to disrupt the Golgi apparatus in...
mammals and fungi (4). Low doses of brefeldin A (20 μg/ml) led to a striking redistribution of the AfMnt1-RFP fusion protein, indicating the disruption of the corresponding organelles (Fig. 9D).

The △afmnt1 mutant shows an increased sensitivity to azole antifungals. We next tested the potential impact of the loss of the afmnt1 gene for the susceptibility of A. fumigatus to antifungals, which represent current therapeutic options in cases of invasive aspergillosis. Using commercially available Etest strips, we evaluated the susceptibility of the D141 wild-type strain, the parental strain, the △afmnt1 mutant, and the complemented strain. The inhibition ellipses obtained with amphotericin B were small and indistinguishable, whereas striking differences were observed with the azoles posaconazole and voriconazole (Fig. 10 and data not shown). The MICs of posaconazole and voriconazole for the △afmnt1 mutant were 0.125 and 0.19 μg/ml, respectively. Identical MICs of 0.38 μg/ml for posaconazole and voriconazole were obtained for the parental and complemented strain. Thus, the deletion of the afmnt1 gene led to an increase in the sensitivity to posaconazole and voriconazole. For the echinocandine caspofungin we observed similar zones of inhibition, but the characteristic trailing growth within these zones (6) clearly was reduced for the △afmnt1 mutant (data not shown), suggesting a slightly increased sensitivity.

Cytokine response of murine macrophages to the △afmnt1 hyphae. For C. albicans it has recently been shown that a camnt1 camnt2 double mutant is partially impaired in its ability to trigger a proinflammatory cytokine response (23). Due to the relatively small number of putative α1,2-mannosyltransferases in A. fumigatus, AfMnt1 is likely to be the functional equivalent of CaMnt1 and CaMnt2 (Fig. 1). We therefore analyzed the cytokine response of murine bone marrow-derived macrophages to hyphae of the △afmnt1 mutant and the corresponding parental and complemented strains. As shown in Fig. 11A, we observed no significant difference in the production of the proinflammatory cytokine TNF-α.

The △afmnt1 mutant is attenuated in a murine infection model. Several lines of evidence indicate that even at 37°C the cell wall of the △afmnt1 mutant is less robust than that of the wild type. Since stress resistance is a characteristic feature of many successful pathogens, we infected mice intravenously with conidia of the △afmnt1 mutant, the corresponding parental strain, and the complemented strain. We observed that mice were significantly more resistant to infection with the △afmnt1 mutant than to infection with the parental strain (P = 0.025) or the complemented strain (P = 0.091) (Fig. 11B). Very similar results were observed in a parallel experiment using B6.CD45.1 mice (P = 0.008 for the wild type and P = 0.056 for the complemented strain). Although the route of infection used in this study does not allow drawing conclusions on the initial phase of infection in the lung, the data nevertheless clearly demonstrate an impairment of the △afmnt1 mutant to establish a successful systemic infection in immunocompetent mice.

**DISCUSSION**

Systemic infections caused by A. fumigatus are a major risk for severely immunocompromised patients, e.g., after stem cell transplantation, and due to the progress in modern medicine, the incidence of invasive aspergillosis steadily increased in the 1990s (21, 33). Although several new antifungals have been approved in recent years, there is still an urgent need to improve and expand the limited set of therapeutic options, and due to its uniqueness, the fungal cell wall has received much interest as a potential source for new therapeutic targets.

Mannosyltransferases add mannosyl residues to N- or O-linked glycans of proteins, but they also are involved in the synthesis of other glycoconjugates of the fungal cell wall. Using the conserved central domains of the members of the Ktr family from S. cerevisiae and C. albicans, we identified three A. fumigatus proteins in the current database that shared high homologies. Obviously, there is a striking difference in the
we generated a knockout mutant of the /H9004Mnt1 seems to be a more appropriate designation.

respective wild-type strains but no differences with respect to nonhomologous recombination events compared to that of the /H9004 derivative of the wild-type strain D141. Strains that lack either Aspergillus K1 has not yet been demonstrated for Sc.

ject of this study.

the latter, the AfMnt1 (AFUA_5G10760) protein, is the sub-

ScKtr2, ScYur1, CaMnt3, and CaKtr2, has no

clusters of closely related proteins. One cluster, comprising ScKtr1, ScKre2, CaMnt1, and CaMnt2. Interestingly, experi-

mental evidence for an α-1,2-mannosyltransferase activity has been published for all proteins of the AfMnt1 cluster (3, 10, 11, 17, 22, 27). This and the high homology of the core domain of AFUA_5G10760 to the α-1,2-mannosyltransferase domain Pfam 01793 strongly suggest that this protein is a bona fide α-1,2-mannosyltransferase. The AFUA_5G10760 gene tenta-

vely was named kre2 according to the homologous ScKRE2/ ScMNT1 gene. However, a cytotoxic effect of yeast killer toxin K1 has not yet been demonstrated for Aspergillus, and therefore AfMnt1 seems to be a more appropriate designation.

To examine the biological function of the AfMnt1 protein, we generated a knockout mutant of the /H9004 strain AfS35, a derivative of the wild-type strain D141. Strains that lack either akuA or akitB show a dramatic reduction in the frequency of nonhomologous recombination events compared to that of the respective wild-type strains but no differences with respect to growth properties or virulence (5, 14).

The deletion of the afmnt1 gene and subsequent comple-

mentation were confirmed by PCR and Southern blotting. For complementation studies, a single copy of the afmnt1 gene (under the control of the gpdA promoter) was introduced into the mutant by targeted insertion. The resulting four strains, the wild-type D141, the parental strain AfS35, the /H9004 mutant, and the complemented mutant (Δafmnt1 afmnt1) were analyzed. At 37°C, all strains grew and sporulated normally, indicating that the Δafmnt1 mutant is not obviously impaired under standard conditions. However, the transmission micros-

copy of hyphae hinted toward a reduced thickness of the cell wall of the Δafmnt1 mutant at 37°C, a phenotype that resembles that recently reported for a C. albicans camnt1 camnt2 double mutant (22). This already suggested a higher sensitivity to cell wall stress, and we indeed observed a reduced growth of the mutant in the presence of calcofluor white and Congo red. This phenotype resembles that of a C. albicans camnt1 camnt2 mutant (22) and a very recently described A. fumigatus mutant that lacks the afmnt1 gene. AfPmt1 encodes an O-mannosyltransferase that initiates the O-glycosylation of proteins (36), a step that precedes the addition of mannosyl residues by an α-1,2-mannosyltransferase. Apart from phenotypes observed with calcofluor white and Congo red, this mutant shares another characteristic with the Δafmnt1 mutant: both are severely im-

paired in growth at higher temperatures (48 to 50°C), a phe-

notype that resembles that of S. cerevisiae mutants with mul-
tiple deletions in PMT genes (7). Interestingly, the growth inhibition at 42°C was dramatic for the Δafmnt1 mutant (36), whereas the growth of the Δafmnt1 mutant was only slightly impaired at 42 and even 44°C. The apparent growth defect of the Δafmnt1 mutant at elevated temperatures could be rescued by osmotic stabilization (36), and the same applies to the Δafmnt1 mutant at 48°C. In both cases, the resulting

FIG. 8. Detection of cytosolic proteins that are released from the hyphal tips of the Δafmnt1 mutant. Conidia of different strains were seeded in 24-well plates containing glass coverslips. The fungi were grown at 37°C for 8 h and then shifted to 48°C. After 4 h, the samples were fixed and treated with the monoclonal antibody B8-C4 and an appropriate Cy3-labeled secondary antibody (red). (A) Δafmnt1 mutant; (B) complemented strain Δafmnt1 afmnt1. The typical breakage of a hyphal tip is indicated by a large arrow. Unusual leakage at the base of a germ tube is indicated by an arrowhead. Note the shrunk cytoplasm in the damaged hyphae indicated by three small arrows. Bars represent 10 μm.

number of these mannosyltransferases, with nine in S. cerevi-
siae, five in C. albicans, and only three in A. fumigatus. One of the latter, the AfMnt1 (AFUA_5G10760) protein, is the subject of this study.

A phylogenetic analysis of the conserved domains of AfMnt1 and the other members of the Ktr family in S. cerevisiae, C. albicans, and A. fumigatus revealed the presence of at least four clusters of closely related proteins. One cluster, comprising ScKtr2, ScYur1, CaMnt3, and CaKtr2, has no A. fumigatus member. AFUA_5G12160 forms a cluster together with ScKtr5 and ScKtr7. AFUA_5G02740 turned out to be especially related to CaKtr4 and ScKTR4, whereas AfMnt1 (AFUA_5G10760) shows the highest homologies to ScKtr3, ScKtr1, ScKre2, CaMnt1, and CaMnt2. Interestingly, experimental evidence for an α-1,2-mannosyltransferase activity has been published for all proteins of the AfMnt1 cluster (3, 10, 11, 17, 22, 27). This and the high homology of the core domain of AFUA_5G10760 to the α-1,2-mannosyltransferase domain Pfam 01793 strongly suggest that this protein is a bona fide α-1,2-mannosyltransferase. The AFUA_5G10760 gene tenta-

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mentation were confirmed by PCR and Southern blotting. For complementation studies, a single copy of the afmnt1 gene (under the control of the gpdA promoter) was introduced into the mutant by targeted insertion. The resulting four strains, the wild-type D141, the parental strain AfS35, the /H9004 mutant, and the complemented mutant (Δafmnt1 afmnt1) were analyzed. At 37°C, all strains grew and sporulated normally, indicating that the Δafmnt1 mutant is not obviously impaired under standard conditions. However, the transmission micros-
colonies appeared white, indicating a dramatically reduced sporulation (36) (Fig. 6). Using electron microscopy, we analyzed Δafm1 colonies grown in minimal medium at 37°C. The Δafm1-1 fusion protein is shown in red, and DAPI-stained nuclei are shown in blue. The localization of Δafm1-1 fusion protein in hyphae is shown in panel A′. The corresponding bright-field image is depicted in panel A. An enlargement of the region indicated by the dotted box is shown as an inset in the upper right part of panel A′. Micrographs of a swollen conidium (B and B′) and a germling (C) are shown. The localization of Δafm1-1 fusion protein in hyphae treated with 20 μg/ml brefeldin A for 6 h is shown in panel D′. The corresponding bright-field image is depicted in panel D. Bars represent 5 μm. The bar in panel A′ is also valid for panels A, D, and D′.

As the reasons for the temperature sensitivity of the Δafm1 mutant have not been further elucidated, we tried to determine them for the Δafm1 mutant. With this mutant, we frequently observed bulky structures at the ends of hyphae grown at 48°C. Closer inspection by scanning electron microscopy revealed an amorphous material that appeared to be released from the fungus. Immunofluorescence analysis revealed the presence of ribosomal proteins in these structures, demonstrating that they consist of released cytoplasm. The striking colocalization of these leakages with the terminal ends of hyphae indicates that the growing tip is the most unstable part of the Δafm1 cell wall. Hyphal growth requires a localized reorganization of the cell wall at the tip to allow the incorporation of new material. During growth, the fungus has to find the delicate balance between the inevitable stability of the cell wall and the need for a dynamic reorganization. A dysfunction of the underlying fine-tuned network of enzymatic activities can result in instability and finally the leakage of the cell. Cell wall-associated
proteins commonly are glycosylated, and defects in protein glycosylation may result in protein misfolding, instability, and/or a reduced enzymatic activity.

One characteristic feature of fungal \( \alpha \)-1,2-mannosyltransferases is their localization in the so-called Golgi equivalents. We have analyzed the localization of an AfMnt1-RFP fusion protein and detected it in compact hyphal organelles both at 37 and 48°C. The expression of the fusion protein completely rescued the temperature-sensitive phenotype of the \( \Delta mnt1 \) mutant, strongly suggesting that it is correctly targeted. The AfMnt1-containing organelles were sensitive to brefeldin A, an agent known to disrupt the ER and the Golgi apparatus. The ER of filamentous fungi is organized as a tubular network (4), a structure that is clearly different from the structures targeted by the AfMnt1-RFP fusion protein. The localization of the AfMnt1-RFP resembles that of a CopA-green fluorescent protein fusion protein that recently was shown to localize in the Golgi equivalents of \( A. \) nidulans (2). Taken together, our data provide evidence for a localization of AfMnt1 in the Golgi apparatus, which is a characteristic feature of \( \alpha \)-1,2-mannosyltransferases.

The reduced thickness of the \( \Delta fmn1 \) cell wall and the increased sensitivity of this mutant to cell wall stressors like calcofluor white suggest a latent instability of the mutant cell wall even at 37°C. An inhibition of the enzymatic activity of AfMnt1 therefore might be an interesting therapeutic option, especially as homologous \( \alpha \)-1,2-mannosyltransferases do not exist in humans. AfPnt1 could be an alternative target, and potent inhibitors already have been identified for Pmt1 of \( C. \) albicans (25). However, in this case, homologous human protein \( O \)-mannosyltransferases do exist and might be a serious obstacle for the development of fungus-specific inhibitors.

We speculated that the latent instability of its cell wall renders the \( \Delta fmn1 \) mutant more susceptible to stress exerted by therapeutic agents or the host immune response. In fact, we observed that the \( \Delta fmn1 \) mutant showed a significantly enhanced sensitivity to posaconazole and voriconazole, two first-line antifungals used for the treatment of invasive aspergillosis. Azoles inhibit the cytochrome P450 enzyme lanosterol demethylase (34), a key enzyme in the biosynthesis of ergosterol, which in turn is a characteristic and essential component of the fungal membrane. Stress applied to the plasma membrane has been shown to activate the fungal cell wall integrity pathway (12, 35), demonstrating that stress targeted at the plasma membrane can be compensated for on the cell wall level. Thus, any stress applied to either the cell wall or plasma membrane is likely to affect the other. This functional interconnection of the structures seems to be pivotal for the maintenance of the fungal cell envelope and may explain the higher sensitivity of the \( \Delta fmn1 \) mutant to azoles.

It has been shown recently that hyphae of a \( C. \) albicans double mutant lacking both \( MNT1 \) and \( MNT2 \) triggered a reduced production of proinflammatory cytokines, e.g., TNF-\( \alpha \). Due to the limited number of members of the Ktr family, AfMnt1 seems to be the functional equivalent of both CaMnt1 and CaMnt2. We therefore challenged murine macrophages with hyphae of the \( \Delta fmn1 \) mutant and the corresponding control strains, but we observed no differences. Mannoproteins are abundant in \( C. \) albicans and form a distinct layer on the surface of the cell wall, whereas these proteins seem to be less abundant and spatially differently organized in \( A. \) fumigatus (1), which might explain the obtained results.

To test whether the reduced robustness of the cell wall of the \( \Delta fmn1 \) mutant has consequences for the resistance to host defense mechanisms, we infected mice intravenously with conidia. We observed an attenuated virulence for the \( \Delta fmn1 \) mutant but not for the complemented strain, indicating that AfMnt1 activity is required for full virulence in this model of a systemic infection. Interestingly, similar results have been reported for the \( camnt1 \) \( camnt2 \) double mutant in a model of a systemic \( C. \) albicans infection (22). We cannot conclude from our data that the \( \Delta fmn1 \) mutant is generally attenuated in virulence, since we have bypassed the initial phase of infection, but our data nevertheless demonstrate that the \( \Delta fmn1 \) mu-

FIG. 10. Susceptibility to antifungal agents. YG agar plates were inoculated with 25,000 conidia of the indicated strains, Etest strips were applied, and plates were incubated at 37°C for 48 h. Representative pictures showing the inhibition ellipses obtained with posaconazole Etest strips are shown. Experiments with all antifungal drugs were performed in triplicate.
tant is impaired in its ability to spread and colonize in infected mice, most likely due to an increased sensitivity to host defense mechanisms.

In conclusion, we found that the AfMnt1 protein is localized in Golgi equivalents and is required for the maintenance of the fungal cell wall. A lack of this protein leads to a thinner hyphal cell wall, an increased sensitivity to cell wall stressors, a growth defect at elevated temperature, and, as a consequence, a dramatically reduced sporulation and a disruption of the cell wall at its most dynamic part, the hyphal tip. The increased sensitivity of the Δafmnt1 mutant to certain antifungals and its attenuation in a systemic mouse model of infection identifies this bona fide α-1,2-mannosyltransferase as a potential drug target. Specific inhibitors could synergize with approved antifungal agents, and if used in combination therapy, they might establish an improved option for the treatment of invasive aspergillosis.

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