ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 2003, p. 588–593 Vol. 47, No. 2
0066-4804/03/$08.00+0 DOI: 10.1128/AAC.47.2.588–593.2003

The Antifungal Protein from *Aspergillus giganteus* Causes Membrane Permeabilization

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Received 8 July 2002/Returned for modification 6 September 2002/Accepted 29 October 2002

We investigated the inhibitory effects of the antifungal protein (AFP) from *Aspergillus giganteus* on the growth of several filamentous fungi. For this purpose, the MICs of AFP were determined and ranged from 0.1 μg/ml for *Fusarium oxysporum* to 200 μg/ml for *Aspergillus nidulans*. The antifungal activity of AFP was diminished in the presence of cations. We were able to show that incubation of AFP-sensitive fungi with the protein resulted in membrane permeabilization using an assay based on the uptake of the fluorescent dye SYTOX Green. No permeabilization by AFP could be detected at concentrations below the species-specific MIC. Furthermore, AFP-induced permeabilization could readily be detected after 5 min of incubation. Localization experiments with fluorescein isothiocyanate-labeled AFP and immunofluorescence staining with an AFP-specific antibody supported the observation that the protein interacts with membranes. After treatment of AFP-sensitive fungi with AFP, the protein was localized at the plasma membrane, whereas it was mainly detected inside the cells of AFP-resistant fungi. We conclude from these data that the growth-inhibitory effect of AFP is caused by permeabilization of the fungal membranes.

The imperfect filamentous fungus *Aspergillus giganteus* was found to secrete two small basic proteins, namely, the antifungal protein (AFP) and α-sarcin (22). α-Sarcin is a cytotoxic protein belonging to the ribotoxins, a family of ribosome-inactivating proteins (2, 6, 7, 13, 17, 24, 30). AFP is a highly basic polypeptide of 51 amino acids with a high content of cysteine, tyrosine, and lysine residues. The isoelectric point was estimated to be 8.8; thus, the protein is positively charged under neutral conditions. AFP is folded into five highly twisted antiparallel strands, defining a small and compact structure with four stabilizing disulfide bridges (3, 20, 29).

Only three small, basic antifungal proteins have been isolated from filamentous fungi to date: AFP from *A. giganteus* (22), PAF from *Penicillium chrysogenum* (18), and Anafp from *Aspergillus niger* (14). Recently, the *naf* gene was isolated from *Penicillium nalgiovense* and is identical to the *paf* gene from *P. chrysogenum* and encodes a protein with transient antifungal activity (8). All these antifungal proteins are secreted and inhibit the growth of numerous filamentous fungi without affecting bacteria and yeast. Most notably, they are significantly similar in regard to their structures, sizes, and basic characters. The PAF protein exhibits 42.6% sequence similarity with the AFP sequence (18).

Not much is known about the modes of action of the antifungal proteins secreted by fungi. It has, however, been shown that defensins, a class of antimicrobial proteins which are quite similar to antifungal proteins in terms of their structures, sizes, and disulfide bridges, interact with anionic phospholipids of bacterial membranes (15). In addition, it has been found that some plant defensins have a receptor-mediated interaction with the fungal plasma membrane (27). However, nothing is known about the mode of action of AFP. The protein was shown to be able to produce an aggregation of large unilamellar vesicles of the acidic phospholipid dimyristoylphosphatidylserine in vitro. No effect on vesicles composed of zwitterionic phospholipids has been detected. It was assumed that the antifungal activity of AFP is related to its ability to interact with anionic phospholipids (13).

In order to gain more information on the cellular target and the mechanism of action of AFP, we examined the interaction of AFP with fungal membranes using an in vivo assay based on the uptake of the fluorogenic DNA dye SYTOX Green. We then determined the site of action by protein labeling and immunofluorescence staining and subsequent detection by fluorescence microscopy. Our results strongly support the concept that the antifungal activity of AFP is due to binding and subsequent permeabilization of the plasma membrane of AFP-sensitive fungi.

MATERIALS AND METHODS

Materials. AFP antibody (0.3 mg of specific immunoglobulin Y [IgY]/ml) and the fluorescein isothiocyanate (FITC)-conjugated antibody (1 mg of IgY/ml) were purchased from Biogenes (Berlin, Germany). The antibody for FITC (0.4 mg of IgY/ml) was purchased from Dako (Glostrup, Denmark). SYTOX Green was obtained from Molecular Probes (Eugene, Oreg.).

Protein purification. Basic proteins from the culture supernatant of *A. giganteus* were isolated by a method modified from that of Wnendt (28). *A. giganteus* was grown for 96 h at 28°C in 2-liter Erlenmeyer flasks, each of which contained 500 ml of culture medium composed of 2% soluble starch, 1% beef extract, 2% peptone, and 0.5% NaCl. After an additional incubation at 37°C for 20 h, the overall yield of all basic proteins secreted by *A. giganteus* was increased by a factor of about 15 (19). These proteins were isolated from the culture supernatant by cation-exchange chromatography. The pH of the culture supernatant was adjusted to pH 7.0 and mixed with 50 ml of carboxymethyl cellulose CM-23 (50 mg/ml in Tris-EDTA [TE; pH 7.0]; Serva, Heidelberg, Germany) for 1 h. The carboxymethyl cellulose was washed twice in a funnel with 500 ml of TE (pH 7.0) to remove unbound proteins. Basic proteins were eluted with 10 ml of TE–1.5 M NaCl (pH 7.0). A stirred ultrafiltration cell with a YM1 membrane (Millipore, Bedford, Mass.) was used to reduce the volume to 3 ml. The eluted proteins were subjected twice to a Sephadex G50 column (1.5 by 50 cm; Amersham, Uppsala,

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Sweden) equilibrated with 0.05 M sodium acetate–0.1 M NaCl to obtain homogenous AFP. Fractions containing AFP were pooled, and the protein concentration was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The polyacrylamide concentration in the separating gel was 15% (wt/vol). Bands were detected by staining with Coomassie brilliant blue R-250 (Serva). Pure AFP was stored in 0.1 M NaCl–0.05 M sodium acetate at 4°C. No loss of activity was detected after 3 months.

MIC assay. The activity of AFP was assayed against different filamentous fungi, yeast, and bacteria. Fungi were cultivated in YPG medium (0.3% yeast extract, 1% peptone, 2% glucose [pH 4.5]). Bacteria were cultivated in Luria-Bertani medium (1% peptone, 0.5% yeast extract, 1% NaCl [pH 7.5]). MICs were determined by adding 100 conidia or cells to 3 ml of culture broth containing AFP at different concentrations ranging from 0.01 to 400 μg/ml. After 48 h of incubation with continuous shaking, the minimal AFP concentration that prevented the growth of a given test organism was determined and was defined as the MIC. The organisms tested are listed in Table 1.

SYTOX Green uptake assay. The SYTOX Green uptake assay was performed by a method described by Thevissen et al. (26). One hundred conidia of the fungi to be tested were cultivated in 96-well Maxisorp F microplates (Nunc, Roskilde, Denmark) containing 150 μl of YPG medium. After 20 h of incubation at 28°C, SYTOX Green was added to a final concentration of 0.2 μM. AFP was added at concentrations ranging from 4 ng/ml to 400 μg/ml. Fluorescence was measured after 5 min and for up to 4 h of incubation with AFP by using a CytoFluor 2350 fluorescence measurement system (Millipore) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Fluorescence values were corrected by subtracting the fluorescence value of a culture incubated only with SYTOX Green. Qualitative detection of SYTOX Green uptake was done by fluorescence microscopy.

FITC labeling of proteins. The pH of 1 ml of the AFP solution (1 mg/ml) was adjusted to pH 9.0 with 1 M $NaHCO_3$. The protein was mixed with 30 μl of FITC solution (0.1 mg/ml in dimethyl formamide) for 1 h at room temperature in the dark. Unincorporated FITC was removed by ultrafiltration in a Centricon YM-3 vial (Millipore). The concentration of the labeled protein was visually estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The absorption at 494 nm was determined, and subsequently, the degree of labeling was calculated by the following equation: dye per protein molecule = (A_{494} × dilution factor)/(68,000 × protein concentration), where 68,000 cm^{-1} M^{-1} is the molar extinction coefficient of FITC at pH 8.0 at 494 nm.

Immunofluorescence. Fungi were grown in six-well PS macroplates (Greiner, Frickenhausen, Germany) containing 4 ml of YPG medium for 20 h at 28°C without agitation. AFP was added, and after an additional 1 h of incubation at 28°C with continuous shaking at 120 rpm, the hyphae were fixed by the addition of formaldehyde to a final concentration of 5%. Incubation for 30 min at room temperature was followed by centrifugation at 2,000 × *g* for 10 min. Mycelia were mixed with 5 ml of fixation solution (5% formaldehyde, 100 mM potassium phosphate buffer [pH 6.5], 0.5 mM $MgCl_2$). After 2 h at room temperature, the hyphae were washed twice with 0.6 M KCl and were resuspended in 12 ml of 0.6 M KCl containing 500 mg of Glucanex (Roth, Karlsruhe, Germany) per ml to obtain spheroplasts. Incubation for 3 h at 28°C with gentle shaking was followed by centrifugation at 1,000 × *g* for 10 min. The spheroplasts were washed twice with 0.6 M KCl and were finally resuspended in 1 ml of 0.6 M KCl. An eight-well microscope slide was prepared for immobilization of the spheroplasts by coating of each well with 10 μl of polylysine (1 mg/ml) and subsequent washing for 10 min with H_2O. Spheroplast solution (20 μl) was added to each well of the microscope slide. The solution was removed after 1 min, and the slide was desiccated. The immobilized spheroplasts were treated with 10 μl of 1% Triton X-100 for 10 min. Subsequently, the slide was washed with 10 ml of phosphate-buffered saline (PBS) containing 1 mg of bovine serum albumin (BSA) per ml. Each well of the slide was incubated with 20 μl of a 1:100 dilution of the AFP-specific antibody for 1 h at room temperature. The slide was washed three times with 10 ml of PBS containing 1 mg of BSA per ml to remove unbound antibody. The secondary FITC-conjugated antibody was used at a 1:1,000 dilution. Treatment of each well with 20 μl of the antibody dilution for 1 h in the dark at room temperature was followed by triple washing with PBS-BSA solution to remove unbound antibody. The fluorescence was detected with a fluorescence microscope.

Fluorescence microscopy. Fluorescence was viewed with an Axioskop fluorescence microscope (Zeiss, Jena, Germany) equipped with a 48 79 09 filter set (Zeiss) for fluorescein detection (excitation wavelength, 450 to 490 nm; emission wavelength, 520 nm).

TABLE 1. MICs of AFP for different microorganisms

Susceptibility and organism	MIC (μg/ml)[a]
Sensitive	
Fusarium sporotrichoides IfGB 39/1601	0.1
Fusarium moniliforme IfGB 39/1402	0.1
Aspergillus niger ATCC 9029	1
Aspergillus niger IfGB 15/1803	1
Aspergillus niger NRRL 372	1
Fusarium equiseti IfGB 39/0701	1
Fusarium lactis IfGB 39/0701	1
Fusarium oxysporum IfGB 39/1201	1
Fusarium proliferatum IfGB 39/1501	1
Fusarium sp. strain IfGB 39/1101	1
Aspergillus awamorii ATCC 22342	2
Fusarium lini IfGB 39/0801	8
Fusarium bulbigenum IfGB 39/0301	10
Fusarium vasinfectum IfGB 39/1301	10
Moderately sensitive	
Fusarium solani IfGB 39/1001	120
Fusarium poae IfGB 39/0901	180
Aspergillus nidulans DSM 969	200
Aspergillus nidulans G191	200
Aspergillus giganteus IfGB 15/0903	>400[b]
Aspergillus giganteus MDH 18894	>400[b]
Fusarium aquaeductuum IfGB 39/0101	>400[b]
Fusarium culmorum IfGB 39/0403	>400[b]
Resistant	
Aspergillus clavatus ATCC 1007	NE[c]
Aspergillus oryzae ATCC 11488	NE
Penicillium chrysogenum ATCC 10002	NE
Bacillus megaterium ATCC 10778	NE
Bacillus subtilis ATCC 6051	NE
Pseudomonas fluorescens IfGB 0301	NE
Escherichia coli ATCC 11775	NE
Saccharomyces cerevisiae AH22	NE

[a] Values are averages of triplicate measurements.
[b] No complete growth inhibition was detected at concentrations of 400 μg/ml.
[c] NE, no effect.

RESULTS

Growth-inhibitory effect of AFP. Several microorganisms including filamentous fungi, bacteria, and yeast were tested for their susceptibilities to AFP. MICs were determined to quantify the growth-inhibitory effect of the protein. No morphological changes were detected for any of the microorganisms tested. As can be seen in Table 1, AFP had no effect on the growth of bacteria and yeast. According to the MICs of AFP for the filamentous fungi, they could be divided into three groups. The first group exhibited high sensitivity to AFP. The MICs ranged from 0.1 μg/ml for the most susceptible fungi *Fusarium moniliforme* and *Fusarium sporotrichoides* to 10 μg/ml for *Fusarium vasinfectum*. The growth of most organisms in this group was inhibited at a concentration of about 1 μg/ml. The second group of fungi showed a moderate sensitivity to AFP. The MICs ranged from 120 μg/ml for *Fusarium solani* to 200 μg/ml for *Aspergillus nidulans*. The growth of the AFP-producing mold *A. giganteus* itself and the growth of *Fusarium aquaeductuum* and *Fusarium culmorum* were slightly inhibited by AFP, but no complete growth inhibition could be detected even at a concentration of 400 μg/ml. In contrast to these organisms, fungi belonging to the third group were resistant to

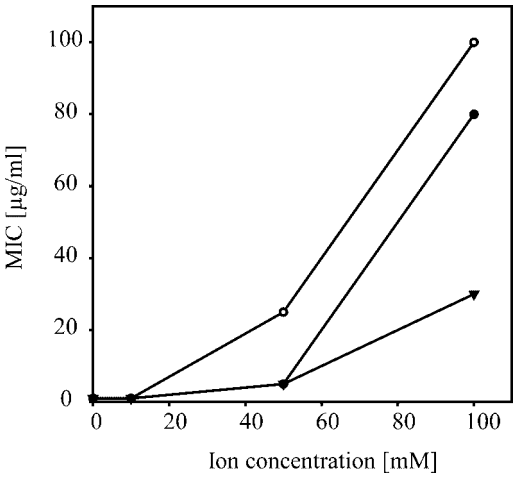

FIG. 1. Effects of different ions on the MIC of AFP for *A. niger*. The MIC of AFP for *A. niger* was tested in the presence of different amounts of KH_2PO_4 (○), KCl (●), and NaCl (▲). The results of a representative experiment are shown.

AFP even at a concentration of 400 μg/ml (*Aspergillus clavatus*, *Aspergillus oryzae*, and *P. chrysogenum*). The AFP-sensitive mold *A. niger* and the AFP-resistant mold *P. chrysogenum* were selected as test organisms for all further tests concerning the mode of action and the localization of AFP.

Cation sensitivity of AFP. As a cation-sensitive action has been described for many antimicrobial and antifungal peptides (9, 27), the growth-inhibitory effect of AFP on *A. niger* and *P. chrysogenum* was tested in the presence of different cations. The AFP resistance of *P. chrysogenum* was not altered by the addition of cations (data not shown), whereas the activity of AFP against *A. niger* was dramatically decreased in the presence of cations (Fig. 1). The presence of 100 mM KH_2PO_4 led to an MIC 100-fold higher than the MIC estimated in medium not supplemented with cations. KCl at a concentration of 100 mM increased the MIC about 80-fold, whereas NaCl at a concentration of 100 mM increased the MIC by a factor of 30. The results show that the antifungal activity of AFP is strongly sensitive to cations.

AFP-induced membrane permeabilization. To check whether AFP has an effect on fungal membranes, we used an assay based on the uptake of the fluorogenic dye SYTOX Green (26). This substance can only penetrate cells that have compromised plasma membranes, and it fluoresces upon binding to DNA. The fungus *A. niger*, which is AFP sensitive, and the fungus *P. chrysogenum*, which is AFP resistant, were tested for AFP-induced membrane permeabilization. Fluorescence could not be detected when each fungus was incubated with 0.2 μM SYTOX Green in the absence of AFP (data not shown). Incubation of *A. niger* with 0.2 μM SYTOX Green and 10 μg of AFP per ml resulted in an uptake of the fluorescence dye and staining of the nuclei (Fig. 2A). This result indicates that the plasma membrane of *A. niger* was permeabilized in the presence of AFP. In contrast, no uptake of SYTOX Green was detected for *P. chrysogenum* after incubation with 0.2 μM SYTOX Green and 100 μg of AFP per ml (Fig. 2C), indicating that AFP did not alter the plasma membrane. Dose-response curves were recorded to further analyze the membrane permeabilization induced by AFP. *A. niger* and *P. chrysogenum* were thus grown in the presence of 0.2 μM SYTOX Green and different amounts of AFP ranging from 4 ng/ml to 400 μg/ml (Fig. 3A). Again, no fluorescence could be detected for *P. chrysogenum*, an AFP-resistant fungus, even at concentrations of 400 μg/ml, confirming that AFP did not permeabilize its plasma membrane. In contrast, treatment of *A. niger* with concentrations of AFP of 10 μg/ml and higher led to membrane permeabilization, as measured by determination of SYTOX Green uptake. No SYTOX Green uptake was detected with AFP at concentrations below 10 μg/ml.

Measurement of the fluorescence after certain times revealed that the plasma membrane of *A. niger* was permeabilized after 5 min. No SYTOX Green uptake was detected for *P. chrysogenum* even after 4 h of incubation with 100 μg of AFP per ml, corroborating the fact that the plasma membrane of *P. chrysogenum* is not altered by AFP.

In order to check whether the cation sensitivity of AFP is due to the fact that AFP does not permeabilize membranes at high salt concentrations, *A. niger* was grown in the presence of 0.2 μM SYTOX Green, 100 mM KCl, and different amounts of AFP. As shown in Fig. 3B, no SYTOX Green uptake was detected in the presence of 100 mM KCl, confirming the result that the antifungal action of AFP is cation sensitive. Furthermore, this result indicates that AFP did not permeabilize the plasma membrane of *A. niger* in the presence of cations.

Localization of FITC-labeled AFP. In order to determine the site of action of AFP, the protein was labeled with FITC and the localization of the FITC-labeled AFP was monitored in *A. niger* and *P. chrysogenum* cells. As labeling of AFP could possibly have a negative effect on the activity of AFP, different molar ratios of FITC to AFP ranging from 0.5 to 10 were tested. The best results were obtained with a threefold molar excess of FITC, which resulted in a degree of labeling of 0.9 ± 0.2 mol of FITC/mol of AFP. AFP labeled in this manner was tested with different sensitive and resistant fungi and showed the same antifungal activity and specificity in comparison to those for unlabeled AFP (data not shown).

A. niger and *P. chrysogenum* were incubated with 1, 10, and 100 μg of FITC-labeled AFP per ml. The localization of AFP was monitored by fluorescence microscopy. As shown exem-

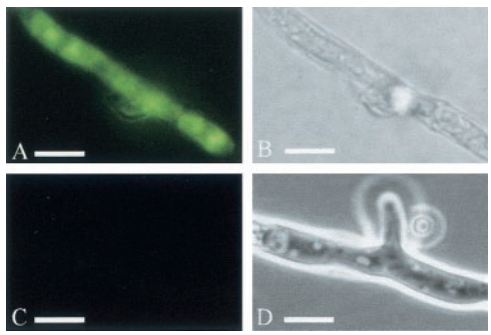

FIG. 2. Detection of AFP-induced SYTOX Green uptake by fluorescence microscopy. *A. niger* (A and B) and *P. chrysogenum* (C and D) were treated with 10 and 100 μg of AFP, respectively, in the presence of 0.2 μM SYTOX Green. After 1 h of incubation, SYTOX Green uptake was detected by fluorescence microscopy. (A and C) Fluorescence microscopy; (B and D) light-field microscopy. Bars, 15 μm.

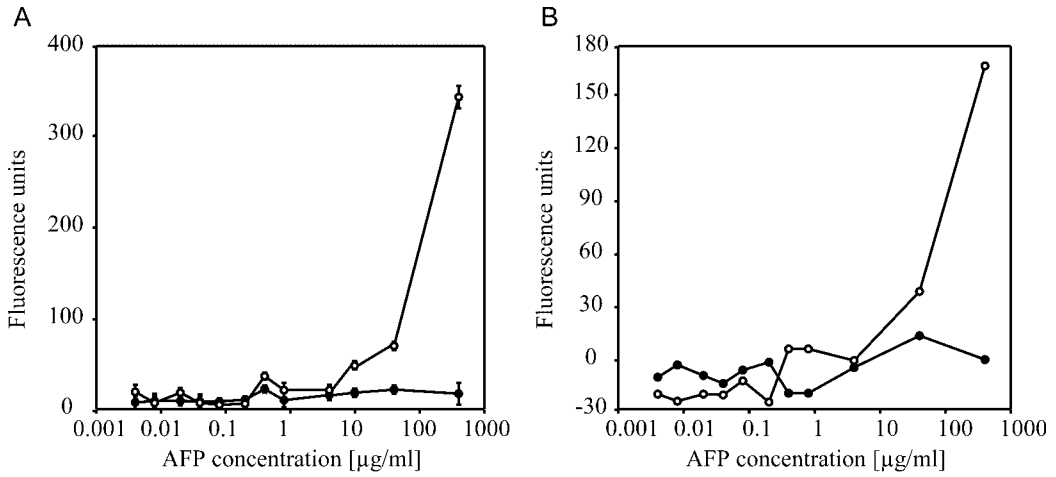

FIG. 3. Detection of AFP-induced SYTOX Green uptake. (A) *A. niger* (○) and *P. chrysogenum* (●) were incubated with different amounts of AFP in the presence of 0.2 μM SYTOX Green. Fluorescence was measured after 1 h of incubation. Data are averages of triplicate measures. (B) *A. niger* was incubated with different concentrations of AFP in the presence (●) or absence (○) of 100 mM KCl. Fluorescence was measured as described above. The results of a representative experiment are shown.

plarily in Fig. 4A (panel A), incubation of *A. niger* with 10 μg of FITC-labeled AFP per ml resulted in fluorescence which was mainly restricted to the membrane or cell wall. No fluorescence was detected inside the cell. The same fluorescence pattern was observed when 1 or 100 μg of FITC-labeled AFP per ml was used (data not shown). To preclude the possibility that the lack of intracellular fluorescence was not due to a low intracellular pH, *A. niger* was treated with 10 μg of FITC-labeled α-sarcin per ml. It is expected that this would result in fluorescent staining of the ribosomes. As can be seen in Fig. 4A (panel C), a bright, intracellular fluorescence of the labeled α-sarcin was detected, indicating that the intracellular pH of *A. niger* had no negative effect on fluorescence. Treatment of the AFP-resistant fungus *P. chrysogenum* with FITC-labeled AFP resulted in uptake of the labeled protein. AFP seemed to accumulate in distinct areas of the cell (Fig. 4A, panel B). This localization pattern was observed with all concentrations of FITC-labeled AFP tested. Internalization of FITC-labeled AFP was not restricted to *P. chrysogenum* but was also observed for other resistant fungi (*A. clavatus* and *A. oryzae*) (data not shown). These results indicate that sensitivity to AFP is due to AFP's interaction with either the plasma membrane or the cell wall.

Localization of AFP by immunofluorescence staining. Direct detection of AFP within AFP-resistant and AFP-sensitive cells

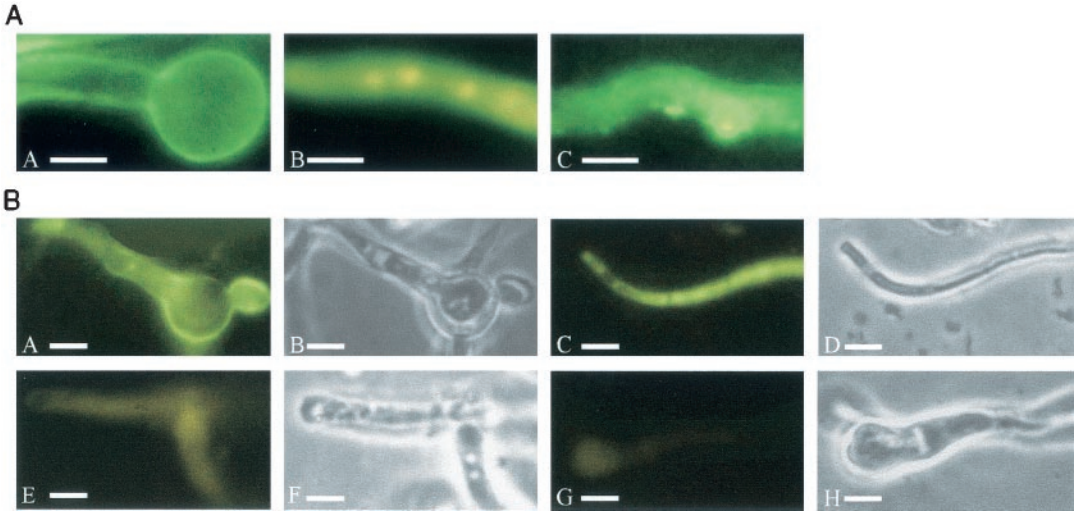

FIG. 4. Localization of AFP within *A. niger* and *P. chrysogenum*. (A) FITC-labeled AFP. *A. niger* (panel A) and *P. chrysogenum* (panel B) were treated with 10 and 100 μg of FITC-labeled AFP ml, respectively. After 1 h of incubation, the labeled protein was detected by fluorescence microscopy. The control was *A. niger* treated with 10 μg of FITC-labeled α-sarcin per ml (panel C). Bars, 15 μm. (B) Immunofluorescence. *A. niger* (panels A and B) and *P. chrysogenum* (panels C and D) were treated with 10 μg of AFP per ml for 1 h. The protein was detected by immunofluorescence staining with an AFP-specific antibody. As negative controls, *P. chrysogenum* (panels E and F) and *A. niger* (panels G and H) were treated with the AFP-specific antibody in the absence of AFP. Panels A, C, E, and G, fluorescence microscopy; panels B, D, F, and H, light-field microscopy. Bars, 15 μm.

was achieved by immunofluorescence staining with an AFP-specific antibody. After treatment with 10 μg of AFP per ml and fixation with formaldehyde, the cell walls of *A. niger* and *P. chrysogenum* were partially degraded with Glucanex. Spheroplasts were treated with an AFP-specific antibody and a FITC-conjugated secondary antibody. Fluorescence microscopic analysis verified the results from the localization experiments done with FITC-labeled AFP. Bright fluorescence of the plasma membrane was detected for *A. niger* spheroplasts (Fig. 4B, panel A), indicating that AFP had bound to the membrane. As a result of AFP uptake, the resistant mold *P. chrysogenum* showed fluorescence inside the hyphae after AFP treatment and immunofluorescence staining (Fig. 4B, panel C). The protein seemed to accumulate in distinct areas of the cells. To preclude nonspecific staining due to cross-reactions of the antibodies with other proteins, both fungi were treated with the AFP-specific antibody and the FITC-conjugated antibody, but without previous AFP treatment. As can be seen in Fig. 4B (panels E and G), no nonspecific staining was detected for either fungus.

DISCUSSION

To gain more information on the mode of action of AFP, we investigated the inhibitory effects of the AFP from *A. giganteus* on the growth of microorganisms by means of assays, monitoring the localization of AFP within resistant and sensitive molds and analyzing the membrane permeabilization effect of AFP.

Our quantitative analyses of the growth-inhibitory effect of AFP gave results similar to those obtained by former qualitative (29) and quantitative (12) procedures. However, it is remarkable that in the latter study no growth-inhibitory effect of AFP on *A. niger* was found, whereas this study and that of Wnendt (28) describe *A. niger* as an AFP-sensitive fungus. The differences observed are presumably due to the different cultivation conditions, different protocols, or different strains used. For example, the dramatic increase in the MICs in the presence of different cations showed that the cultivation medium has a great influence on the activity of AFP. In addition, we also tested an agar dilution method which yielded MICs about 20 times higher than those obtained by the broth microdilution method, demonstrating that MICs are dependent on the choice of protocol.

On the basis of the results of the SYTOX Green assay, we propose that the growth-inhibitory effect of AFP is due to binding and subsequent permeabilization of fungal plasma membranes. An interaction with anionic lipid components of the plasma membrane has been hypothesized to explain the antimicrobial effects of insect and mammalian defensins (4, 5, 11). In contrast, a receptor-mediated interaction has been found for some plant defensins (27). A nonspecific interaction of AFP with anionic membrane components seems to be unlikely because the growth of bacteria whose outer plasma membrane layer is mainly composed of anionic phospholipids is not affected by AFP. However, it is possible that the bacterial cell wall is responsible for the resistance to AFP. Studies of *Staphylococcus aureus* have shown that the structure of teichoic acids, which are major components of the cell wall of gram-positive bacteria, is responsible for the high tolerance of *S. aureus* to defensins, protegrins, and other antimicrobial peptides. Mutants with altered teichoic acids were more sensitive to antimicrobial proteins (23).

The very specific antifungal activity of AFP, which is restricted to only a few fungi mainly belonging to the genera *Fusarium* and *Aspergillus*, together with the broad distribution of MICs, which ranged from 0.1 to 200 μg/ml, could be an indication of a receptor-mediated interaction of AFP with a membrane-based target. Fungi that exhibit this postulated receptor are highly sensitive to AFP, whereas fungi that lack this target are resistant. It has been shown in this connection that the sphingolipid mannose (inositol phosphate)-2-ceramide of *Saccharomyces cerevisiae* acts as a receptor for the plant defensin DmAMP1 from dahlias. Mutants lacking this sphingolipid are resistant to this protein (25). Moderate sensitivity may be explained by nonspecific binding to targets which show similarities to the actual fungal receptor.

Localization experiments with AFP-sensitive and AFP-resistant fungi confirmed that sensitivity to AFP is due to an interaction with a membrane-based target. The protein was localized within intact hyphae and spheroplasts by FITC labeling and immunofluorescence staining, respectively. Both methods revealed that AFP binds to the plasma membrane of AFP-sensitive fungi. However, an interaction with remaining cell wall fragments cannot be completely excluded. It is interesting in this regard that Liu et al. (16) recently reported that AFP has an affinity for binding to chitin, which is a main constituent of fungal cell walls. AFP1, an antifungal protein from *Streptomyces tendae*, was found to bind to chitin, which caused severe alterations in cell morphogenesis (1). Additionally, binding of alpha-thionins from barley and wheat to polysaccharides containing beta-1,3-glucan, which are present in fungal cell walls, has been reported (21). Using the SYTOX Green assay, we were able to show that AFP causes the membranes of AFP-sensitive fungi to become permeable. Interestingly, detectable membrane permeabilization measured by the SYTOX Green assay occurs only at AFP concentrations which are significantly higher than the MIC (e.g., permeabilization for *A. niger* was detected at 10 μg of AFP per ml, whereas the MIC was estimated to be 1 μg/ml). On the one hand, it is conceivable that the SYTOX Green assay might not be sensitive enough to detect membrane permeabilization at lethal concentrations of AFP. On the other hand, the antifungal activity of AFP might not be due to pore formation but, rather, may be due to AFP binding to cell wall or membrane components (e.g., chitin), which additionally contributes to the growth-inhibitory effect of AFP.

Membrane permeabilization by AFP could be partially explained by the two-state model described by Huang (10). According to this model, AFP would bind to membranes but would stay in a functionally inactive state. However, this binding may hinder transport processes within AFP-sensitive fungi and lead to a growth-inhibitory effect. The conformation of AFP would change at a species-specific protein concentration, and AFP would be able to enter the membrane and form pores. However, to date there is no proof of a concentration-dependent change in the conformation of AFP.

The localization experiments revealed that AFP is internalized by AFP-resistant fungi. It is most likely that AFP is taken up by these fungi and is subsequently subjected to normal degradative processes. Alternatively, it may also be possible

that AFP is stored in vacuoles due to its high level of resistance to proteases (12). This would explain the accumulation of AFP observed in distinct areas of AFP-resistant fungi.

The growth-inhibitory effect of AFP has been found to be cation sensitive. We conclude from the results of the SYTOX Green uptake assay that this sensitivity is due to an interaction of cations with the negatively charged membrane target site of AFP. We were able to show that in the presence of 100 mM KCl no SYTOX Green had entered the cells of AFP-sensitive fungi, probably due to a lack of permeabilization of the plasma membrane. The presence of large amounts of cations probably leads to saturation of the membrane-based target of AFP with cations. As a result of this interaction, the target site is no longer accessible for AFP. The much stronger effect achieved with KCl compared to that achieved with NaCl might suggest that potassium has a higher affinity for the structure of the AFP target. However, an interaction with a specific ion-selective channel is not likely since the growth-inhibitory effect of AFP is altered by many different cations. Cation sensitivity has been described for basic antimicrobial proteins which are thought to act via electrostatic interaction with negatively charged membrane components (15) as well as for proteins which bind to specific receptors (27). It is believed that cations interfere with the interaction of the antimicrobial protein with its cellular target, but to date there is no experimental proof for this hypothesis.

Although the exact mechanism of action of AFP has not yet been resolved, the results reported here indicate a plasma membrane-based action of AFP. There are strong indications of receptor-mediated binding of AFP. Characterization of this putative receptor or target site will facilitate the understanding of the specificities of many antifungal proteins.

ACKNOWLEDGMENTS

This work was partially supported by grants from the "Stiftung Industrieforschung" (grant T26/98) and the "Max-Buchner-Forschungsstiftung" (grant 2259).

We thank Roslin Bensmann for checking the English.

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