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Glycosylphosphatidylinositol-Anchored Ecm33p Influences Conidial Cell Wall Biosynthesis in *Aspergillus fumigatus*

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***ECM33* encodes a glycosylphosphatidylinositol-anchored protein whose orthologs in yeast are essential for sporulation. *Aspergillus fumigatus* Ecm33p is unique and has an apparent mass of 55 kDa. Disruption of *A. fumigatus ECM33* results in a mutant with several morphogenetic aberrations, including the following: (i) a defect in conidial separation, (ii) an increase in the diameter of the conidia of the mutant associated with an increase in the concentration of the cell wall chitin, (iii) conidia that were sensitive to the absence of aeration during long-term storage, and (iv) conidia that were more resistant to killing by phagocytes, whereas the mycelium was more easily killed by neutrophils.**

The fungal cell wall is composed of interlinked polysaccharides. Enzymes involved in the biosynthesis of linear glucan and chitin, the main cell wall polysaccharides encountered in the fungal kingdom, have been identified (5). In contrast, enzymes responsible for providing cell wall stability through their activities in the branching and cross-linking of these linear polysaccharides in a three-dimensional rigid skeleton have not been discovered. During a search for such enzymes, the first β1,3-glucanosyltransferase activity identified in fungi was discovered in *Aspergillus fumigatus* (14). This protein is glycosylphosphatidylinositol (GPI) anchored to the plasma membrane (21) so that its cellular location correlates with its enzymatic function, since linear neosynthesized polysaccharides are extruded through the plasma membrane and modified within the cell wall space. Disruption of the gene encoding this activity in the filamentous fungus *A. fumigatus*, but also in the yeasts *Saccharomyces cerevisiae* and *Candida albicans*, has resulted in mutants with a reduced growth phenotype and an altered cell wall composition (22, 23, 29, 30, 34). Because the primary modifications of the skeletal-wall polysaccharide are identical in yeasts and molds, it is expected that the enzymes responsible for such activities will be highly homologous. Since the only transferase identified to date is a GPI-anchored protein, an analysis of GPI-anchored proteins of *S. cerevisiae* and *A. fumigatus* was undertaken with the hope of identifying GPI-anchored proteins common to yeasts and molds that would have putative polysaccharide cross-linking enzymatic activities. Comparative genomic and proteomic analyses have identified at least four families of GPI-anchored proteins common to *S. cerevisiae* and *A. fumigatus* that are at least partially involved in fungal cell wall reconstruction (2, 4).

This study investigates Ecm33p, a GPI-anchored protein that was originally identified in *S. cerevisiae* in a screen of Tn*3* transposon-mutagenized cells for altered sensitivity to calcofluor white (CFW) (19). Although the biochemical function of this protein is not known, many phenotypes of the *ecm33* (YBR078w) mutant suggest a putative role of *ECM33* in cell wall assembly. Moreover, *ECM33* belongs to the *S. cerevisiae SPS2* and *Schizosaccharomyces pombe meu10* families, which play essential roles in the sporulation of these two yeast species, in particular, the formation of the ascospore cell wall (15, 37). These data have led us to investigate the role of the *ECM33* ortholog in *A. fumigatus*. In this report, we describe the disruption of the *ECM33* gene in *A. fumigatus* (Af*ECM33*) and the phenotype of the mutant. We show that mutation of the Af*ECM33* gene results in a conidium phenotype that is associated with a differential sensitivity of the mutant to phagocytes.

MATERIALS AND METHODS

Strains, plasmids, and culture media. The *Aspergillus fumigatus* wild-type (WT) and clinical isolate (CBS 144-89) was maintained on 2% malt agar slants, and the Af*ecm33* mutant was maintained on the same culture medium supplemented with 200 μg/ml of hygromycin B (Sigma). Sabouraud liquid medium (2% glucose, 1% mycopeptone; Biokar, Beauvais, France) was used for growth and mycelium DNA extraction (18 h of growth at 37°C), and Sabouraud solid medium (2% agar) was used for germination experiments at 37°C. For transformation experiments, minimal medium (MM) (glucose, 1%; ammonium tartrate, 0.092%; KCl, 0.052%; $MgSO_4$-$7H_2O$, 0.052%; KH_2PO_4, 0.152%; trace element solution, 1 ml/liter; pH 6.8) was used (8). All cloning steps were performed in *Escherichia coli* DH5α (Biolabs). Cells were grown at 37°C in LB medium (2.5% Luria broth base) supplemented with 100 μg/ml ampicillin if required. The following plasmids were used: pCR2.1 vector (Topo TA Cloning kit; Invitrogen, Carlsbad, CA) to clone PCR products, pUC18 plasmid (Invitrogen) to clone Af*ECM33*, and pAN7.1 and pAN8.1 plasmids (22, 31) to disrupt the Af*ECM33* gene and complement the Afecm33 mutant.

Oligonucleotides, cloning procedures, and DNA manipulations. DNA manipulations were done following an established protocol (33). The sequences of the oligonucleotides used in this study are listed in Table 1. Genomic DNAs of *A. fumigatus* transformants were prepared according to the method of Girardin et al. (12). For PCR experiments, the samples in a 50-μl reaction volume containing 20 μM of each deoxynucleoside triphosphate, 50 pmol of each primer, 100 ng of cDNA or genomic DNA, and 1 unit of *Taq* DNA polymerase (Amersham Pharmacia Biotech) were subjected to 30 cycles of amplification consisting of the following steps: 5 min of denaturation at 95°C, 30 cycles of amplification (1 min of denaturation at 95°C, 1 min of annealing at 50°C or 55°C depending on the primer pair used, and 1 min of elongation at 72°C), and 5 min of elongation at 72°C.

The protein homologous to *S. cerevisiae* Ecm33p was initially identified by

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TABLE 1. Oligonucleotides used in this study

Oligonucleotide	DNA sequence[a]
ECM33-a	5′-GGGAGCTCTCAAATCGCAGAGTGAGC-3′
ECM33-b	5′-AAGCGGCCGCCCACACTGCCTCGCC-3′
ECM33-c	5′-TGATCTGCCTTCCCTGA-3′
ECM33-d	5′-ATGATATACATCAGCT-3′
ECM33-e	5′-CAAACAAAACCACATGCG-3′
ECM33-h	5′-GGAATTCCACTCTCCCTCTTTCTCT-3′
ECM33-i	5′-CGGAATCCTCAGCGAGATACCGTTCA-3′
ECM33-j	5′-GCTCTAGAAAAGGATGGCCAAGACTA-3′
ECM33-k	5′-CCCAAGCTTCTCTGATATCTGTTATGC-3′
ECM33-l	5′-ATGACATTACTGACCTTGCT-3′
ECM33-m	5′-ATGGCTTTCCTCAAATACGC-3′
ActinRT1	5′-CATTGTCATGTCTGGTGGTAC-3′
ActinRT2	5′-GGAGGAGCAATGATCTTGACC-3′

[a] Primers ECM33-h, ECM33-i, ECM33-j, and ECM33-k contained, respectively, EcoRI, BamHI, XbaI, and HindIII sites at the 5′ end (underlined). Primers were synthesized by PROLIGO Inc.

BLAST analysis in the Genome Therapeutic Corporation (GTC) database of *A. fumigatus* before the genome of *A. fumigatus* was sequenced (4). Two oligonucleotides (ECM33-a and ECM33-b) deduced from the *A. fumigatus* GTC database genome sequence (access kindly provided by J. M. Bruneau, Aventis, Romainville, France) were used to amplify by PCR a 500-bp DNA genomic fragment (ab-probe) (Fig. 1a). The ab-probe was used to screen a cosmid DNA genomic library of *A. fumigatus* provided by P. Borgia (3). The cosmid 47H3, which contained the ab-probe sequence, was subcloned and sequenced. The positions of introns and start and stop codons were determined after amplification of a λgt11 (Invitrogen Corp.) *A. fumigatus* cDNA library (a kind gift from M. Monod, CHUV, Lausanne, Switzerland) by PCR using primers deduced from the genomic DNA sequence (ECM33-c/ECM33-e, ECM33-l/ECM33-i, and ECM33-m/ECM33-i). This analysis showed that the position of the ECM33-b primer was outside the open reading frame, due to a wrong annotation in the GTC database.

Disruption of the Af*ECM33* gene and construction of a complemented *ecm33*::*ECM33* strain. The three-step strategy described in Fig. 1 was used to produce an *A. fumigatus* strain with a nonfunctional copy of the Af*ECM33* gene disrupted by the *E. coli* hygromycin B phosphotransferase gene marker (*HPH*). Primers ECM33-h and ECM33-i and primers ECM33-j and ECM33-k were used to obtain hi and jk PCR fragments of 720 bp and 770 bp, respectively. A 3,914-bp BglII-XbaI fragment containing the *HPH* gene flanked by the TRPC terminator and the GPD promoter of *Aspergillus nidulans* was obtained from the pAN7.1 plasmid (31). The hi PCR fragment, the *HPH* marker, and the jk PCR fragment were cloned successively in that order in pUC18.

Transformation of *A. fumigatus* using pUC18/Af*ecm33* was performed using the protoplast procedure previously described (25). Protoplasts were obtained after treatment with Glucanex enzyme (Novo Nordisk Ferment Ltd., Switzerland) for 2.5 h at 30°C with gentle agitation. After an overnight expression of the *HPH* gene, the transformants were selected on MM supplemented with 1 M sucrose and 200 μg/ml hygromycin B.

Transformants (127) were first tested by PCR to verify the integration of the *HPH* marker gene at the Af*ECM33* locus by using the oligonucleotides ECM33-c and ECM33-e (Fig. 1a), and then genomic DNAs of the PCR-selected transformants were digested by BamHI, HindIII, and NcoI for Southern blotting using the hi PCR fragment to confirm the integration of *HPH* at the right locus. The presence or absence of AfEcm33p was verified by Western blotting using an anti-AfEcm33p hyperimmune rabbit antiserum. Anti-AfEcm33p hyperimmune rabbit antiserum was produced by Millgen (Toulouse, France) following immunization with the peptide CKAAEPNPTTKDGSSG coupled by glutaraldehyde treatment to thyroglobulin. An increase in specific antibodies was determined by Western blotting; sufficient titers of antibody were obtained after 3 months of immunization. For Western blotting experiments, mycelial extracts were obtained in the following way: after 24 h of growth in Sabouraud medium, the mycelium was disrupted in 0.2 M Tris HCl, pH 8.0, 20 mM EDTA, 1 mM phenylmethylsulfonyl fluoride buffer in a CO_2-cooled MSK Braun homogenizer using 0.5-mm glass beads. After verification of cell breakage under light microscopy, the mycelial slurry was centrifuged at 4,000 × *g*, leading to a 4,000 × *g* intracellular (IC) supernatant and cell wall (CW) pellet. Both fractions were tested by Western blotting after extensive washing of the pellet.

To complement the Af*ecm33* mutant, the cosmid 47H3 (containing the Af*ECM33* gene) and the plasmid pAN8.1 (containing the phleomycin resistance marker) were used to cotransform the Af*ecm33* mutant by the protoplast procedure described above. After overnight expression of the phleomycin gene, the transformants were selected on MM supplemented with 1 M sucrose and 40 μg/ml phleomycin. Restoration of the wild-type conidial phenotype was searched for among the transformants, and the ectopic integration of an intact copy of the *ECM33* gene was verified by Southern blotting as described above.

Analysis of the mutant phenotype. (i) Conidial germination. *A. fumigatus* conidia of the WT, Af*ecm33* mutant, and *ecm33*::*ECM33* revertant strains, grown at room temperature for 6 days on 2% malt agar slants, were recovered in 0.05% Tween 20 water. Homogeneous conidial suspensions of each strain were collected following filtration through a 40-μm-pore-size filter (Falcon) and then through sterile filter paper (MN617; Macherey-Nagel, Germany). If necessary, conidia were labeled with fluorescein isothiocyanate (FITC) (Sigma) or Texas Red (Texas Red-X; succinimidylester mixed isomers; Molecular Probes) (17, 28): freshly harvested conidia (2×10^7 conidia/10 ml of 0.1 M Na carbonate buffer, pH 10.2) were incubated with the fluorescent dye at a final concentration of 0.1 mg/ml at 37°C for 45 min and washed by centrifugation three times in 0.1% Tween 20-PBS. Five microliters of a conidial suspension at 10^6/ml was spotted on Sabouraud agar and incubated at 37°C. The percent germination was assessed microscopically every hour for 14 h. If needed, the lengths of germ tubes were measured under a light microscope (DL; Leica) coupled to a cool-snap Cf monochrome camera (Photometrics; Roper Scientific, France). Images were obtained with Metavue software (Universal Imaging Corporation, Dowingstown, PA). Conidia of different ages from slants stored for up to 2 months either under normal aeration conditions or in the absence of aeration were tested. For that

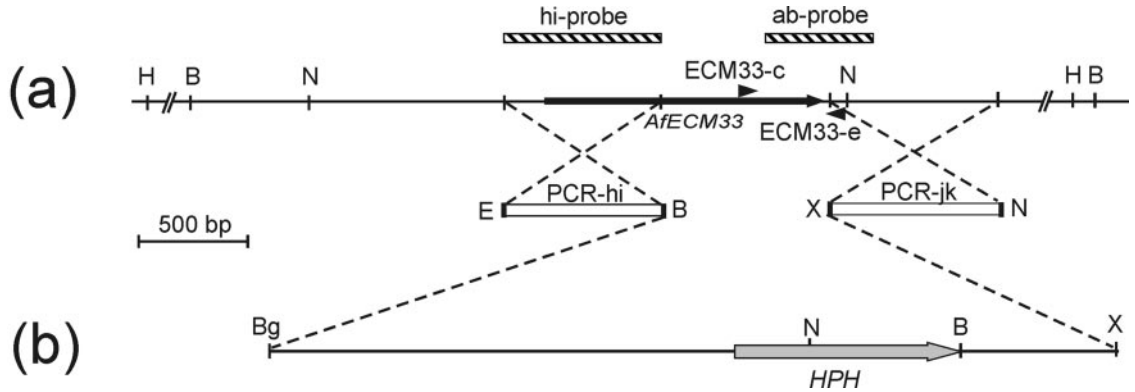

FIG. 1. Disruption of the Af*ECM33* gene (black arrow) by double-crossover events. (a) Restriction maps of the Af*ECM33* deletion constructs and cloning steps during Af*ECM33* gene deletion. Restriction enzyme abbreviation: B (BamHI), N (NcoI), E (EcoRI), X (XbaI), H (HindIII), and Bg (BglII). See Materials and Methods for the amplification of PCR hi and jk fragments, and ab and hi probes. (b) Restriction map after the integration of the *HPH* gene marker (gray box) at the Af*ECM33* locus.

purpose, tubes were inverted and partly dipped into paraffin oil to avoid the entrance of air.

(ii) Analysis of the cell wall phenotype. To measure the amount of chitin in the conidial cell wall, conidia were disrupted in water in a CO_2-cooled MSK Braun homogenizer using 0.5-mm glass beads. After verification of cell breakage under light microscopy, the mycelial slurry was centrifuged at 4,000 × *g*, and the cell wall pellet was extensively washed with 0.5 M NaCl and water and freeze-dried. Total hexosamine was measured after hydrolysis with 8 N HCl for 4 h at 100°C (11). The hexosamine content was estimated as the amount of chitin per conidium. To visualize the presence of chitin in the cell wall, *A. fumigatus* conidia of wild-type and Af*ecm33* strains were labeled with calcofluor white at a final concentration of 100 μg/ml. Conidia were observed with a Leica DL fluorescence microscope as described above.

To test the susceptibility of the *ecm33* mutant to various cell wall inhibitors, 6×10^3 6-day-old conidia were inoculated in 200 μl 1% yeast extract medium containing twofold dilutions of the cell wall-perturbing agents Congo Red (0 to 1 mg/ml), CFW (0 to 1 mg/ml), nikkomycin (0 to 500 μg/ml), caspofungin (0 to 100 μg/ml), mulundocandin (0 to 200 μg/ml), Lilly 303366 (0 to 200 μg/ml), glucanex (0 to 5 mg/ml), and polyoxin (0 to 250 μg/ml) in enzyme-linked immunosorbent assay plates. The plates were incubated at 37°C for 48 h, and the MICs were determined by quantification of the growth estimated as the optical density at 600-nm absorbance in a microtiter plate reader.

(iii) Conidial cell wall ultrastructure. Conidia were processed for transmission electron microscopy as previously described (17, 28). Briefly, the conidia were fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 M phosphate buffer and washed in the same buffer. Postfixation (30 min) in aqueous 1% osmium tetraoxide (OsO_4) in 0.1 M cacodylate buffer, pH 7.4, was followed by several washes (5 min each) in sodium acetate buffer (50 mM, pH 5, at 0 to 2°C). Dehydration in an ethanol gradient preceded infiltration in Epon resin. Ultrathin (50- to 60-nm) sections were stained with 4% uranyl acetate followed by lead citrate. The sections were examined using a Jeol GEM-1010 transmission electron microscope.

(iv) Sensitivity of the Af*ecm33* mutant to phagocytes. The susceptibility of the conidia to alveolar macrophages (AM) and polymorphonuclear phagocytes (PMNs) and of the mycelium to PMNs was evaluated under different experimental setups either in vitro or in vivo.

(v) Killing of swollen conidia by AM of the MH-S cell line. To obtain swollen conidia, FITC conidia were incubated in RPMI medium (RPMI 1640 Glutamax medium; Gibco, Invitrogen Corp.) supplemented with 10 mM sodium pyruvate, 1 mM HEPES, 50 U/ml penicillin (Gibco) and streptomycin (Gibco), and 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Invitrogen Corp.) for 3 h at 37°C in an atmosphere of 5% CO_2. AM from the MH-S murine cell line were cultured in RPMI plus 10% FBS medium at 37°C under 5% CO_2. The MH-S cells were plated 24 h prior to each experiment. For the killing assay, 5×10^4 FITC-labeled swollen conidia were added to 5×10^5 MH-S cells (ratio, 1:10) and incubated at 37°C in RPMI plus 10% FBS medium for 6 h. After disruption of the cells by water, the conidia were incubated overnight on Sabouraud agar at 37°C. Conidial killing was estimated as a percentage of germinated conidia counted under a light fluorescence Leica DL microscope [(number of nongerminated conidia/total number of conidia phagocytosed by the macrophages) × 100] (17, 28).

(vi) Killing of resting conidia by mouse AM. FITC-labeled conidia were used to infect immunocompetent OF1 mice (Janvier, France) anesthetized with an intramuscular injection of 0.1 ml of a solution containing 10 μg/ml ketamine (Mérial, France) and 2 μg/ml xylazine (Bayer Leverkusen, Germany) per mouse. For each mouse, 25 μl (10^7 conidia) of 0.1% Tween 20-PBS FITC-labeled conidial suspension was inoculated intranasally, using an automatic pipetting device. After 24 h, AM were harvested from the mouse lungs with 15 ml of ice-cold Ca^{2+}- and Mg^{2+}-free PBS (Gibco, Invitrogen Corp.) through an 18-gauge plastic catheter inserted into the trachea after the mouse was killed with 100 μl of pentobarbitol. The cells were separated from the lavage fluid by centrifugation at 400 × *g* for 8 min at 4°C, and conidial germination was assessed as described above.

(vii) Killing of conidia by human neutrophils. Human PMNs were isolated from the peripheral blood of healthy volunteers using dextran sedimentation and centrifugation through Lymphocyte Separation Medium (Mediatech Cellgro, Virginia). The percentage of neutrophils was estimated to be greater that 90%. The cells were suspended in RPMI 1640 medium, and 0.2 ml of medium containing 5×10^5 PMNs/ml and 5×10^4 conidia of *A. fumigatus*/ml was incubated at 37°C for 6 h. The cells were then lysed with an aqueous solution containing 0.5% Tween 20 and homogenized before being plated on petri dishes containing 2% malt agar. CFU were counted, and the results were estimated as the percentage of killing according to the following formula: (number of CFU conidia at zero time [T_0] − number of CFU conidia after 6 h of incubation with PMN/number of CFU conidia at T_0) × 100.

(viii) Killing of mycelium by human neutrophils. Human PMNs were isolated as described above and suspended in RPMI 1640 medium. A conidial suspension of *A. fumigatus* was adjusted to 2.5×10^4/ml in RPMI 1640, and the suspension was dispensed into 96-well microtiter plates and incubated for 20 h at 37°C (0.2 ml/well) in order for conidia to germinate. The plates were centrifuged in situ, the supernatants were aspirated, and 0.2 ml of PMNs (2.5×10^5/ml) was added to each well containing germinated conidia. One control lacking PMNs was also used. In another control, plates without PMNs were incubated with H_2O_2 (0.25 to 2.5 mM). After 24 h at 37°C under CO_2, the plates were centrifuged, the supernatants were aspirated and discarded, and deionized water was added to lyse the neutrophils. This step was repeated. Then, 0.2 ml of Na-methoxy-nitrosulfophenyl-tetrazolium-carboxanilide (XTT) at 1 mg/ml in 1% phenazine methosulfate (Tox2 kit; Sigma) per well was added, and the cultures were incubated for 20 h at 37°C. Absorption at 450 nm was determined with a 96-well plate reader. Readings at 690 nm (the reference wavelength) for each well were also recorded and subtracted from the absorbance at 490 nm. The percent growth inhibition for each strain was calculated using the formula (A_{450} of fungi incubated without PMNs − A_{450} of fungi incubated with PMNs)/A_{450} of fungi incubated without PMNs × 100.

(ix) Mycelial growth in the lungs of immunocompromised mice. Previous studies have shown that the best comparison of the pathobiologies of a mutant and a parental strain occurs when the mutant and parental strains are inoculated together into the same mouse immunosuppressed by an intraperitoneal injection of 25 mg of cortisone acetate (Sigma) at day 4 and day 0 before intranasal inoculation (day 0) (1). On day 0, 25 μl of a 0.05% Tween 20 aqueous solution containing 5×10^6 Texas Red-labeled wild-type conidia and 5×10^6 FITC-labeled mutant conidia was inoculated intranasally into an OF1 mouse. Bronchoalveolar lavages were performed 10 h after conidial inhalation, as described previously (28). After centrifugation (at room temperature for 10 min at 1,400 rpm), germ tubes of the two strains were differentially identified under a light fluorescence Leica DL microscope, and their lengths were measured.

Statistical analysis. All experiments were repeated at least three times, and the statistical significance of the results was determined by one- or two-way variance analysis using JMP5 of SAS (Cary, NC). The results were expressed as means ± standard error values.

RESULTS

Analysis of the Af*ECM33* gene and construction of the Af*ecm33* mutant. The *A. fumigatus ECM33* homolog, Af*ECM33*, is 1,321 base pairs long and contains two introns of 69 and 56 bp (starting at nucleotides 61 and 972, respectively). The gene encodes a predicted protein of 398 amino acids corresponding to a 44-kDa polypeptide. The predicted AfEcm33p has a hydrophobic amino terminus characteristic of a secretory signal sequence and a hydrophobic carboxy terminus characteristic of GPI-anchored proteins. The protein was previously identified in a proteomic analysis of GPI-anchored proteins of *A. fumigatus* (4). BLAST analysis with the Institute for Genomic Research database (http://www.tigr.org) indicated a single homolog of Ecm33p in the *A. fumigatus* genome.

ECM33 was found in all yeast and mold fungal genomes sequenced to date (*S. cerevisiae*, *S. pombe*, *C. albicans*, *Candida glabrata*, *Magnaporthe grisea*, *Neurospora crassa*, and *Fusarium graminearum*, as well as other *Aspergillus* spp.) with different numbers of orthologs per species. In Fig. 2, a comparative tree shows the sequences of *A. fumigatus ECM33* with six other yeast *ECM33* orthologs (four in *S. cerevisiae* and two in *S. pombe*) that have been functionally analyzed. In *S. cerevisiae*, Ecm33p belongs to a family which contains four members that are separated into two clusters (Sps2p and YCL048wp; Pst1p and Ecm33p) and that are associated with a sporulation function, at least for the first cluster (A. M. Neiman, personal communication; 6). The two orthologs identified and studied in

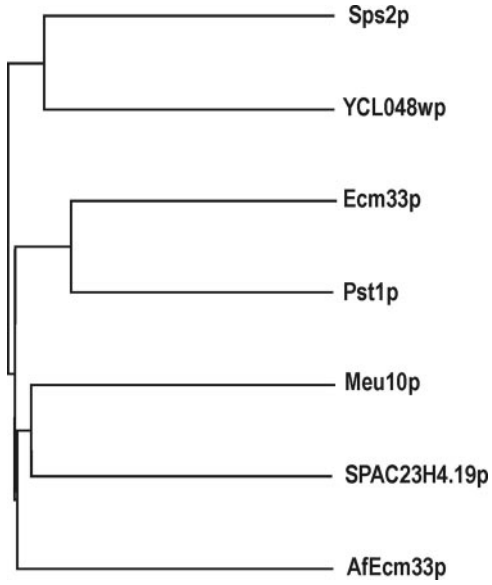

FIG. 2. Dendrogram composed of *A. fumigatus* Ecm33p and six yeast orthologs: the four members of the *SPS2* family in *S. cerevisiae* (Sps2p, Pst1p, Ecm33p, and YCL048wp) and the two homologs (Meu10p and SPAC23H4.19p) of *S. pombe*.

S. pombe (*meu10* and SPAC23H4.19) are also involved in ascospore formation (37). The sizes of the Ecm33p proteins in yeast and molds were similar, approximately 400 amino acids. The levels of identity between proteins of the different species varied from 23% to 57%, with the highest similarity found for Ecm33p and Pst1p of *S. cerevisiae*. *A. fumigatus* Ecm33p was more closely related to *S. pombe* Meu10p and SPAC23H4.19p than to the *S. cerevisiae* orthologs, a common observation in all sequence comparisons of these three species. However, the insulin-like domain that was considered to be important functionally in *S. pombe* (37) was not found in *A. fumigatus*.

The strategy illustrated in Fig. 1 was used to produce an *A. fumigatus* strain with a nonfunctional disrupted copy of Af*ECM33*. Southern blot data (Fig. 3a), using three different restriction enzymes (BamHI, HindIII, and NcoI) and the ^{32}P-labeled hi PCR fragment, confirmed the integration of the hygromycin gene at the Af*ECM33* locus. Western blot analysis indicated that the apparent mass of AfEcm33p, estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was 55 kDa (Fig. 3b). The protein was present in about the same amount in the 4,000 × *g* CW and IC fractions. Western blot data confirmed the gene disruption, since no reactive band was detected by the anti-AfEcm33p antisera in the mutant extracts (CW or IC) (Fig. 3b).

Phenotype analysis of the Af*ecm33* mutant. (i) Characteristics of the Af*ecm33* mutant conidia. When 6-day-old conidia of the Af*ecm33* mutant were observed by phase-contrast microscopy, they appeared less refractory than the wild-type and revertant conidia (Fig. 4a). Moreover, the conidial diameter of the Af*ecm33* mutant was 1.6 times greater than the diameter of the wild-type conidia (Fig. 4a and 5). Restoration of the conidial phenotype was used to search for revertants among transformants resistant to phleomycin and hygromycin. Restoration of the small-conidium phenotype was associated with the ectopic integration of a copy of the WT *ECM33* gene, as verified by Southern blotting (data not shown). Swelling of the conidia from the wild-type, mutant, and revertant strains occurred

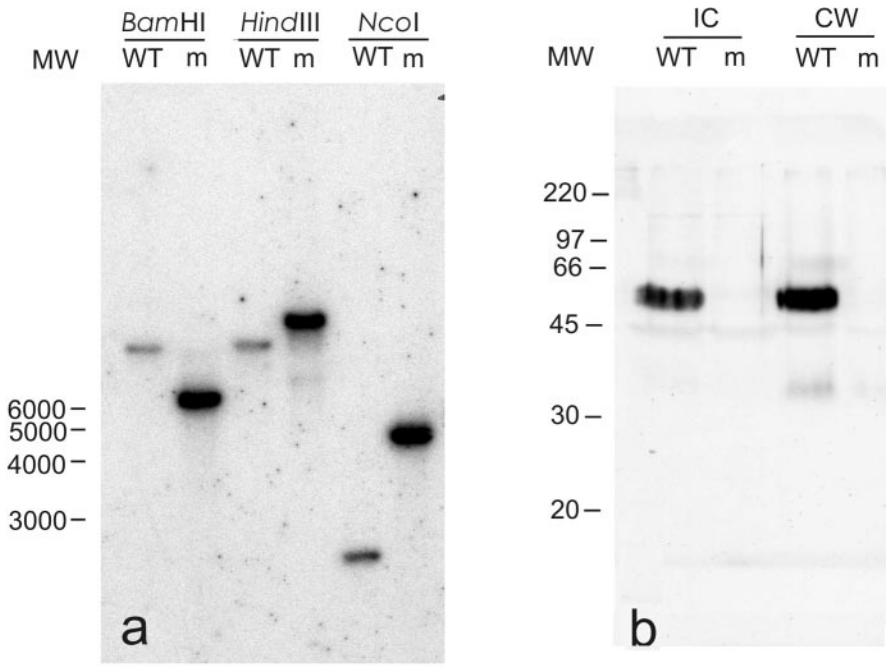

FIG. 3. Southern and Western blot analyses of the Af*ECM33* gene and protein. (a) Southern blot of the Af*ecm33* mutant (m) and WT strains. Genomic DNA of each strain was digested with BamHI, HindIII, or NcoI and hybridized with the radioactively labeled hi probe (see Fig. 1 for the identification of the probe). (b) Western blots of the IC and CW fractions obtained after 4,000 × *g* centrifugation from the WT strain and the Af*ecm33* mutant (m) after incubation of electrotransferred proteins with an anti-AfEcm33p hyperimmune antiserum.

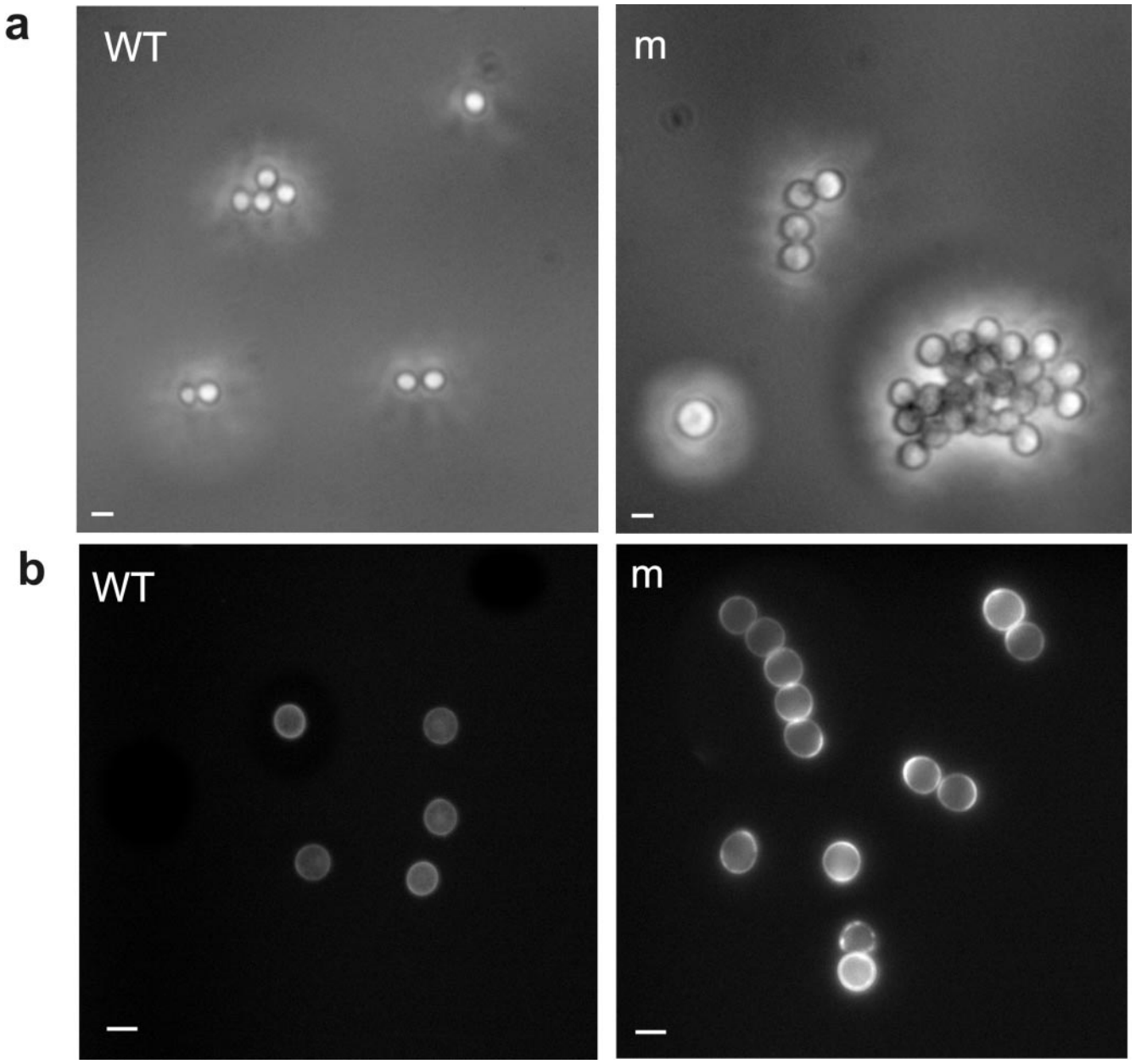

FIG. 4. Light microscopy of 6-day-old *A. fumigatus* WT and Af*ecm33* mutant (m) conidia under phase-contrast (a) or fluorescence imaging microscopy after labeling of the conidia with CFW (b). Note that the conidia of the mutant are larger, less refractile (a), and more labeled by CFW (b) than the conidia of the wild-type strain. Bars, 2 μm.

(although to a lesser extent for the mutant strain), but mutant conidia reached the same diameter before germinating (Fig. 5). No significant difference in the percentages of conidial germination was seen for the Af*ecm33* mutant, the wild type, and the *ecm33*::*ECM33* revertant after 11 h on Sabouraud agar medium at 37°C (data not shown).

In addition to a difference in the morphologies of the conidia, a separation defect was also noticed in the mutant compared to the revertant and the wild-type strains (Fig. 4a and b). The highest number of conidia per chain that were recovered with a 0.5% Tween 20 solution of 2-month-old slant cultures was 2.7 ± 0.3 in the mutant, whereas the lowest average conidial numbers per chain were seen in the wild-type and reconstituted strains (1.2 ± 0.1 and 1.2 ± 0.04, respectively; $P < 0.01$) (Fig. 4a). Transmission electron microscopy confirmed that the conidia often attached through their melanin layers, and detachment of the conidia from neighboring conidia on the same chain was incomplete (Fig. 6). An increase in calcofluor staining at the surfaces of the mutant conidia suggested that the incomplete separation of the conidia from the conidial chains was associated with an increase in chitin deposition in the cell walls of the conidia (Fig. 4b). Chitin determinations showed that the amount of chitin present in the conidia of the mutant was three times higher than in the

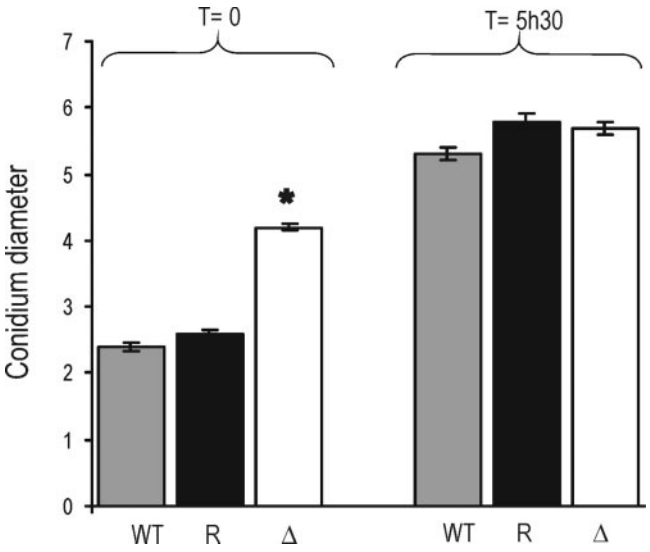

FIG. 5. Measurements of the resting (T = 0) and swollen (after 5 h 30 min of incubation at 37°C in Sabouraud medium) (T = 5h30) conidia of the parental strain (WT), the *ecm33* mutant (Δ), and the *ecm33*::*ECM33* revertant (R). Means ± standard errors are indicated. The asterisk indicates that the conidium diameter of the Af*ecm33* mutant is significantly different from the diameters of the conidia of the parental and revertant strains.

wild-type and revertant strains: 70 ± 11, 51 ± 1, and 233 ± 97 fg of hexosamine was seen per conidium of the wild type, *ecm33*::*ECM33* revertant, and *ecm33* mutant, respectively ($P < 0.01$). Accordingly, the *ecm33* mutant was more susceptible to chitin synthase inhibitors than the wild-type and revertant strains. The MICs for polyoxin D and nikkomycin were, respectively, 62.5 μg/ml and 16 μg/ml for the conidia of the mutant and >125 μg/ml and 125 μg/ml for the same inhibitors with the wild-type and revertant conidia. The inhibition was specific for chitin synthesis inhibitors, since the conidia of the mutant and wild-type strains were equally sensitive to the cell wall-disturbing agents Congo red (MIC, 125 to 250 μg/ml), caspofungin (MIC, 30 ng/ml) and Lilly 303366 (3 ng/ml), and only slightly more susceptible to CFW (MIC, 15 to 30 μg/ml). These data suggested a modification of the cell wall composition of the mutant conidia. Accordingly, conidia of the Af*ecm33* mutant were killed following storage for 2 months at room temperature in the absence of aeration, whereas the wild-type and revertant strains were not killed. Under normal aerated conditions, no differences in the viabilities of the conidia of the two strains were observed during >3 months of storage. In spite of the modifications of the conidial morphology, no quantitative difference was seen between the global mycelial growth and total conidium formation in the Af*ecm33* mutant and the *ecm33*::*ECM33* revertant in 2% agar, 2% glucose plus 1% peptone, 2% malt, or 1% yeast extract medium (data not shown).

(ii) Sensitivity to phagocytes. The sensitivities of the conidia and mycelium to killing by phagocytes are shown in Fig. 7. *ecm33* conidia were able to survive phagocytic killing by AM and PMNs better than WT parental and revertant strains. After 24 h in the lungs of immunocompetent mice, 83 to 89% of wild-type and revertant conidia were killed by AM, whereas only 53% of the mutant conidia were killed (Fig. 7). Similar results were seen after 6 h of phagocytosis of conidia by cells from the MH-S cell line: in vitro, 9.6% ± 3.1% and 19.8% ± 2.8% of the mutant and wild-type swollen conidia were killed. Similarly, PMNs killed 43 to 53% of the conidia of the parental and revertant strains after 6 h of incubation, whereas only 27% of the mutant conidia were killed under the same conditions (Fig. 7). In contrast to conidia, the mutant germ tubes were more sensitive to human neutrophils than those of the wild-type and revertant strains (Fig. 7): 43.5% ± 1.7%, 22% ± 1.6%, and 26% ± 3% XTT inhibition, respectively, were obtained with the *ecm33* mutant, wild-type, and *ecm33*::*ECM33* revertant strains. All these differences were statistically significant ($P < 0.01$). The differences in susceptibility among strains were not correlated with growth differences in the lung. Indeed, 10 h after inoculation, the lengths of the germ tubes of Texas Red-labeled wild-type conidia and FITC-labeled mutant conidia injected simultaneously into the same immunocompromised mice were not significantly different (average for 50 germ tubes, 190 ± 55 versus 271 ± 37 μm, respectively, for the wild-type strain and Af*ecm33* mutant).

DISCUSSION

In *S. cerevisiae*, genes from the *SPS2* family are expressed during meiosis and ascospore formation. Homozygous haploid null *SPS2* and *YCL048w* mutants of *S. cerevisiae* sporulate as efficiently as wild-type strains with normal spore viability, suggesting that these genes are not essential for sporulation (26, 27). However, the *YCL048w*/*SPS2* homozygous diploid null mutant produced aberrant, blebbed spores surrounded by abnormal cell walls (7), showing that these two *ECM33* homologs indeed play essential roles in ascospore formation. In contrast, deletion of *ECM33* and its closer homolog *PST1* does not lead to a sporulation defect in *S. cerevisiae*. Moreover, if *SPS2* expression is induced during sporulation, *ECM33* and *PST1* expression are repressed during sporulation, suggesting that in spite of sequence similarities, these two clusters of genes do not share similar functions (http://genome-www.stanford.edu/). *meu10*, an *ECM33* ortholog in *S. pombe*, is essential for the formation of mature viable ascospores. Deletion of *meu10* results in an increase in the number of ascospores with an elongated morphology and abnormal nuclei at meiosis (37). Most interestingly, Meu10p is required for mature cell wall formation. In *meu10* mutants, (i) the thickness of the wall is not homogeneous; (ii) the spore wall is fragile, as some appear to be broken, allowing the cytoplasmic material to leak out; and (iii) immunochemical studies have suggested a delocalization of β1-3 glucans of the inner layer in the *meu10* mutant. Although β1-3 glucan is mislocalized in the *meu10* mutant, a study with a β1-3 glucan synthase mutant has shown that localization of Meu10p in the cell wall is not affected by the absence of β1-3 glucan (37). Localization at the cell wall level is essential, since removal of the 117 amino acids at the N terminus has shown that Meu10p is no longer localized to the cell wall and ascospores are not viable. In *S. cerevisiae*, sps2p and YCL048wp, sps 2p and YCL048wp are required to organize the β-glucans into a coherent layer. As a result of improper β-glucan organization, the spore wall layers appear mixed rather than stratified (7). This phenotype is very remi-

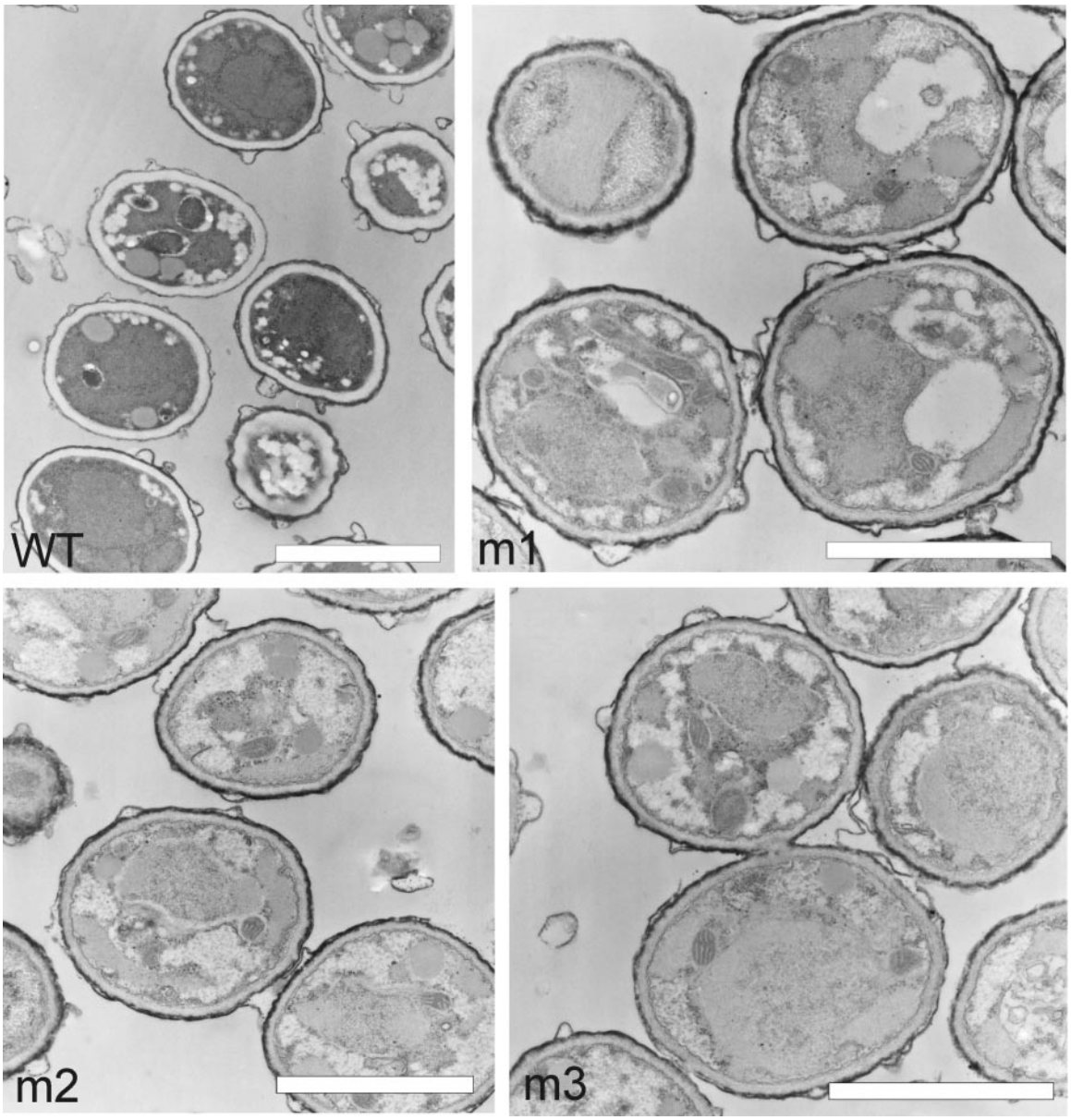

FIG. 6. Electron microscopy images of 6-day-old conidia from *A. fumigatus* WT and the Af*ecm33* mutant (m1 to m3) showing the incomplete separation of the conidia apparently caused by melanin bridging between adjacent conidia. The conidia of the revertant looked like those of the wild-type strain (data not shown). Bars, 2 μm.

niscent of the *S. pombe meu10* mutant phenotype. Similarly, the modifications of the conidial cell wall with the improper separation of the conidia in *A. fumigatus* could result from the mislocalization of the glucan layer. In *A. nidulans*, an *Aspergillus* species that has a sexual reproduction cycle, a unique *ECM33* gene has been found. However, an *ecm33* mutant has not been constructed in this species to investigate whether *ECM33* plays a role in sexual reproduction in molds, as in yeasts.

ECM33 was initially identified as a calcofluor white-sensitive mutant during a screen of a transposon-mutagenized yeast library (19). The *ecm33* mutant had other phenotypes often encountered after disruptions of cell wall-encoding genes that result in a weakened cell wall, among which are (i) a reduction in the mannose/glucose ratio; (ii) a hypersensitivity to zymolyase, hygromycin, and caffeine; (iii) an increased release of β1-3/1-6 glucans in the culture medium; (iv) an increased phosphorylation of Slt2p, indicating the activation of the cell wall integrity pathway; and (v) disorganization of the mannoprotein outer layer and defects in N-glycosylation (9, 19, 24). These data would suggest that Ecm33p is directly involved in cell wall biosynthesis. Other phenotypes of the *ecm33* mutant, such as the temperature sensitivity of the growth phenotype (36) or hypersensitivity to oxidative stress (16), suggest only a membrane defect. Meu10p from *S. pombe* and Pst1p and Ecm33p of *S. cerevisiae* have a domain that is homologous to the mam-

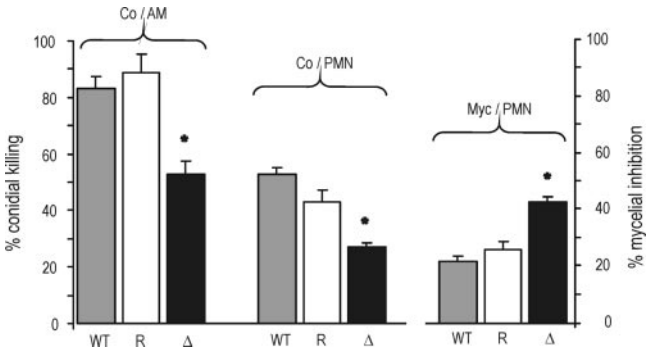

FIG. 7. Sensitivity of the WT, the *ecm33* mutant (Δ), and the *ecm33*::*ECM33* revertant (R) strains to phagocytes. Killing of resting conidia (Co) and mycelium (Myc) by mouse AM and human PMNs was estimated as percent germination and percent mycelial-growth inhibition using the XTT assay. Values are expressed as means plus standard errors. The asterisks indicate that the values obtained with the *ecm33* mutant were significantly different from the ones obtained with the parental and revertant strains.

malian insulin receptor, suggesting that Ecm33p and its homologs could function as a receptor to sense environmental information, such as the nutrient conditions of the medium. Moreover, the absence of any of the *ECM33* sequences in the CAZYME database (http://afmb.cnrs-mrs.fr/CAZY/) suggests that this protein does not enzymatically modify a glycosylated molecule (B. Henrissat, personal communication). In *C. albicans*, deletion of ECM33p impacts cell wall integrity and morphogenesis (20). Mutant yeasts are more rounded and bigger than the wild-type yeasts. They exhibit cell wall defects and a marked tendency to flocculate. Moreover, the yeast-to-hypha transition is inhibited only in solid media, not in liquid media. These results are very reminiscent of those obtained with *A. fumigatus* with higher cell volumes of the conidia and variability of the mycelial phenotype based on the environmental growth conditions. Because the biochemical function of Ecm33p is unknown in *A. fumigatus* and other fungi, it is impossible to say that the differences in the cell walls of the Ecm33p mutants are due to a direct effect of the gene deletion rather than a secondary downstream event resulting from the perturbation of sensing mechanisms.

The Ecm33p proteins of *A. fumigatus*, *Candida albicans*, and *S. cerevisiae* are GPI anchored (4, 6, 9, 10, 13, 24, 35). Ecm33p of *S. cerevisiae* has a dibasic KK motif upstream from the GPI cleavage site that is a signature sequence of proteins that are localized at the membrane. Anchoring to the plasma membrane is essential for Ecm33p function: replacement of the G406 residue (to which GPI binds) with another amino acid residue leads to the loss of a complementation function of the temperature-sensitive phenotype (36). The GPI anchoring of the *Ecm33* orthologs of *S. pombe* has not been investigated. Hydropathy plots, however, showed that Meu10p has hydrophobic C and N termini, suggesting that Meu10p is also GPI anchored to the membrane. Moreover, the GPI anchor also seems functionally important in this protein, since removal of a 40-amino-acid residue at the C terminus (the putative GPI domain) is sufficient to decrease ascospore viability, whereas the cell wall localization of the protein is conserved. The dibasic KK residue is found in Ecm33p of *C. albicans* but is not found in AfEcm33p. Indeed, membrane-anchored GPI proteins of *A. fumigatus*, such as Gelps, Crhps, and acid phosphatase (I. Mouyna, unpublished data; 2), do not possess a dibasic protein, indicating that the dibasic rule does not apply to all fungi, at least not to *A. fumigatus*. The analysis of the *ecm33* mutant in *A. fumigatus* is another example of the diversity of the functions of GPI proteins in this filamentous fungus. Some of the GPI proteins have enzymatic activities, such as phosphatase, phospholipase B, or proteases that are involved in fungal nutrition (2, 4, 18). Others are associated with cell wall construction, such as the glucanosyltransferase encoded by *GEL* or the *CRH* family gene products (14, 21, 32).

This is the first demonstration that conidia and mycelia of a mutant and a parental strain of *A. fumigatus* exhibit different susceptibilities to phagocytes. Since previous studies have shown that reactive oxidants are essential for the killing of this fungus by phagocytes (28), the different susceptibilities of the two fungal morphotypes could be associated, at least partly, with a higher sensitivity of mycelia to reactive oxidants. In agreement with the PMN data, the germ tubes of the mutant were indeed more susceptible to reactive oxidants in vitro than those of the wild-type and revertant strains (93% germination for the wild type and revertant versus 33% for the mutant after 8.5 h in Sabouraud medium in the presence of 1.5 mM H_2O_2). The higher resistance of mutant conidia to the phagocyte remains unexplained but could be due to major differences in cell wall organization that would lead to a lower permeability of the mutant cell wall to phagocyte toxic metabolites or a lower sensitivity to host enzymes, such as chitinases, that are present in high concentrations in the lung macrophages. A detailed chemical analysis of the mycelial and conidial mutant and wild-type strains is under way to explain these differences.

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