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SUN Proteins Belong to a Novel Family of β -(1,3)-Glucan-modifying Enzymes Involved in Fungal Morphogenesis^{*[5]}

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Amandine Gastebois^{†1,2}, Vishukumar Aimananda^{†1}, Sophie Bachellier-Bassi^{§¶}, Audrey Nesseir^{§¶}, Arnaud Firon^{§¶3}, Anne Beauvais[‡], Christine Schmitt^{||}, Patrick England^{**}, Rémi Beau[‡], Marie-Christine Prévost^{||}, Christophe d'Enfert^{§¶}, Jean-Paul Latgé^{‡4}, and Isabelle Mouyna[‡]

From the [†]Institut Pasteur, Unité des *Aspergillus*, Département Parasitologie et Mycologie, 25 rue du Docteur Roux, F-75015 Paris, the [§]Institut Pasteur, Unité Biologie et Pathogénicité Fongiques, Département Génomes et Génétique, 25 rue du Docteur Roux, F-75015 Paris, the [¶]Institut National de la Recherche Agronomique (INRA), USC2019, 25 rue du Docteur Roux, F-75015 Paris, the ^{||}Institut Pasteur, Plate-Forme de Microscopie Ultrastructurale, Département Biologie Cellulaire et Infection, 25 rue du Docteur Roux, F-75015 Paris, and the ^{**}Institut Pasteur, Plate-Forme de Biophysique des Macromolécules et de Leurs Interactions, Département Biologie structurale et chimie, 25 rue du Docteur Roux, F-75015 Paris, France

Background: SUN proteins are involved in yeast morphogenesis, but their function is unknown.

Results: SUN protein plays a role in the *Aspergillus fumigatus* morphogenesis. Biochemical properties of recombinant SUN proteins were elucidated.

Conclusion: Both *Candida albicans* and *Aspergillus fumigatus* SUN proteins show a β -(1,3)-glucanase activity.

Significance: The mode of action of SUN proteins on β -(1,3)-glucan is unique, new, and original.

In yeasts, the family of SUN proteins has been involved in cell wall biogenesis. Here, we report the characterization of SUN proteins in a filamentous fungus, *Aspergillus fumigatus*. The function of the two *A. fumigatus* SUN genes was investigated by combining reverse genetics and biochemistry. During conidial swelling and mycelial growth, the expression of *AfSUN1* was strongly induced, whereas the expression of *AfSUN2* was not detectable. Deletion of *AfSUN1* negatively affected hyphal growth and conidiation. A closer examination of the morphological defects revealed swollen hyphae, leaky tips, intrahyphal growth, and double cell wall, suggesting that, like in yeast, *AfSun1p* is associated with cell wall biogenesis. In contrast to *AfSUN1*, deletion of *AfSUN2* either in the parental strain or in the *AfSUN1* single mutant strain did not affect colony and hyphal morphology. Biochemical characterization of the recombinant *AfSun1p* and *Candida albicans* Sun41p showed that both proteins had a unique hydrolysis pattern: acting on β -(1,3)-oligomers from dimer up to insoluble β -(1,3)-glucan. Referring to the CAZy database, it is clear that fungal SUN proteins represent a new family of glucan hydrolases (GH132) and play an important morphogenetic role in fungal cell wall biogenesis and septation.

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[5] This article contains supplemental Table S1 and Figs. S1–S4.

¹ Both authors contributed equally to this work.

² Present address: Laboratoire des Interactions Plantes-Microorganismes, Institut National de la Recherche Agronomique, 24 Chemin de Borde-Rouge – Auzeville, CS 52627, 31326 Castanet-Tolosan cedex, France.

³ The recipient of a postdoctoral fellowship of Institut Pasteur (Bourse Roux). Present address: Institut Pasteur, Unité de Biologie des Bactéries Pathogènes à Gram-positif, 25 rue du Docteur Roux, F-75015 Paris, France.

⁴ To whom correspondence should be addressed. Tel: 33-1-40613518; Fax: 33-1-40613419; E-mail: jplatge@pasteur.fr.

In *Saccharomyces cerevisiae*, four paralogous SUN genes, namely *SIM1*, *UTH1*, *NCA3*, and *SUN4* (1–3), are classified into two groups. Members of group I encode proteins with a conserved C-terminal region of ~250 amino acids corresponding to the SUN domain or Pfam-PF03856 (4) comprising four Cys residues in a Cys-X₅-Cys-X₃-Cys-X₂₄-Cys motif (5). The N-terminal region is less conserved, ranging from 80 to 168 amino acids with a signal peptide and a low complexity region rich in serine and threonine residues. On the contrary, in the group II members, the SUN domain (especially the Cys-rich motif) and the low complexity region are degenerated.

Mounting evidences strongly suggest that the yeast SUN proteins play a role in cell wall biogenesis, septum integrity and cell separation (3, 6). In *S. cerevisiae*, Sun4p is localized in the cell wall and is required, together with Uth1p, for septation (3). In *Schizosaccharomyces pombe*, deletion of the single class I SUN gene, *psu1*, is associated with cell wall defects during cell separation, resulting in swollen cells that eventually undergo lysis (7). In *Candida albicans*, inactivation of *SUN41* leads to a defect in the separation of daughter cells from mother cells, whereas simultaneous inactivation of *SUN41* and *SUN42* is lethal in the absence of osmotic protection. Cell wall defects seen in this double mutant are mainly localized in the region surrounding the septa in mother yeast cells and subapical hyphal compartments (8–10). Proteomic analyses have shown that Sun41p and Sun42p are found in the *C. albicans* cell wall and secretome (9, 11, 12). However, no biochemical function has been associated with any of these SUN proteins, and no direct role in cell wall biosynthesis has been demonstrated.

SUN proteins have not been studied in the filamentous ascomycetes. Here, we report the characterization of the SUN genes of *Aspergillus fumigatus*. The *A. fumigatus* genome harbors two SUN genes: the class I *AfSUN1* and the class II *AfSUN2*. In the present study, we showed that only *AfSUN1* plays a role during morphogenesis. Using the recombinant Sun1p of *A. fumigatus*

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and the orthologous Sun41p of *C. albicans*, it is shown for the first time that SUN proteins bind and hydrolyze β -(1,3)-glucan in a very specific manner.

EXPERIMENTAL PROCEDURES

Strains and Culture Media

A. fumigatus mutants constructed in this study were derived from the strain CEA17 Δ ku80 (13); all strains were maintained on 2% malt agar slants supplemented, when necessary, with 150 μ g/ml hygromycin B (Sigma) and/or 20 μ g/ml phleomycin (InvivoGen). Minimal medium was used for the transformation experiments (14). Cultures were grown in liquid Sabouraud medium (2% glucose containing 1% mycopeptone) for DNA extraction as well as phenotypic analyses or in YPD (1% yeast extract, 2% Bacto peptone, 1% glucose) for RNA extraction. *C. albicans* used in this study was the strain BWP17 (15), which was maintained at 30 °C on YPD.

RNA Extraction, Reverse Transcription, and Quantitative PCR

For RNA extraction, the *A. fumigatus* parental strain was grown in YPD medium and incubated at 37 °C for different times (0, 4, 8, 30, 36, 43, and 48 h). Conidia and mycelia were disrupted, and RNA was isolated as described earlier (16) and stored at -20 °C. 1 μ g of total RNA was reverse-transcribed using the Bio-Rad Iscript cDNA synthesis kit following the instructions of the manufacturer. Quantitative PCR assays were performed as described previously (17) using primers Sun1q1, Sun1q2, Sun2q1, Sun2q2, and TEF1a-TEF1b (as control). *FKS1* (β -(1,3)-glucan synthase gene) was used for the comparison (primers used were FKS1a-FKS1b; supplemental Table S1).

Construction of *A. fumigatus* Deletion and Complementation Cassettes by Fusion PCR

The deletion and complementation cassettes used in this work were constructed by fusion PCR as described earlier (18). Primer positions are illustrated in supplemental Fig. S1, and the primer sequences are shown in supplemental Table S1. The *Escherichia coli hph* gene, coding for hygromycin B phosphotransferase, obtained from the plasmid pAN7-1 (19) was used to replace *SUN1*. *SUN2* was replaced by a *lox* disruption cassette-borne *ble* marker (encoding a phleomycin binding protein), obtained from the plasmid pSK341 (a kind gift from S. Krapmann, Georg-August-University Göttingen, Germany). In a first round of PCR, flanking regions 1 and 2 (amplicons 1 and 3, respectively) were amplified from the CEA17 Δ ku80 genomic DNA prepared according to Girardin *et al.* (20), and selection markers (*HPH* and *BLE*; amplicon 2) were amplified using plasmids pAN7-1 and pSK341, respectively, and 60-bp chimeric oligonucleotides (primers SunB, SunC, SunD, and SunE). PCR was performed as follows: 30 cycles of amplification for 30 s at 95 °C and 3 min at 68 °C (Advantage 2 polymerase, Clontech). The resulting three PCR products were gel-purified and used as templates for a second PCR using the SunA and SunF primers. The PCR parameters were the same as described above except for a 6-min annealing and extension step.

A *SUN1* complementation cassette was also constructed using the fusion PCR method (18) as described above. This

cassette contained the 5'-flanking region of *AfSUN1*, the *AfSUN1* gene, the actin terminator, the phleomycin resistance marker, and the 3'-flanking region of *AfSUN1* (supplemental Fig. S1C).

A. fumigatus Transformation

The fusion PCR products (1–2 μ g) were used to transform either CEA17 Δ ku80 conidia or Δ sun1 conidia using the electroporation method described by Sanchez *et al.* (21) with modifications (22, 23). Transformants were selected on minimal medium agarose (0.7%) + 150 μ g/ml hygromycin B for *AfSUN1* deletion or minimal medium agarose + 20 μ g/ml phleomycin for *AfSUN2* deletion and incubated at room temperature for 1 week. Genomic DNAs from hygromycin- or phleomycin-resistant transformants and the parental strain were prepared as described by Girardin *et al.* (20), digested with restriction enzymes (Roche Applied Science), and verified by Southern blot analysis (supplemental Figs. S2 and S3). For Southern blot analysis, 5 μ g of digested genomic DNA was loaded on a 0.7% agarose gel, blotted on a nylon membrane (Hybond N⁺, GE Healthcare), and revealed using probes ³²P-labeled using the Rediprime kit (Amersham Biosciences).

A. fumigatus Growth

Growth kinetics were performed by inoculating 10⁵ conidia in 50 ml of Sabouraud liquid medium in a shaking incubator (37 °C, 150 rpm). Mycelia were collected by filtration under vacuum after 24, 36, and 48 h and dried at 80 °C overnight, and dry weights were recorded. For mycelial growth on the plates, colony diameters were measured. Germination tests were performed by placing 5 μ l of a conidial (10⁶) suspension on Sabouraud solid medium at 37 °C and counting the number of germinated conidia every hour. For microscopic observation, the parental as well as the mutant strains were grown in 24-well plates in 1 ml of Sabouraud medium with 10⁴ conidia per well and incubated for 24 h at 37 °C without shaking.

Sensitivity to Antifungal Drugs

Sensitivity to Calcofluor white and Congo red was tested as described previously (17). The effect of echinocandins (caspofungin and micafungin) was checked by E-test as per the manufacturer's instructions (bioMérieux). Conidial suspensions (2 \times 10⁷) were spread on 1% yeast extract agar plates, and the E-test strip was placed. After a 48-h incubation at 37 °C, minimum inhibitory concentrations were determined as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip.

Electron Microscopy Analysis

Conidia (2 \times 10⁷) were incubated in 40 ml of Sabouraud liquid medium for 36 and 44 h at 37 °C. The mycelium was collected and fixed at 4 °C for 2 days in 4% glutaraldehyde in 0.1 M cacodylate, pH 7.4. After several washings with 0.1 M cacodylate buffer, samples were post-fixed for 45 min in the same buffer containing 2% osmium tetroxide (Merck, Darmstadt, Germany). During dehydration in a graded ethanol series, the 50% (v/v) ethanol incubation was prolonged for a period of 1 h, and 1% of 3-glycidoxypropyl trimethoxysilane (SPI-CHEM,

West Chester, PA) was added during this incubation. Then, samples were embedded in Epon resin. Contrasted ultrathin sections (60 nm) were observed under a JEM 1010 transmission electron microscope (under Jeol, Tokyo, Japan).

Expression of AfSUN1 and CaSUN41 in *Pichia pastoris* and Purification of Recombinant AfSun1p and CaSun41p

P. pastoris GS115 strain (Invitrogen) and the expression vector pHLS1 (Invitrogen) were used to express recombinant AfSun1p and CaSun41p (r-AfSun1p and r-CaSun41p, respectively).⁵ The open reading frame of AfSUN1 fused to a histidine tag (His₆ tag) was obtained after PCR amplification on *A. fumigatus* CEA17Δku80 cDNA with primers Sunprot1 and Sunprot2-HIS containing XhoI and BamHI restriction sites, respectively (supplemental Table S1). The open reading frame of CaSUN41 fused to a His₆ tag was also obtained by PCR amplification with primers Sun41-fw-ATG and Sun41-rev-HIS, both containing an XhoI restriction site (supplemental Table S1). The resulting PCR products were digested with XhoI and BamHI (in the case of AfSUN1) or XhoI (in the case of CaSUN41) and cloned in pHLS1 digested by the same enzymes, yielding pHLS1-AfSUN1 and pHLS1-CaSUN41, respectively. pHLS1-AfSUN1 and pHLS1-CaSUN41 were linearized by BglII or StuI, respectively, purified with phenol-chloroform extraction, and precipitated with ethanol before transformation of *P. pastoris* by the lithium chloride method (Invitrogen). Transformants were plated on a histidine-deficient medium and screened on minimal methanol medium (Invitrogen) for the insertion of the construct in the AOX1 locus of *P. pastoris*. Both recombinant proteins were obtained after culturing the transformed *P. pastoris* in buffered complex methanol medium (Invitrogen) at 30 °C for 72 h followed by the addition of methanol (1%) to the culture medium every 24 h. Purification of secreted r-AfSun1p and r-CaSun41p was performed using ProBond nickel beads following the manufacturer's instructions (Invitrogen), and the eluates containing recombinant proteins were dialyzed against sodium acetate buffer (pH 6.5).

Deglycosylation of r-AfSun1p

N-Deglycosylation was performed using a recombinant N-glycosidase F (Roche Applied Science) according to the manufacturer's instructions. Total deglycosylation was carried out using the trifluoromethanesulfonic acid reagent (24). 10 μg of purified protein was freeze-dried and kept under vacuum in presence of P₂O₅. The sample was treated with 50 μl of a trifluoromethanesulfonic acid/anisole solution (2:1, v/v) in an ice bath under argon atmosphere for 3 h. The reaction was stopped by the addition of 60% pyridine in an ethanol/dry ice bath until pH 6.0 was achieved. Deglycosylated protein samples were dialyzed against water and analyzed by SDS-PAGE.

⁵ The abbreviations used are: r-AfSun1p, recombinant AfSun1p; r-CaSun41pAl, recombinant CaSun41p; alkali-insoluble; AS, alkali-soluble; p-NPG, p-nitrophenyl-β-D-glucopyranoside; PABA, p-aminobenzoic acid; DP, degree of polymerization; GH, glycoside hydrolase.

Cell Wall Analysis

A. fumigatus was grown in liquid Sabouraud medium at 37 °C for 20 or 36 h, mycelia were harvested, washed with water, and disrupted using glass beads in a FastPrep (MP Biomedicals), and the disrupted suspension was centrifuged (3000 × g, 10 min). The cell wall fraction (pellet) was washed with water and boiled in 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM EDTA, 2% SDS and 40 mM β-mercaptoethanol (15 min, ×2). The sediment obtained after centrifugation (3000 × g, 10 min) was washed with water and incubated in 1 M NaOH containing 0.5 M NaBH₄ at 65 °C (1 h, ×2). The insoluble pellet obtained upon centrifugation (3000 × g, 10 min) was washed with water to neutrality and freeze-dried (alkali-insoluble (AI) fraction). In the supernatant, excess of NaBH₄ was removed using acetic acid followed by dialysis against water and freeze-drying (alkali-soluble (AS) fraction). The monosaccharide composition in the AI and AS fractions was determined by gas-liquid chromatography (GLC; Perichrom PR2100 (Saulx-les-Chartreux, France) equipped with flame ionization detector and fused silica capillary column (30 m × 0.32 mm inner diameter) filled with BP1)) as described earlier (25).

Analysis of the r-AfSun1p and r-CaSun41p Activities

The activity of both recombinant proteins was checked on the fungal cell wall components obtained as described above. *C. albicans* cell wall fractions were obtained as described in Wang *et al.* (26). In brief, the reaction mixture contained alkali-insoluble (AI) or alkali-soluble (AS) fractions extracted from the fungal cell wall (200 μg), r-AfSun1p (1 μg) or r-CaSun41p (1 μg) in a total volume of 100 μl (acetate buffer, 50 mM, pH 6.0). Following incubation at 37 °C overnight, reducing sugars released from the solubilized cell wall materials were analyzed using the p-aminobenzoic acid (PABA) method. Briefly, 50 μl of the solubilized material was boiled for 10 min with 950 μl of PABA reagent (50 mM sodium sulfite, 250 mM NaOH, 25 mM sodium citrate, 10 mM calcium chloride, and 10 mg × ml⁻¹ PABA), the optical density was measured at 414 nm, and the amount of reducing sugar was calculated using glucose as the standard. Further, enzyme kinetic studies were performed using AI fraction and the PABA method.

Activities of the recombinant proteins were checked on p-nitrophenyl-β-D-glucopyranoside (p-NPG) as the substrate in acetate buffer (pH 5.5, 50 mM) at 37 °C (27). Enzyme activity was also checked on laminarioligosaccharides of varying degrees of polymerization (DPs) as substrates (28) (checked using 1 μg of the enzyme with 0.1–30 μM substrate concentrations; for further assays, 20 μM substrates were used as above this concentration, there was saturation in the reaction kinetics; assays were performed in acetate buffer (50 mM, pH 5.5) at 37 °C). The resultant product was analyzed on a CarboPac PA200 anion-exchange column (high-performance anion-exchange chromatography, 3.2 × 250 mm; Dionex) using NaOH (50 mM) and NaOAc (500 mM) in 50 mM NaOH as the eluents A and B, respectively. The column was pre-equilibrated for 20 min with 98% A + 2% B. Following sample injection, a gradient run (flow rate 0.35 ml/min) was performed as follows: 0–2 min, isocratic step (95% A + 2% B), 2–15 min, 98% A + 2% B, 65% A + 35% B;

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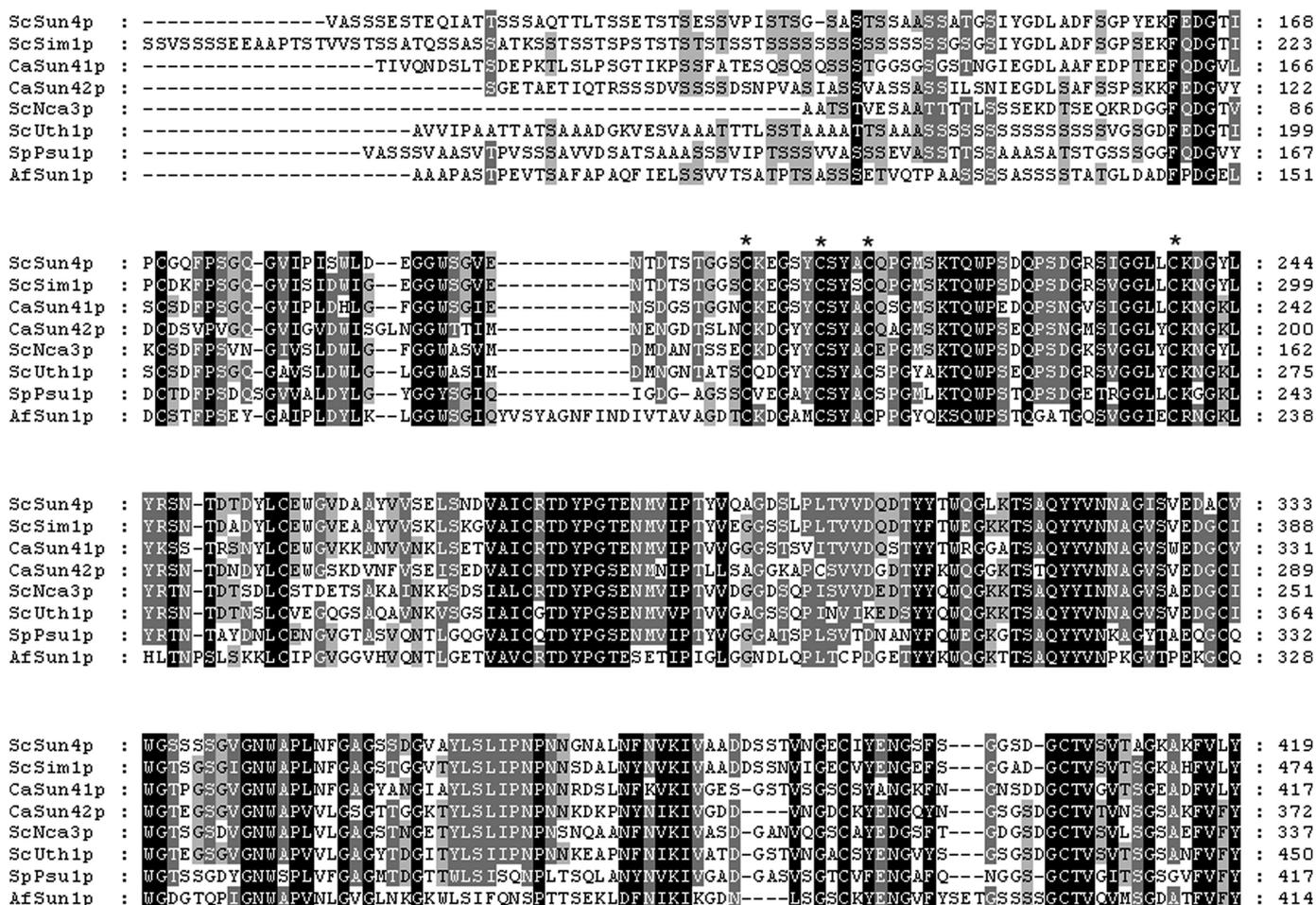


FIGURE 1. ClustalX alignment of SUN domain of group I proteins of *S. cerevisiae* (ScSun4p, ScSim1p, ScNca3p, and ScUth1p), *C. albicans* (CaSun41p and CaSun42p), *S. pombe* (SpPsu1p), and *A. fumigatus* (AfSun1p, AFUA_7G05450). Cysteines of the putative iron binding domain are indicated with an asterisk. Amino acids shared by all eight proteins have a black background, amino acids shared by six or seven proteins have a dark gray background, and amino acids shared by five proteins have a light gray background.

15–35 min, 65% A + 35% B, 40% A + 60% B; 35–37 min, 40% A + 60% B, 100% B; and 37–40 min, 100% B. The products were detected on a pulsed electrochemical detector.

Substrate Binding Assays

Pull-down Assay—10 μ g of r-AfSun1p was incubated with 100 μ g of cell wall β -(1,3)-glucan in a total volume of 100 μ l of acetate buffer (50 mM, pH 5.5; 37 $^{\circ}$ C for 1 h). Upon centrifugation, protein concentration in the supernatant was determined by Bradford assay. The sediment obtained was washed twice with wash buffer (10 mM Tris (pH 7.5), 500 mM NaCl, 0.02% Tween 20) and then boiled with SDS sample buffer. Protein in the sample buffer as well as in the supernatant was analyzed by SDS-PAGE (15% gel).

Surface Plasmon Resonance Assay—SPR assays were performed on a Biacore X100 biosensor instrument (GE Healthcare). Recombinant AfSun1p diluted in 50 mM acetate buffer (pH 4.5) containing 100 μ M EDTA to a concentration of 25 μ g/ml was coupled to the surface of a CM5 chip (Biacore, Inc., Piscataway, NJ) by standard amine chemistry (*N*-hydroxysuccinimide-1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, Biacore, Inc.) to a level of \sim 2000 response units. The remaining active sites were quenched using 1 M ethanolamine. A reference

flow cell consisting of an activated and quenched surface without r-AfSun1p was created to normalize readings. The running solvent was acetate buffer (50 mM, pH 5.5) containing 100 μ M EDTA at a flow rate of 10 μ l/min. Laminarioligosaccharides of DPs 2, 6, 12, and 23 were diluted to a final concentration of 200 μ M in the running buffer. Both injection and dissociation of the laminarioligosaccharides were analyzed for 570 s at 37 $^{\circ}$ C. Regeneration of the surface was performed by injecting 50 mM glycine-NaOH, pH 9.5, for 60 s at a flow rate of 10 μ l/min; β -(1,6)-oligosaccharides of DP10 were used as the control.

RESULTS

Identification of the SUN Genes in *A. fumigatus*—A BLAST search against the *A. fumigatus* genome identified two SUN domain-containing proteins encoded by AfSUN1 (AFUA_7G05450) and AfSUN2 (AFUA_1G13940). As for the other eukaryotes (8), *A. fumigatus* contained only one class I SUN protein Sun1p. AfSUN1 displayed a 1245-bp open reading frame (ORF) coding for a protein of 415 amino acids with a predicted molecular mass of 43.5 kDa. The AfSun1p SUN domain, containing the canonical Cys-X₅-Cys-X₃-Cys-X₂₄-Cys motif, spanned residues 111–414 (Fig. 1). The sequence contained a signal peptide at the N terminus (amino acids residues

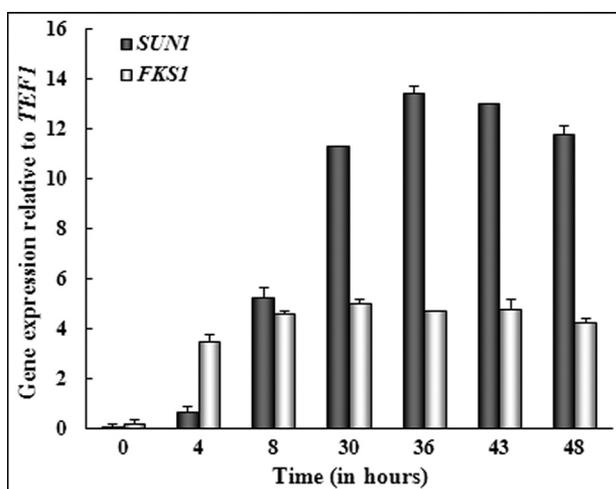


FIGURE 2. Expression level of the *AfSUN1* and *AfFKS1* gene during growth of *A. fumigatus*. Gene expression was determined by real time RT-PCR in resting conidia (0 h), swollen conidia (4 h), germinated conidia (8 h), and mycelia (30, 36, 43, and 48 h). Error bars indicate S.D.

1–19; SignalP prediction) (29), suggesting that *AfSun1p* is a secreted protein. It also showed three putative *N*-glycosylation sites at positions 80, 242, and 377 (NetNGlyc 1.0 Server).

In contrast, *AfSun2p* showed the characteristics of group II SUN domain proteins with a degenerated motif located at the N-terminal end of the protein (residues 17–301). The 1413-bp *AfSUN2* ORF encoded a protein with 471 amino acid residues with a calculated molecular mass of 48.6 kDa. *AfSun2p* also displayed a signal peptide at the N terminus (corresponding to the amino acid residues 1–21). In addition, *AfSun2p* showed all the characteristics of glycosylphosphatidylinositol-anchored proteins: a hydrophobic *N*-terminal region (amino acid residues 1–17), a C-terminal region (452–471 amino acids) rich in serine and threonine, and a cleavage site for a carboxyl-peptidase and a ω -site on the 446-amino acid residues (30). However, unlike *AfSun1p*, *AfSun2p* did not show any *N*-glycosylation site.

Expression of the *AfSUN* Genes—*AfSUN1* expression was detected starting 4 h after the initiation of spore germination and thereafter during mycelial growth. Quantitative PCR analysis showed a linear increase in the *AfSUN1* expression during growth until 36 h beyond which there was a slow decrease (Fig. 2). In contrast, *AfSUN2* expression was not detectable in the conditions tested.

Genetic Inactivation of *AfSUN* Genes—To analyze the functions of *AfSUN1* and *AfSUN2*, deletion mutants were constructed by replacing each gene with an antifungal resistance cassette in the parental *A. fumigatus* strain, CEA17 Δ *ku80*. PCR and Southern blot analyses showed correct and unique integration of the drug resistance cassettes at the target locus for *AfSUN1* and *AfSUN2* (supplemental Fig. S2). Deletion of *AfSUN2* in the Δ *AfSun1* mutant strain was also performed. PCR and Southern blot analysis showed correct and unique integration of the phleomycin resistance cassette at the *AfSUN2* locus in the Δ *AfSun1* mutant background (supplemental Fig. S2). *AfSUN1* gene complementation was performed in the Δ *AfSun1* mutant strain by reintroduction of the wild-type *AfSUN1* gene into the genome. RT-PCR confirmed expression of the *AfSUN1*

gene in the complemented Δ *sun1* mutant strain (supplemental Fig. S3).

***AfSUN1* Is Required for Normal Growth and Correct Hyphal Morphogenesis**—A modest but significant reduction in the dry mass was observed after 36 h of growth of the Δ *AfSun1* and Δ *AfSun1/AfSun2* mutants in the Sabouraud liquid medium at 37 °C (Fig. 3A). Mycelial growth of these mutants was also affected on the agar medium. After 36 h of incubation on Sabouraud solid medium, diameters of the Δ *AfSun1* and Δ *AfSun1/AfSun2* mutant colonies were 1.5 times smaller than that of the parental strain at 37 °C and 2 times smaller at 50 °C (Fig. 3B). In contrast, no difference could be observed for the Δ *AfSun2* mutant when compared with the parental strain (Fig. 3B). The growth defects on agar plates were independent of the pH and the presence of osmotic stabilizers (sorbitol or NaCl; data not shown). In agreement with the growth defects, an alteration of the mycelial morphology was seen in the mycelium of the Δ *AfSun1* mutant. After 16 h of growth, the hyphae of the Δ *AfSun1* mutant showed leaky hyphal tips (~15–20%), intrahyphal growth, and short intercalary cells with closely arranged septa and swollen appearance (Fig. 3C, row i); after 48 h (Fig. 3C, row ii), there was an increase in the severity of the morphological alterations with an increased number of leaky tips (~35–40%), whereas the parental strain hyphae were normal. In the Δ *AfSun1/AfSun2* double mutant, no additional defect in hyphal integrity and morphogenesis was observed when compared with the Δ *AfSun1* mutant strain (data not shown).

Morphological defects of the Δ *AfSun1* mutant were further confirmed by electron microscopy analysis. Intrahyphal growth was shown by the doubling of the cell wall in the Δ *AfSun1* mutant (Fig. 3C, row iii). Interestingly, a high number of Woronin bodies were seen at the septal region of the Δ *AfSun1* mutant, suggesting that a defect in the closure of the septal pores could be at the origin of intracellular hyphae.

Conidiation was also affected with a 2-fold reduction for the Δ *AfSun1* and the Δ *AfSun1/AfSun2* mutants when compared with the parental strain (data not shown). However, conidia produced were normal, and there were also no differences in the germination of the single and double Δ *AfSun* strains and the parental strain spores (data not shown). Reintroduction of the *AfSUN1* in the Δ *AfSun1* mutant restored parental phenotypes (data not shown).

Biochemical Function of the SUN Proteins *AfSun1p* and *CaSun41p*—The phenotypes (swollen and vacuolated hyphae with double cell wall, and leaky tips; Fig. 3) observed during vegetative growth suggested a cell wall defect in the Δ *AfSun1* and Δ *AfSun1/AfSun2* mutant strains. However, when cell wall analysis was performed at different times of growth (20 and 36 h), no differences were seen in the cell wall composition (alkali-insoluble/alkali-soluble ratio or monosaccharide composition) of the Δ *AfSun1* mutant or the parental or *AfSUN1*-complemented strains (supplemental Fig. S4; data not shown). These results suggested that *Sun1p* does not have a direct effect on overall cell wall polysaccharide biosynthesis.

To investigate further the function of *AfSun1p*, a recombinant protein r-*AfSun1p* carrying a His₆ tag was expressed in the yeast *P. pastoris* and affinity-purified (see “Experimental Procedures”). The recombinant r-*AfSun1p* was highly glycosylated

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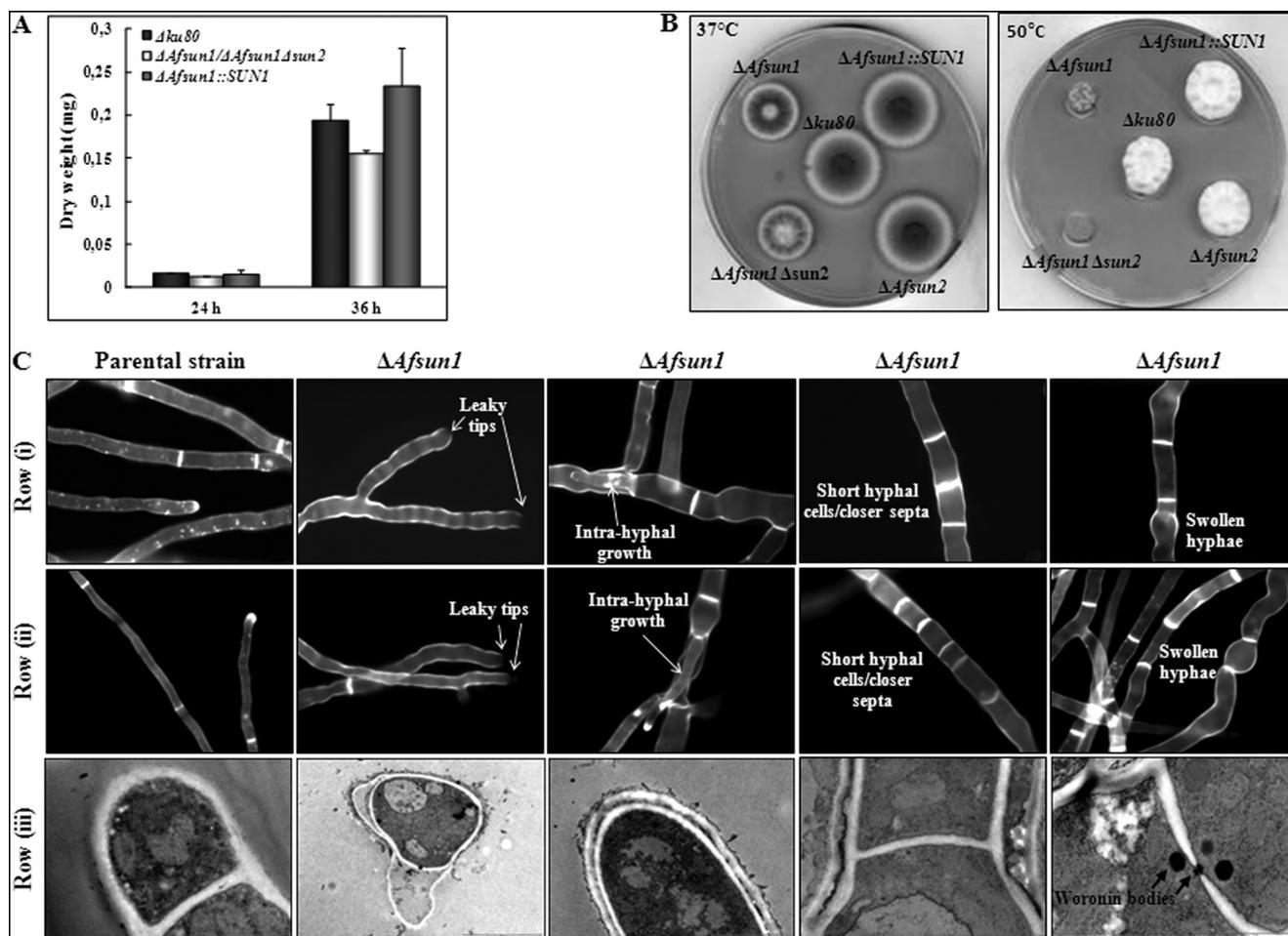


FIGURE 3. Growth of the $\Delta Afsun1$, $\Delta Afsun2$, $\Delta Afsun1/Afsun2$ mutant, and the complemented $\Delta Afsun1::SUN1$ strains. A, after 24 and 36 h of growth at 37 °C in Sabouraud liquid medium. Error bars indicate S.D. B, after 36 h of growth at 37 °C and 64 h at 50 °C on Sabouraud agar medium. C, hyphal morphology of the $\Delta Afsun1$ mutant and the parental strains after incubation in Sabouraud liquid medium at 37 °C for 16 h (row i) and 48 h (row ii) ($\times 100$) (cell walls are stained with Calcofluor) and (row iii) transmission electron microscopic observations of the parental and the mycelial sections of the $\Delta Afsun1$ mutant strain after 36–48 h of growth. Note the doubling of the cell wall and the presence of Woronin bodies in the $\Delta Afsun1$ mutant hyphae.

and had an apparent molecular mass of 68.5 kDa. Upon deglycosylation, r-AfSun1p migrated on a SDS-PAGE at 44 kDa (data not shown) in agreement with the predicted molecular mass of AfSun1p.

When incubated with the AS and AI fractions obtained from the parental *A. fumigatus* cell wall, r-AfSun1p showed a hydrolytic activity only toward the AI fraction (7–9% reducing sugars released; determined by the PABA method), which consists mainly of β -(1,3)-glucan and chitin. However, r-AfSun1p did not show any activity toward pure chitin. Further characteristics of the β -(1,3)-glucan hydrolysis were studied using the AI fraction as the substrate. r-AfSun1p was active in the pH and temperature ranges of 5.0–7.0 and 25–45 °C, with pH and temperature optima of 5.5 and 37 °C, respectively. The specific activity was 3.2 nmol of reducing equivalent/ $\text{min}^{-1} \mu\text{g}^{-1}$ protein. When incubated with curdlan (a linear β -(1,3)-glucan) and schizophyllan (a branched polysaccharide having β -(1,6)-side chain on every third glucose residue of the β -(1,3)-backbone) (31), r-AfSun1p showed hydrolytic activity only toward curdlan (12–16% reducing sugars released, as determined by the PABA method), indicating that AfSun1p acted only on linear β -(1,3) glucans.

To further characterize the mode of action and the minimum size of the β -(1,3)-glucan required for r-AfSun1p activity, p-NPG as well as soluble laminarioligosaccharides of varying DPs were used as the substrates. There was no hydrolytic activity by r-AfSun1p when p-NPG was used as substrate, confirming that AfSun1p is not a β -glucosidase. The products formed using laminarioligosaccharides as the substrates were analyzed on an anion-exchange chromatography column (Dionex). The smallest substrate for r-AfSun1p was found to be laminaribiose (DP2). Surprisingly, along with the release of glucose, there was also the formation of laminaritriose in minor quantity (as confirmed based on the retention time of a standard laminaritriose on the Dionex), suggesting an associated, but minor, transferase activity of r-AfSun1p in addition to its exo- β -(1,3)-glucanase activity (Fig. 4A). When laminarioligosaccharides of higher DP (DPs 6 and 12) were used as the substrates, r-AfSun1p also showed exo- β -(1,3)-glucanase and minor transferase activities (Fig. 4, B and C). With the course of time, there was an increase in the release of glucose by r-AfSun1p; however, the amount of substrate degraded never exceeded 10% of the initial concentrations. It was verified that this limited hydrolytic activity was not due to the presence of the product of

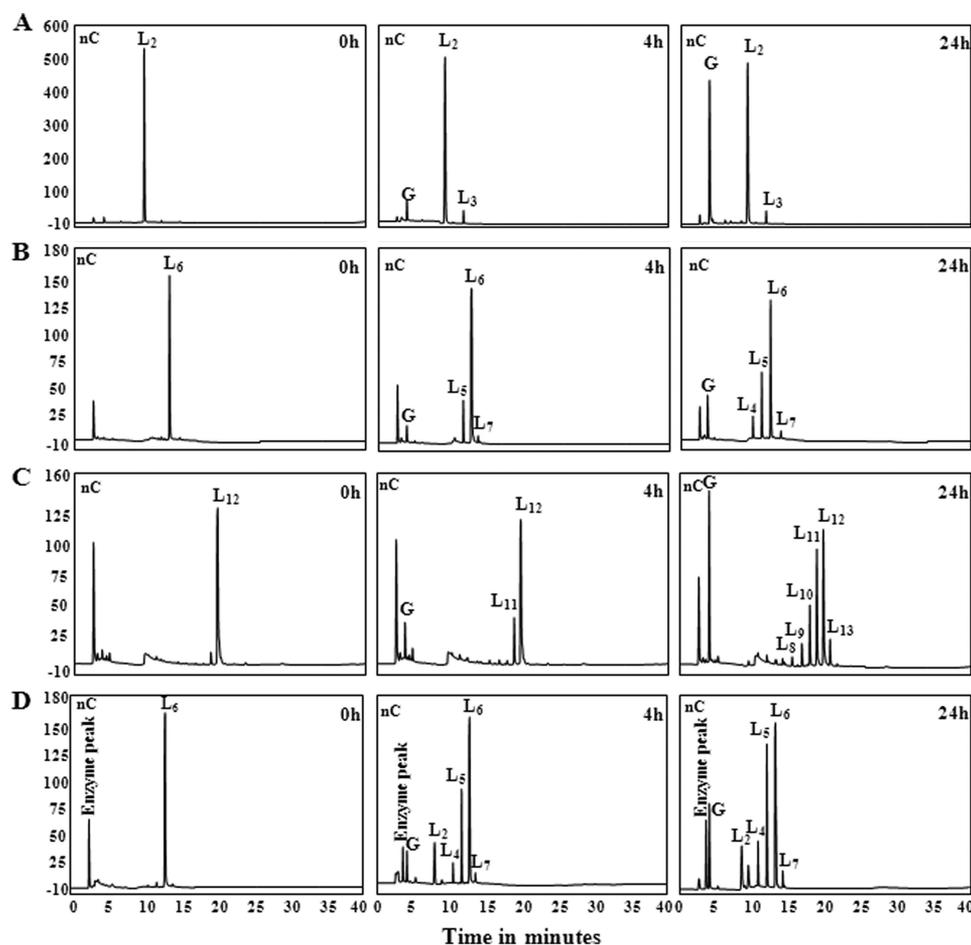


FIGURE 4. **Recombinant SUN proteins activity on the laminarioligosaccharides.** A–C, Dionex (anion-exchange chromatographic) profiles of the products formed by the action of the recombinant *AfSun1p* on the soluble β -(1,3)-oligosaccharides of DP2 (A), DP6 (B), and DP12 (C). D, recombinant *Sun41p* from *C. albicans* (*r-CaSun41p*) showed enzymatic activity similar to *AfSun1p*; here, the action of *r-CaSun41p* on β -(1,3)-oligomer of DP6 is presented. Reactions were performed in acetate buffer (50 mM, pH 5.5), at 37 °C; recombinant enzymes (1 μ g) were incubated with 20 μ M of individual laminarioligosaccharides for 4 and 24 h. nC, nanocoulombs.

hydrolysis because the addition of external glucose had no effect on the observed activity of *r-AfSun1p* (data not shown).

P. pastoris was used to produce recombinant *AfSun1p*; hence, the possibility of this expression system contaminating the observed *AfSun1p* glucanase activity was checked. Firstly, histidine-tagged *r-AfSun1p* was affinity-purified, which showed a single band on the SDS-PAGE when revealed by silver staining, and secondly, another histidine-tagged recombinant protein, *r-AfAspF1* (a ribonuclease; AFUA_5G02330), when affinity-purified following the same protocol, did not hydrolyze laminarioligosaccharide. Moreover, the endogenous $\text{exo-}\beta$ -(1,3)-glucanase produced by *P. pastoris* is reported to hydrolyze chromogenic substrate *p*-NPG (32), whereas *r-AfSun1p* did not act on *p*-NPG. All these confirmed that the observed $\text{exo-}\beta$ -(1,3)-glucanase and minor transferase activities were exclusively associated with *AfSun1p*.

Substrate binding assays also showed a weak but specific binding toward β -(1,3)-glucan. Upon pull-down assay, ~20% of *r-AfSun1p* was bound to the cell wall β -(1,3)-glucan (Fig. 5). In accordance with the Dionex data, the surface plasmon resonance assay displayed a low binding of *r-AfSun1p* to laminaribiose, and this binding affinity increased with an increase in the size of the β -(1,3)-oligomers (DPs 6 and 12). However, a

decrease in the binding affinity with the β -(1,3)-oligomer DP23 when compared with that of DP12 could be due to decrease in the solubility of DP23 when compared with DP12. *r-AfSun1p* did not show binding affinity toward β -(1,6)-linked oligosaccharide of DP10, confirming the specific binding of *r-AfSun1p* with the β -(1,3)-linked substrates (Fig. 5B).

Similar to *r-AfSun1p*, recombinant *CaSun41p* showed activity in the pH range 5.0–6.0 with an optimum at 5.5 and between 35 and 40 °C (here the alkali-insoluble fraction obtained from *C. albicans* cell walls was used as the substrate). The specific activity was found to be 4.4 nmol of reducing sugar equivalent/ $\text{min}^{-1} \mu\text{g}^{-1}$ proteins. No hydrolysis of *p*-NPG could be observed, and the activities toward laminarioligosaccharides were similar to those observed with *r-AfSun1p* (Fig. 4D). These results suggested that SUN proteins from both *A. fumigatus* and *C. albicans* displayed similar enzymatic activity.

DISCUSSION

In this study, for the first time, we report the role of *SUN* family genes in a filamentous ascomycete, *A. fumigatus*. Although two genes belonging to the *SUN* family were found in *A. fumigatus*, only *AfSUN1* had a morphogenetic role, at least in the growth conditions tested. Deletion of *AfSUN1* resulted in a

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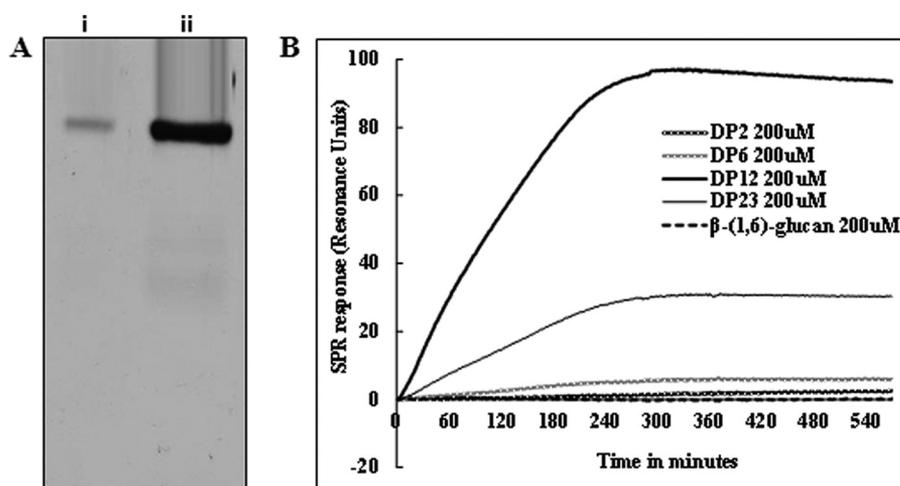


FIGURE 5. **Substrate binding assay.** *A*, pull-down assay wherein ~80% of the *r-AfSun1p* was found in the supernatant (*lane ii*) and only ~20% was bound to the insoluble β -(1,3)-glucan (*lane i*) upon incubation of *r-AfSun1p* with β -(1,3)-glucan at 37 °C for 1 h. *B*, surface plasmon resonance assay, which further confirmed the binding of *r-AfSun1p* to the β -(1,3)-oligomers of different DPs; here a β -(1,6)-linked oligomer were used as the control.

variety of cell wall phenotypes, suggesting a role of *AfSun1p* in cell wall polarization and integrity. In particular, the appearance of intrahyphal hyphae suggested a role of *AfSun1p* during cell wall septation. This is further supported by the occurrence of many Woronin bodies in the septal region of the $\Delta AfSun1$ hyphae, an indication of difficulties in completion/closure of the septum (33). Similar phenotypes, especially intrahyphal hyphae, were observed in the *csm*-chitin synthase mutants of *A. fumigatus* (34, 35). It was suggested that these chitin synthases could function as repair enzymes in septated compartments of the old hyphae. A role of SUN proteins in septation is also in agreement with the data obtained in other fungal species. Deletion of the *S. pombe* group I *SUN* gene, *psu1*, is lethal and associated with cell wall defects during cell separation (7). Similarly, simultaneous inactivation of the *C. albicans* *SUN41* and *SUN42* is synthetically lethal, leading to lysis of the mother cells after septation (8). A putative role in septum formation has also been proposed for *ScSUN4* and *ScUTH1* of *S. cerevisiae* (3). In *Williopsis saturnus* var. *mrakii*, the SUN family protein *Wmsu1p* has been reported to be involved in the cell wall structure (36, 37).

However, differences do exist among these fungal species in the phenotypes observed for the group I SUN protein mutants. *AfSun1p* is not essential for growth in contrast to *SpPsu1p* and the *CaSun41p/CaSun42p* pair. In *A. fumigatus* (present study) and in *S. pombe* (7), the defective phenotypes could not be rescued by the osmo-protectants (data not shown) as observed in *C. albicans* (8).

Moreover, functions of the group II and group I SUN proteins are obviously different. In *A. fumigatus*, we could not identify conditions upon which the group II *AfSUN2* gene is expressed. Consistently, deletion of *AfSUN2* did not result in any phenotypic difference relative to the parental strain. Several group II SUN proteins have been identified in yeasts: *S. cerevisiae* (*Ymr244w*), *S. pombe* (*Adg3p*), and *C. albicans* (*Orf19.5896*) (8). However, only *Adg3p* of *S. pombe* has been studied, and the corresponding mutant strain showed a slight delay in cell separation (38).

In databases, SUN proteins have been annotated as β -glucanase on the basis of sequence homologies with *BglAp* of *Candida wickerhamii* (39). However, these SUN proteins did not show similarity with already described glycoside hydrolases (GH) in the CAZyme database (40), and hence, they will be assigned to a new GH family (GH132). The *CwBGLA* and *CwBGLB* genes were identified during the search for proteins reacting with a polyclonal antibody raised against a purified extracellular β -glucosidase. However, the β -glucosidase activity has been demonstrated only for *BglBp*, which does not carry a SUN domain (39). Thus, until now, SUN proteins could not be considered as β -glucosidases as the biochemical evidence for such an activity was lacking. Here, we have characterized, for the first time, an enzymatic activity that is similar in SUN proteins from two different fungal species, *A. fumigatus* and *C. albicans*. *AfSun1p* and *CaSun41p* bind to and act on linear β -(1,3)-glucans in a specific manner. *AfSun1p* showed a hydrolytic activity only against β -(1,3)-glucan. Branching on the β -(1,3)-glucan negatively affected such hydrolytic activity. Moreover, earlier, Zverlov *et al.* (41) showed a laminariase activity associated with *Thermotoga neapolitana* *BglBp*; however, it also showed hydrolytic activity on the *p*-NPG, whereas *r-AfSun1p* acted on laminaribiose (Fig. 4A) and not on *p*-NPG, confirming a distinct role associated with this protein. The mode of action of the SUN proteins is unique. It is clear from the biochemical data obtained that our understanding of the enzymatic function of this protein is far from being complete. For example, in contrast to *AfSun1p*, true exo-glucanases of *A. fumigatus* degraded 100% of the substrate after 1 h of incubation (42). Similarly, when pull-down assay was performed with β -(1,3)-glucan binding receptors such as *GNBP3* (43), 100% of the receptor is pulled down by the β -(1,3)-glucan, whereas with *AfSun1p*, it is only ~20%. Several reasons could be put forward to explain these discrepancies: (i) the recombinant proteins used are highly glycosylated, and this moiety could impact on the enzymatic function due to steric hindrance problems; and (ii) the activity has been tested only *in vitro*, and the lack of another cofactor may impede the identification of

the true biochemical function of the protein. For example, in *S. cerevisiae* and *S. pombe*, the machinery constructing the septum is composed of several proteins acting together (44, 45). We have verified that the addition of external chitohexaose or *N*-acetylglucosamine did not result in any additional product formation, confirming that r-*AjSun1p* has no glucan-chitin cross-linking activity. Moreover, except for a single transferred product, there was no major transferase activity associated with r-*AjSun1p* during the course of time, ruling out its β -(1,3)-glucan elongase activity (Fig. 4).

Although the biological function of these proteins *in vivo* is not fully understood, this study showed that these proteins bind and act on β -(1,3)-glucans and are probably involved in the septum closure. Consistent with this hypothesis, inactivation of genes involved in degradation of the primary septum (*CaACE2* or *CaCHT3*) in *C. albicans* or physiological conditions preventing the activity of chitinases that act at the primary septum rescue the lethality associated with simultaneous inactivation of *CaSUN41* and *CaSUN42* (data not shown). This may suggest that in the cell wall, group I SUN proteins act to provide building blocks to other enzymes that are necessary for cell wall biogenesis and/or counteracting the activity of cell wall-degrading enzymes. This also highlights the need to develop approaches to study cell wall biosynthetic enzymes *in situ* because global chemical approaches are not able to pinpoint structural changes occurring at very specific locations such as the septum.

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SUN Proteins Belong to a Novel Family of β -(1,3)-Glucan-modifying Enzymes Involved in Fungal Morphogenesis

Amandine Gastebois, Vishukumar Aimanianda, Sophie Bachellier-Bassi, Audrey Nesseir, Arnaud Firon, Anne Beauvais, Christine Schmitt, Patrick England, Rémi Beau, Marie-Christine Prévost, Christophe d'Enfert, Jean-Paul Latgé and Isabelle Mouyna

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