Applied and Environmental Microbiology, Apr. 2007, p. 2128–2134 Vol. 73, No. 7
0099-2240/07/$08.00+0 doi:10.1128/AEM.02497-06

The Antifungal Protein AFP from *Aspergillus giganteus* Inhibits Chitin Synthesis in Sensitive Fungi[▽]

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Received 25 October 2006/Accepted 21 January 2007

The antifungal protein AFP from *Aspergillus giganteus* is highly effective in restricting the growth of major human- and plant-pathogenic filamentous fungi. However, a fundamental prerequisite for the use of AFP as an antifungal drug is a complete understanding of its mode of action. In this study, we performed several analyses focusing on the assumption that the chitin biosynthesis of sensitive fungi is targeted by AFP. Here we show that the N-terminal domain of AFP (amino acids 1 to 33) is sufficient for efficient binding of AFP to chitin but is not adequate for inhibition of the growth of sensitive fungi. AFP susceptibility tests and SYTOX Green uptake experiments with class III and class V chitin synthase mutants of *Fusarium oxysporum* and *Aspergillus oryzae* showed that deletions made the fungi less sensitive to AFP and its membrane permeabilization effect. In situ chitin synthase activity assays revealed that chitin synthesis is specifically inhibited by AFP in sensitive fungi, indicating that AFP causes cell wall stress and disturbs cell integrity. Further evidence that there was AFP-induced cell wall stress was obtained by using an *Aspergillus niger* reporter strain in which the cell wall integrity pathway was strongly induced by AFP.

Filamentous fungi represent an increasing threat for patients suffering from sustained immunosuppression. The medically important fungi include species of the genus *Aspergillus*, among which *Aspergillus fumigatus* is considered to be the most prominent aspergillosis-causing organism (6). Moreover, some filamentous fungi, such as *Fusarium oxysporum*, are considered major causes of plant diseases and crop loss (1, 13, 17). However, the antifungal drugs commonly used to treat fungal infections are limited and have considerable disadvantages; hence, novel and more efficient antifungal agents are badly needed (46). In the development of new antifungal strategies, antifungal compounds that target the cell wall which is unique to fungi are highly preferable. In addition, the ultimate aim is to obtain a drug that combines major desirable properties, such as sustainability, high efficacy, restricted toxicity, and low cost of production.

A naturally derived molecule that has all of the characteristics listed above is the antifungal protein AFP from *Aspergillus giganteus*. AFP is abundantly secreted by *A. giganteus* and exhibits high levels of antifungal activity against species of the genera *Aspergillus* and *Fusarium*, with minimal protein concentrations necessary for total inhibition ranging from 1 to 20 μM, but it does not affect the growth of yeasts or bacteria (41). In addition, AFP has been demonstrated to have neither cytotoxic nor immunogenic effects on different types of mammalian cells (38), which is indicative of its excellent potential for medical applications. Based on their degrees of susceptibility to AFP, filamentous fungi are classified into sensitive species (e.g., *A. fumigatus*, *Aspergillus niger*, *F. oxysporum*, and *Fusarium moniliforme*), moderately sensitive species (e.g., *Aspergillus nidulans*), and resistant species (e.g., *Penicillium chrysogenum*) (41).

AFP is a small protein that is composed of 51 amino acids (aa). Five antiparallel β strands define a β barrel topology which is stabilized by four disulfide bridges, providing remarkable resistance to heat and protease degradation (21). The positive net charge of AFP can be attributed to the presence of 12 lysine residues, which are partially involved in the formation of a protein domain (K9, K10, and K32) referred to as the cationic site. This site, in conjunction with a hydrophobic domain (Y29, V30, Y45, and V50), accounts for the amphipathic character of AFP (5). Electron microscopic analyses have demonstrated that AFP accumulates at distinct areas within the cell wall of the AFP-sensitive fungus *A. niger*. In addition, small amounts of AFP localized at the plasma membrane and severe membrane alterations have been observed (40). In accordance with this, it has been shown that AFP readily permeates the plasma membranes of sensitive fungi (41). However, no AFP targets localized within the cell wall or plasma membrane have been identified so far.

Fungal cell walls are dynamic structures that are very complex and are responsible for maintaining the shape and the integrity of the cell. Factors such as polarized growth, development, environmental stimuli, and stress cause fungi to change the composition and architecture of their cell walls (18, 32). Chitin, a polymer consisting of β-1,4-linked *N*-acetylglucosamine (GlcNAc) units, is one of the main structural components in fungal cell walls, and increased chitin biosynthesis is essential in the compensatory response to cell wall stress in yeasts and filamentous fungi (19, 22, 33, 43). Interestingly, the amount of chitin in yeasts and the amount of chitin in filamentous fungi are markedly different. While the chitin in *Saccharomyces cerevisiae* accounts for ~1 to 2% of the cell wall

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▽ Published ahead of print on 2 February 2007.

dry mass, the proportion in filamentous fungi ranges from 10 to 30% of the cell wall dry mass (14, 19).

The synthesis and deposition of chitin involve a complex network of biochemical and biophysical events, in which multiple membrane-bound glycosyltransferases (chitin synthases) play a central role. The initial steps comprise the trafficking of zymogenic chitin synthase clusters to the plasma membrane via microvesicles termed chitosomes (4, 36). Upon fusion with the plasma membrane, the inserted chitin synthase units are proteolytically activated. Newly synthesized chitin chains are subsequently translocated across the plasma membrane, where they finally coalesce to form microfibrils (8). Most fungi contain multiple chitin synthases, and the genes encoding these enzymes (*chs*) have been divided into six classes on the basis of sequence similarity (34). The different chitin synthases have distinct cellular functions during fungal growth which underlie the regulation of gene products that are ultimately involved in their temporal regulation and spatial localization (37). Filamentous fungi express chitin synthases that belong to classes I to VI, whereas yeasts contain only three chitin synthases, which are grouped into classes I, II, and IV (34). The observations that (i) most AFP localizes within the cell wall of *A. niger*, (ii) class III and V chitin synthases are found exclusively in filamentous fungi, (iii) yeasts (e.g., *S. cerevisiae*) are AFP resistant, and (iv) AFP can be purified by chitin affinity chromatography (24) prompted us to assume that chitin and/or chitin biosynthesis is targeted by AFP and that this interaction may be involved in determining the AFP susceptibility of sensitive fungi.

In this study, we showed that the N-terminal domain of AFP (aa 1 to 33) is responsible for efficient binding of AFP to chitin but is not sufficient for inhibition of the growth of sensitive fungi. In situ chitin synthase activity assays, AFP susceptibility studies, and SYTOX Green uptake experiments were performed with wild-type strains and class III and class V chitin synthase mutants, and the results demonstrated that chitin synthesis is specifically inhibited by AFP in sensitive fungi. Finally, we showed that AFP causes cell wall stress and triggers the cell wall integrity (CWI) pathway in the AFP-sensitive fungus *A. niger*.

MATERIALS AND METHODS

Strains and culture conditions. All strains used in this work are listed in Table 1. *A. giganteus* was cultivated in Olson medium (2% soluble starch, 1% beef extract, 2% peptone, 0.5% NaCl). *A. fumigatus*, *A. niger* IfGB 15/1801, *F. oxysporum*, and *Aspergillus oryzae* strains were grown in YPG medium (0.3% yeast extract, 1% peptone, 2% glucose; pH 4.5). *A. niger* strains N402 and RD6.47 (10) were grown in minimal medium (3) or complete medium consisting of minimal medium supplemented with 1% yeast extract and 0.5% Casamino Acids.

Microscopy. A total of 5×10^5 conidia of *A. niger* strains N402 and RD6.47 were inoculated into 5 ml of liquid minimal medium supplemented with 0.003% yeast extract and grown until small germ tubes were visible (5 h at 37°C) on coverslips, which were placed into petri dishes containing the liquid medium. After AFP (1, 5, 10, 20, 50, or 100 μg/ml), caspofungin (10 μg/ml), or the same volume of water (negative control) was added, the petri dishes were incubated for 2 h at 30°C. Germlings that adhered to the coverslips were observed using an Axioplan 2 (Zeiss) equipped with a DKC-5000 digital camera (Sony). Both light images (obtained using differential interference contrast settings) and fluorescence images (obtained using green fluorescent protein [GFP] settings) were captured with a ×40 objective. For GFP images, a fixed exposure time of 2 s was used.

Preparation of AFP and sAFP. *A. giganteus* was grown for 96 h at 28°C in Olson medium. After incubation at 37°C for an additional 20 h, the *A. giganteus* fermentation was stopped. Isolation and purification of AFP were carried out using the procedure described by Theis et al. (41). sAFP was a short version of AFP consisting of aa 1 to 33 and was synthesized by SynPep (Dublin, CA) as follows. One of the naturally occurring disulfide bridges, spanning from Cys^7 to Cys^{33}, was included with the aim of providing a means of protein stabilization. The remaining cysteine residues at positions 14, 26, and 28 were replaced by serine.

TABLE 1. Strains used in this study

Strain	Genotype	Reference
A. giganteus IFGB15/0903	Wild type	41
A. fumigatus IFGB15/0809	Wild type	41
A. niger IFGB15/1801	Wild type	41
A. niger N402	Wild type	10
A. niger RD6.47	P*agsA*::*h2b*::*egfp*::T*trpc*	10
F. oxysporum 4287	Wild type	25
F. oxysporum D1	*chsV*::*hph*	25
A. oryzae HowB101	Δ*pyrG*	30
A. oryzae CM100	*chsB*::*pyrG*	30
A. oryzae CM101	*csmA*::*pyrG*	30

Susceptibility assay. Fungi were cultivated in YPG medium (pH 4.5) consisting of 0.3% yeast extract, 1% peptone, and 2% glucose. One thousand conidia were added to 150 μl of culture medium containing AFP at concentrations ranging from 1 to 400 μg/ml. After 48 h of incubation at 28°C with continuous shaking, the minimal AFP concentration that prevented the growth of a test organism was determined. Growth was evaluated by determining the optical density at 600 nm. Experiments were carried out in triplicate. Before and after completion of the susceptibility assays, culture supernatants were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and analyzed to determine the presence of AFP and sAFP.

SYTOX Green uptake assay. SYTOX Green nucleic acid stain was obtained from Molecular Probes (Eugene, OR). The SYTOX Green uptake assay was carried out by using the method described by Theis et al. (41). In brief, 100 conidia were cultivated in 150 μl YPG medium for 20 to 40 h at 28°C. AFP and SYTOX Green were added to final concentrations of 100 μg/ml and 0.2 μM, respectively. Fluorescence was quantified immediately after addition of SYTOX Green and AFP. Measurements were obtained for 210 min using a CytoFluor 2350 fluorescence measurement system (Millipore) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. The fluorescence values were corrected by subtracting the fluorescence value for samples incubated in the absence of AFP. Triplicate experiments were performed.

Chitin-binding assay. Chitin from crab shells (practical grade) was obtained from Sigma (St. Louis, MO). Chitin was regenerated by the procedure described by Koo et al. (20). One hundred microliters of regenerated chitin was shown to bind a maximum of 10 μg AFP. Thus, 100-μl aliquots of regenerated chitin were incubated with 10 μg of the protein analyzed. The chitin matrix was treated successively with washing buffer (10 mM Tris-HCl, 1 mM EDTA; pH 7.0) and elution buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.0], 1.5 M NaCl). One hundred microliters of each solution was applied to the matrix. After centrifugation, the supernatants were removed thoroughly and subjected to SDS-PAGE. The protein contents of individual samples were determined using the Kodak 1D image analysis software (Image Station 440CF). Proteins that were identified in samples and were isolated before application of the washing buffer were designated "nonbound protein fractions." Proteins that were identified in samples after application of the elution buffer were designated "bound protein fractions." Triplicate experiments were performed.

Chitin synthase activity assay. A modified protocol described by Choi and Cabib (7) was used to prepare spheroplasts from germinated conidia of filamentous fungi. First, 1.4 ml of 0.1 M EDTA and 24 μl of 2-mercaptoethanol were added to 1 g of mycelium. The volume was adjusted to 3.5 ml using distilled water, and the sample was subsequently incubated at 30°C for 30 min with slight shaking. Subsequently, the cells were washed using 5 ml of 0.8 M sorbitol and resuspended in 6.7 ml of solution A (0.57 ml of citrate buffer [pH 6.3], 67 μl of 0.1 M EDTA, 0.8 M sorbitol); 10 to 40 mg/ml Glucanex was added to solution A. Incubation at 30°C for 30 min with gentle shaking was followed by centrifugation and resuspension of the cells in 30 ml of chilled 0.05 M Tris-HCl (pH 7.5). Additional incubation on ice for 5 min was followed by centrifugation and resuspension of spheroplasts in 1 ml of assay buffer (0.05 M Tris-HCl [pH 7.5], 33% glycerol). The chitin synthase assay was carried out by using the protocol described by Crotti et al. (9), with slight modifications. The reaction mixtures (total volume, 50 μl) contained 32 mM Tris-HCl (pH 7.5), 4.3 mM magnesium acetate, 1.1 mM

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1.    1     ------------ATYNGKCYKKDNICKYKAQSGK-TAICKCY-VKKC-     33
2.    655   AWQVNTAYTAGQLVTYNGKTYK----CLQPHTSLAGWEPSNVPALWQLQ     699
3.    772   AWSAGTVYNTNDKVSHNQLVWQ----AKYWTQGNEPSRTADQ---WKLV     813
4.    392   TWSSSTAYNGGATVAYNGHNYQ----AKWWTQGNVPSSSTGDGQPWADL     438
5.    435   AWNSTTTYVAGDRVTHQQKVYE----AKWWTQGEEPGA-SD---VWKAI     475
6.    35    EWSQSSAYNGGAQVQKSQQAFE----AKWWTQADPVTH-SGQ---WDDW     75
7.    39    EWQSDTIYTGGDQVQYNGSAYQ----ANYWTQNNDPEQFS-----YAVV     78
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FIG. 1. Alignment of amino acid sequences of AFP and bacterial chitin-binding proteins. 1, N-terminal domain of AFP; 2, chitinase A1 of *Bacillus circulans* strain WL-12; 3, chitinase A of *Aeromonas punctata*; 4, chitinase A1 of *Aeromonas* sp. strain 10S-24; 5, chitin-binding protein CbpI of *Alteromonas* sp. strain O-7; 6, chitinase-like enzyme ChiD of *Alteromonas* sp. strain O-7; 7, chitodextrinase EndoI of *Vibrio furnissii*. Residues that are identical are indicated by bold type.

uridine diphospho-*N*-acetyl-D-[U-^{14}C]glucosamine ([^{14}C]GlcNAc) (Amersham Biosciences, Buckinghamshire, United Kingdom), 2 μl of trypsin (5 mg/ml), and 20 μl of spheroplasts. The mixtures were incubated at 30°C for 90 min. Then 2 μl of a soybean trypsin inhibitor solution, in which the inhibitor concentration was 1.5 times the concentration of the trypsin, was added. After addition of 10% trichloroacetic acid and filtration, the insoluble chitin formed was assayed by determining the radioactivity using a liquid scintillation counter. The chitin synthase assay was performed in the presence and in the absence of 1 μg/ml AFP. Triplicate experiments were performed.

RESULTS

The N-terminal domain of AFP is responsible for chitin binding. Recently, Liu et al. (24) reported that AFP can be purified by chitin affinity chromatography. However, the identities of the amino acids or domains of AFP involved in chitin binding are not known. Therefore, we compared the amino sequence of AFP with the amino sequences of several chitin-binding proteins from plants, invertebrates, and fungi. However, no significant sequence homologies were found (data not shown). Instead, the N-terminal domain of AFP comprising residues 1 to 33 exhibited considerable homology with bacterial chitin-binding proteins (Fig. 1). Notably, remarkable sequence similarities were observed when we compared residues 14 to 19 of AFP with the AKWWTQ sequence, a motif that is well conserved in bacterial type 3 chitin-binding domains (28).

Judging from these in silico data, the N-terminal domain of AFP might indeed represent a putative chitin-binding domain. To prove this hypothesis, in vitro chitin binding assays were carried out using native AFP and sAFP. Ten micrograms of AFP or sAFP was incubated with the chitin matrix, and protein samples obtained before washing and after elution from the chitin matrix were subjected to SDS-PAGE. The amount of bound protein was determined using the Kodak 1D image analysis software (Fig. 2). Both AFP and sAFP exhibited very strong and similar binding activity with chitin (92 and 91%, respectively), suggesting that the N-terminal domain of AFP determines the chitin affinity of the protein, whereas the C-terminal part of AFP (aa 34 to 51) is dispensable for chitin binding.

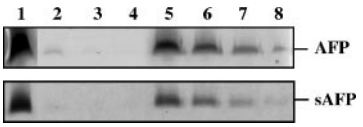

FIG. 2. Chitin-binding affinity of AFP and sAFP. Ten micrograms of protein was mixed with the chitin matrix. The proteins in the bound and eluted fractions were subjected to SDS-PAGE and quantified as described in Materials and Methods. Lane 1, 10 μg protein; lane 2, amount of protein that did not bind to the matrix; lanes 3 and 4, amount of protein that eluted after washing buffer was applied two times; lanes 5 to 8, amount of protein that eluted after elution buffer was applied four times.

sAFP is less bioactive. With the aim of determining the MIC of sAFP, the growth of selected AFP-sensitive strains was evaluated using susceptibility assays. Compared with the MICs of AFP, the MICs of sAFP were dramatically higher (Table 2). Neither sAFP nor native AFP was degraded during cultivation of the test strains (data not shown), eliminating the possibility that the lower bioactivity of sAFP was due to protein degradation. The potency of sAFP relative to the potency of AFP was also assessed using a SYTOX Green uptake assay. SYTOX Green is a fluorogenic dye which can penetrate only compromised membranes and fluoresces upon binding to nucleic acids (42). This assay has been used previously to demonstrate that AFP-induced plasma membrane permeabilization occurs, and it revealed that the degree of AFP activity can be correlated with the extent of fluorescence (41). Figure 3 shows the results for AFP-sensitive fungi, which were incubated in the presence of AFP or sAFP. As demonstrated by the results for *F. oxysporum*, clear membrane permeabilization was observed with AFP, whereas no membrane-permeabilizing effect was observed with sAFP. Congruently, the plasma membrane of *A. niger* was compromised by AFP but not by sAFP (data not shown). These results demonstrate that sAFP is basically inactive and indicate that just binding of AFP to chitin is not sufficient for antifungal activity.

Chitin synthase mutants of *F. oxysporum* and *A. oryzae* are less susceptible to AFP. Next, we studied whether the presence of class III and class V chitin synthases in filamentous fungi is involved in the susceptibility of sensitive fungi to AFP. For this purpose, the AFP-sensitive fungi *F. oxysporum* and *A. oryzae* (MIC of AFP, 1 μg/ml) where chosen. We analyzed mutants of these species which had deletions in genes coding for class III chitin synthases (*chsB* mutant of *A. oryzae*) and class V chitin synthases (*csmA* mutant of *A. oryzae* and *chsV* mutant of *F. oxysporum*). As shown in Fig. 4, the growth of the *chs* mutants in the presence of 50 and 100 μg/ml AFP was affected considerably less than the growth of the corresponding wild-type strains was affected. Even at an AFP concentration of 400 μg/ml, residual growth was observed for the *chs* mutants (data not shown), indicating that the mutations made the fungi con-

TABLE 2. MICs of AFP and sAFP for selected strains[a]

Protein	MIC (μM)		
	F. oxysporum	*A. niger*	*A. fumigatus*
AFP	1.5	1.5	15
sAFP	130	>1,000	>1,000

[a] All of the fungi analyzed were wild-type strains.

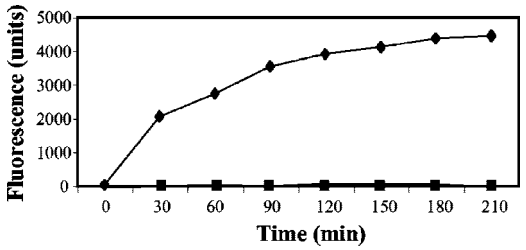

FIG. 3. SYTOX Green uptake by *F. oxysporum* in the presence of AFP or sAFP. Strains were incubated in the presence of 0.2 μM SYTOX Green and 100 μg/ml AFP for 120 min. Fluorescence values were corrected with the baseline fluorescence, which was determined by incubating the fungi in the absence of AFP. ◆, incubation in the presence of AFP; ■, incubation in the presence of sAFP. The results of a representative experiment are shown.

siderably less sensitive to AFP. In order to exclude the possibility that the results obtained were due to the morphological abnormalities observed particularly in the *chsV* mutant under low-osmotic conditions (25), the experiments were repeated using growth medium supplemented with 1 M sorbitol. In this analysis, the *chs* mutant strains exhibited the same reduced sensitivity to AFP, and no significant differences were observed when the results were compared to the results obtained using nonstabilized medium (data not shown).

To confirm the results obtained in the susceptibility assays, the membrane-permeabilizing effect of AFP on *chs* mutants and wild-type strains of *F. oxysporum* and *A. oryzae* was analyzed by performing SYTOX Green uptake assays. As expected, the plasma membranes of the wild-type strains of *F. oxysporum* and *A. oryzae* were permeabilized by AFP equally well, whereas the plasma membranes of the *chsV*, *csmA*, and *chsB* mutants were not compromised by AFP (Fig. 5).

Chitin synthesis is inhibited by AFP in sensitive fungi. Interestingly, all *chs* mutants described above exhibited reduced susceptibility to AFP; however, only the *chsV* mutant of *F. oxysporum* had a reduced amount of chitin compared to the wild-type strain (25). Therefore, we assumed that the chitin content of the cell wall alone does not determine AFP susceptibility. To analyze whether AFP specifically targets chitin synthesis, chitin synthase activities were measured in

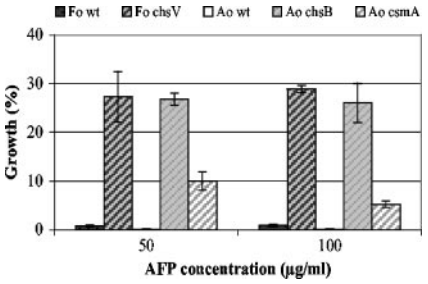

FIG. 4. Analysis of growth inhibition of chitin synthase mutants of *F. oxysporum* (Fo) and *A. oryzae* (Ao). Susceptibility assays were carried out with wild-type (wt) and *chsV* mutant *F. oxysporum* strains, as well as with *A. oryzae* wild-type, *chsB* mutant, and *csmA* mutant strains. Fungi were cultivated in the presence of two AFP concentrations. Growth is expressed as percentages compared with the negative control, which consisted of the fungi cultivated in the absence of AFP. The errors bars indicate standard deviations for triplicate experiments.

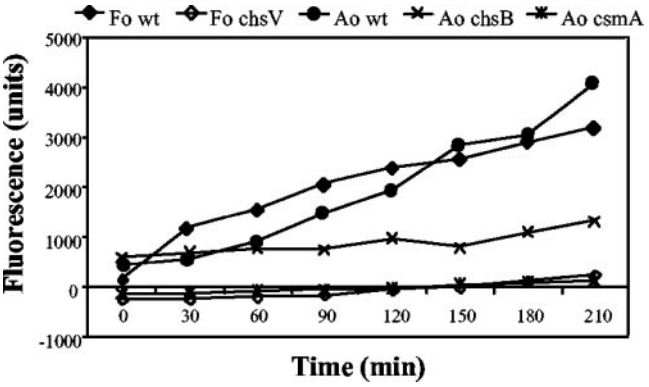

FIG. 5. SYTOX Green uptake assay analyzing membrane permeabilization in *chs* mutants of *F. oxysporum* (Fo) and *A. oryzae* (Ao). SYTOX Green uptake was measured as described in the legend to Fig. 2. The strains used were *F. oxysporum* wild-type and *chsV* mutant strains and *A. oryzae* wild-type, *chsB* mutant, and *csmA* mutant strains. The results of a representative experiment are shown.

situ. Spheroplasts from young, germinated conidia of selected strains were incubated with the chitin precursor [^{14}C]GlcNAc in the absence or presence of 1 μg/ml AFP. If chitin synthesis is inhibited by AFP, the inhibition should have been reflected in reduced enzyme activities. As shown in Table 3, the chitin synthase activities in the AFP-sensitive strains of *F. oxysporum*, *A. oryzae*, and *A. niger* were significantly reduced in the presence of AFP, suggesting that chitin synthesis in AFP-sensitive fungi is generally inhibited by AFP. Interestingly, the moderately sensitive *chsV* mutant of *F. oxysporum* exhibited elevated levels of chitin synthase activity when it was treated with AFP, indicating that it is possible that chitin synthases belonging to other classes are strongly induced to compensate for the lack of *chsV*.

AFP induces the CWI pathway in *A. niger*. It can be assumed that by interfering with the synthesis of chitin, AFP causes cell wall stress and disturbs the integrity of cells of sensitive fungi. In this context, it has recently been shown that the antifungal drugs calcofluor white (an inhibitor of chitin assembly [35]) and caspofungin (an inhibitor of β-1,3-glucan synthesis [16]) specifically trigger the CWI pathway in *A. niger* (11, 12). In response to these cell wall-acting drugs, the transcription factor RlmA becomes activated and induces expression of α-1,3-glucan synthase (*agsA*) so that the organism can withstand cell wall-threatening conditions. To analyze activation of the CWI

TABLE 3. Relative chitin synthase activities in selected fungal strains[a]

Strain	Chitin synthase activity		MIC (μg/ml)
	Without AFP	With AFP	
A. niger wild type	5.71 (0.01)	1.61 (0.01)	1
F. oxysporum wild type	3.61 (0.01)	2.25 (0.02)	1
A. oryzae wild type	5.24 (0.04)	3.15 (0.02)	1
F. oxysporum chsV mutant	2.72 (0.01)	4.28 (0.01)	>400

[a] Spheroplasts of *A. niger*, *A. oryzae*, and *F. oxysporum* wild-type strains and an *F. oxysporum chsV* mutant were incubated with [^{14}C]GlcNAc in the absence or presence of 1 μg/ml AFP. Chitin synthase activities are expressed in picomoles of [^{14}C]GlcNAc incorporated per minute per unit of optical density of spheroplasts. The values in parentheses are standard deviations. The MICs of AFP are included for reference.

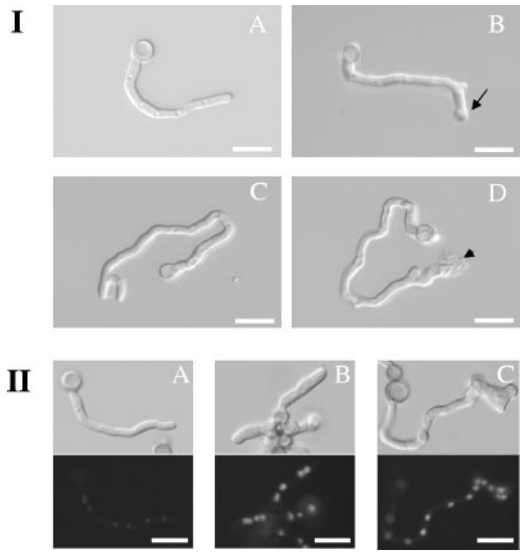

FIG. 6. Microscopic analysis of *A. niger* strains treated with AFP. The strains were pregrown and subsequently stressed with AFP or caspofungin, as described in Materials and Methods. (I) Differential interference contrast images of nontreated germlings of N402 (A) and germlings of N402 treated with 1 μg/ml AFP (B to D). A swollen hyphal tip is indicated by an arrow, and a burst hyphal tip and outflow cell material are indicated by an arrowhead. (II) Differential interference contrast and corresponding fluorescence images of RD6.47 germlings not treated with either AFP or caspofungin (A), RD6.47 germlings treated with 10 μg/ml caspofungin (B), and germlings of RD6.47 treated with 10 μg/ml AFP (C). Bars, 10 μm.

pathway in *A. niger*, the reporter strain RD6.47 was constructed, in which the *agsA* promoter is fused to a nucleus-targeted GFP (H2B::eGFP). Consequently, the effects of compounds that act on cell wall integrity can be monitored based on the increase in nuclear fluorescence (10). To investigate whether AFP causes cell wall stress and induces the CWI pathway, spores of RD6.47 and of the corresponding wild-type strain, strain N402, were grown on coverslips until small germlings were visible. Subsequently, the germlings were stressed for 2 h with AFP concentrations ranging from 1 to 100 μg/ml and analyzed microscopically. As shown in Fig. 6I, treatment of N402 with 1 μg/ml AFP resulted in an arrest of polarized growth, caused swelling of hyphal tips (panel B), and provoked apical and subapical branching (panels B and C), indicating that AFP causes cell wall stress predominately at the hyphal apex and inhibits maintenance of polarity. Moreover, AFP-treated germlings tended to burst exclusively at the swollen tips, resulting in leakage of the hyphae (Fig. 6I, panel D) and cell death. The same morphological alterations were observed for strain RD6.47 treated with 1 μg/ml AFP (data not shown) and became much more pronounced when both strains were stressed with AFP concentrations up to 100 μg/ml (data not shown). Like the nuclear fluorescence in the caspofungin control, nuclear fluorescence was found to be strongly induced in germlings of RD6.47 treated with 10 μg/ml AFP, demonstrating that AFP triggers a remodeling of the cell wall (Fig. 6II, panel C). Remarkably, nuclei were distributed equally throughout the hyphae of AFP-treated cells but accumulated in the spherical apex. Therefore, it can be assumed that AFP has little if any effect on nuclear division but that the linear correlation between the nuclear concentration and hyphal length, as reported previously for *A. nidulans* (15), cannot be maintained due to the provoked arrest in polar growth.

DISCUSSION

With the aim of obtaining detailed insight into the mode of action of AFP, we conducted several analyses focusing on the assumption that chitin and/or chitin biosynthesis might be targeted by AFP in sensitive fungi. In vitro chitin-binding assays showed that AFP attaches to chitin very efficiently, confirming previously published data for the isolation of AFP by chitin affinity chromatography (24). Binding studies using sAFP suggested that the N-terminal part of AFP (aa 1 to 33) is sufficient for efficient binding of AFP to chitin. We caution, however, that the C14S, C26S, and C28S substitutions introduced into sAFP could have a positive effect on the chitin-binding affinity of the truncated protein. Further studies involving more detailed analysis of the binding properties of AFP with chitin, as well as site-directed mutagenesis, should provide more insight into this interaction.

Remarkably, the AFP sequence CKYKAQ (aa 14 to 19) exhibits conspicuous similarity with the conserved AKWWTQ motif found in bacterial type 3 chitin-binding domains. As residues 14 to 19 are arranged in a linear stretch (5), they might be accessible for promotion of interactions with chitin. It is conceivable that this kind of assembly facilitates binding to chitin oligomers or microfibrils rather than to single GlcNAc units. Relevant support for this speculation may be found in the AVR4 elicitor from *Cladosporium fulvum*, which is also proposed to contain a linearly arranged chitin-binding site. It has been demonstrated that in AVR4 this site interacts exclusively with $(GlcNAc)_3$ repeats. Hevein, a chitin-binding protein from *Hevea brasiliensis*, in contrast, contains a small binding pocket, which is assumed to provide enough contact to sustain interactions with GlcNAc units alone (44).

An alternative attractive explanation for the affinity of binding of AFP to chitin is its three-dimensional structure, which resembles an oligonucleotide/oligosaccharide-binding (OB) fold (26). The OB fold is a closed five-strand β-barrel that presents a face for binding to different ligands, such as RNA, single-stranded DNA, oligosaccharides, and proteins (2, 31). For example, binding to specific oligosaccharides, either oligosaccharide moieties of membrane-localized gangliosides or glycoproteins, has been demonstrated for the AB_5 class of bacterial toxins (for a review, see reference 27). The binding face of the OB fold consists of β strands 2 and 3, as well as loops 1, 2 (or 3), and 4 (2). Based on this folding pattern, one would expect that the truncated sAFP version (lacking β strands 4 and 5) should still be able to bind to chitin, given that the topology of sAFP resembles that of the native AFP. The finding that sAFP and AFP bind to chitin to the same extent opens the possibility that the OB topology of AFP might mediate binding of AFP to chitin. It should be stressed, however, that the OB fold structure of AFP does not rule out a possible function of the CKYKAQ motif in chitin binding, as this stretch encompasses part of β strand 2 and loop 2 and is also present in sAFP.

The fact that sAFP does not permeabilize plasma membranes of AFP-sensitive fungi and is remarkably less potent or even not potent for inhibiting the growth of these organisms suggests that binding of AFP to chitin alone does not necessarily result in perturbation of cell integrity. However, it is conceivable that via the interaction with chitin AFP is kept in close proximity (or may even be targeted) to cellular structures that could harbor additional AFP targets. One process that could be affected by AFP is chitin biosynthesis. Yeasts do not possess *chs* genes encoding class III, V, and VI enzymes, and it is thought that enzymes belonging to these classes play specific roles in the highly polarized tip growth of filamentous fungi (reference 39 and references therein). Our intention was to find out whether the lack of these classes of chitin synthases simultaneously results in reduced susceptibility to AFP. Therefore, a class V mutant of *F. oxysporum* (*chsV*) and class V and class III mutants of *A. oryzae* (*csmA* and *chsB*, respectively) were analyzed. AFP susceptibility assays clearly showed that the *chs* mutants were less sensitive to AFP than the corresponding wild-type strains and that the mutants were not permeabilized by the APF concentration tested. The reduced AFP sensitivity of the *chs* mutants supports the hypothesis that class III and V chitin synthases are targets of AFP. This result, however, implies that AFP applied to wild-type hyphae should provoke a phenotype similar to that observed for *chs* mutant strains. Indeed, as shown for *A. niger* (Fig. 6), AFP disturbs the normal pattern of polarized growth, a phenotype that has also been reported for the *F. oxysporum chsV* mutant (25) and for the *chsB* and *csmA* mutants of *A. oryzae* (30). However, an alternative explanation for the reduced susceptibility of the *chs* mutants tested is that their cell walls are significantly remodeled to complement the defect and that other putative AFP targets may not be accessible in the mutants. In this respect it is interesting that only small amounts of AFP can be found within the cell wall and plasma membrane of the AFP-resistant fungus *P. chrysogenum*, whereas large amounts of AFP accumulate within the cell wall and plasma membrane of the sensitive fungus *A. niger* (40). This observation could indicate the presence (or absence) of specific binding structures that determine the efficiency of AFP binding.

Further evidence that AFP targets chitin synthesis was obtained from in situ measurements of chitin synthase activities. We observed that AFP-sensitive fungi, such as wild-type strains of *A. niger*, *F. oxysporum*, and *A. oryzae*, all exhibit reduced chitin synthase activities in the presence of AFP. As the synthesis of chitin involves a cascade of events, there are a number of possible explanations for the decreased enzyme activities in AFP-sensitive fungi: AFP may (i) disturb the transport of chitosomes toward the hyphal apex, (ii) restrict the release of chitin synthases from chitosomes, (iii) prevent the proper localization and anchoring of chitin synthases to the plasma membrane, (iv) inhibit chitin synthase activity itself (e.g., via binding to chitin precursor molecules or via binding to polymerized chitin), and/or (v) disturb the incorporation of chitin microfibrils into the cell wall and linkage to cell wall-localized β-glucans. As AFP causes severe distortions of the plasma membrane of sensitive fungi (29, 40) and targets processes required for maintenance of a stable polarity axis (this study), we propose that AFP potentially interferes with the polarized trafficking of chitosomes and class III and V chitin synthases and/or with the insertion of the class III and V chitin synthases into the plasma membrane. It is also imaginable that a membrane-localized interaction between chitin synthases and AFP causes stretching of the plasma membrane and results in a loss of membrane integrity.

AFP induces cell wall stress by disturbing plasma membrane integrity and inhibiting chitin synthesis in sensitive fungi. In addition, AFP was shown to induce the CWI pathway in *A. niger*. It is imaginable that the CWI pathway is also induced in moderately sensitive or resistant fungi, as increased chitin synthase activity was observed for the *F. oxysporum chsV* mutant strain when it was treated with AFP. It can be assumed that chitin synthases belonging to other classes were much more strongly induced to compensate for the inhibition of class III and class V chitin synthases. A similar response has been shown for *S. cerevisiae*, in which chitin synthase activities increased considerably in the presence of calcofluor white (35).

Our data demonstrate that AFP does not affect nuclear division in *A. niger*, even at a concentration that is 100-fold higher than the MIC (Fig. 6 and data not shown). This result is in agreement with the results of our previously published transmission electron microscopy study, in which we did not observe nuclear localization of AFP when *A. niger* was incubated with the MIC of AFP. Only when a 300-fold-higher concentration of AFP was used was AFP localized intracellularly in collapsed, dead cells (40). In this regard, it has been reported that AFP interacts with DNA and RNA under in vitro conditions, and when it was used at an 80-fold-higher concentration that caused damage of the plasma membrane, AFP was localized in the hyphae of *Magnaporthe grisea* (26, 29). The OB fold structure of AFP explains its interaction with nucleic acids; however, it is highly questionable whether this interaction can explain the highly species-specific antifungal activity of AFP. Like AFP, sAFP is able to bind to DNA and RNA under in vitro conditions (unpublished data), emphasizing the conclusion that the affinity of AFP for nucleic acids cannot explain its specific antifungal effect. We therefore suggest that the species-specific determinants of AFP susceptibility reside in the cell wall and plasma membrane of filamentous fungi. However, once the membrane has been permeabilized and the cell integrity has been disturbed by AFP, the protein might enter the cell and, supported by its OB topology and basic character, might bind to negatively charged molecules, such as nucleic acids.

In summary, we concluded that the N-terminal domain of AFP determines the affinity of binding of AFP to chitin, which, supported by the OB topology of the protein, may facilitate efficient binding to chitin in filamentous fungi. This binding to chitin may then target the protein to cellular structures within the cell wall and the plasma membrane that could harbor additional AFP targets. Inhibition of chitin synthesis at the hyphal apex, probably via inhibition of class III and V chitin synthases, interrupts maintenance of polar growth, causes cell wall stress, and induces the CWI pathway as a compensatory response. As class V chitin synthases play crucial roles in pathogenicity in *F. oxysporum* (25), *Wangiella dermatididis* (23), and *Ustilago maydis* (45), AFP can be considered a promising candidate for future antifungal therapies. In addition, further studies addressing the mode of action of AFP probably will

provide new insight into the regulation of polar growth of filamentous fungi.

ACKNOWLEDGMENTS

This work was partially supported by a grant from the Bundesministerium für Wirtschaft und Arbeit, Arbeitsgemeinschaft industrieller Forschungsvereinigungen "Otto von Guericke" (grant 13305N).

We are grateful to Barbara Walewska for excellent technical assistance. We thank Isabel Roncero and Jens Nielsen for kindly providing the *F. oxysporum* and *A. oryzae* strains, respectively.

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