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ORIGINAL PAPER

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Characterization of the *Penicillium chrysogenum* antifungal protein PAF

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Abstract The filamentous fungus *Penicillium chrysogenum* abundantly secretes the small, highly basic and cysteine-rich protein PAF (*Penicillium* antifungal protein). In this study, the antifungal activity of PAF is described. PAF inhibited the growth of a variety of filamentous fungi, including opportunistic human pathogenic and phytopathogenic fungi, whereas bacterial and yeast cells were unaffected. PAF reduced the conidial germination and hyphal extension rates in a dose-dependent manner and induced severe changes in cell morphology that resulted in crippled and distorted hyphae and atypical branching. Growth-affected hyphae suffered from oxidative stress, plasma membrane leakage, and metabolic inactivity, which points to an induction of multifactorial effects in sensitive fungi. In contrast to other known antifungal proteins, the effects of PAF were only partially antagonized by cations.

Key words Antifungal activity · *Penicillium chrysogenum* · Morphology · Membrane leakage · Oxidative stress

Introduction

Many organisms produce antimicrobial proteins to protect themselves from infectious microbial agents. First reports date from the 1970s, when peptides with antimicrobial properties were isolated from plants. Since then, proteins with similar functions have been found in diverse organisms, such as bacteria, fungi, plants, insects, birds, arthropods, amphibians, and mammals (Ganz 1994; Tao et al. 1990; for reviews see Dimarcq et al. 1998; Garcia-Olmedo et al. 1998; Raj and Dentino 2002; Tossi et al. 2000). Many of these proteins are active against a broad spectrum of microorganisms, including bacteria, yeasts and filamentous fungi. Although the amino acid composition and tertiary structure of these antifungal proteins vary, most are secreted, small in size, highly basic, and contain a large number of cysteine residues, which are involved in specific disulfide-bond formation to stabilize protein structure.

In a previous study, we purified the secreted antifungal protein PAF from the penicillin-producing strain *Penicillium chrysogenum* Q176 (Marx et al. 1995). Only a few similar antifungal peptides from ascomycetes are known, and these are not related to antifungal proteins from other species. The antifungal proteins AFP from *Aspergillus giganteus*, ANAFP from *Aspergillus niger,* and NAF from *Penicillium nalgiovense* show an amino acid identity with PAF of 42, 37, and 100%, respectively. These proteins inhibit the growth of different taxonomically related filamentous fungi (Geisen 2000; Lee et al. 1999; Marx et al. 1995; Theis et al. 2003; Wnendt et al. 1994). However, apart from the determination of inhibition spectra and some studies addressing the interaction of *A. giganteus* AFP with the plasma membrane (Theis et al. 2003) and with nucleic acids (Martinez Del Pozo et al. 2002), little is presently known of their precise mechanism of cytotoxicity.

In this report, detailed information is presented for the first time about the properties of the antifungal protein PAF. We found detrimental effects of PAF on conidial germination, hyphal growth and cell morphology of a variety of economically important filamentous fungi, including human and plant pathogens, and we show that growth inhibition might be due to multifactorial effects. Furthermore, we give evidence that the formation of reactive oxygen species by PAF might contribute to or even induce detrimental effects, such as disintegration of the cytoplasmic membranes and metabolic inactivation of hyphal cells.

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Materials and methods

Strains and media

In vitro tests were carried out with 21 microbial species, all of which have been deposited in the University of Innsbruck, Department of Molecular Biology: *Aspergillus flavus* (ATCC 9643), *Aspergillus fumigatus* (ATCC 46645), *A. giganteus* (AG 090701), *Aspergillus nidulans* (FGSC4), *A. niger* (CBS 120.49), *Aspergillus terreus* (304), *Botrytis cinerea* (BC 080801), *Cochliobulus carbonum* SB111 (ATTC 90305), *Fusarium oxysporum* (FO 240901), *Fusarium sambucinum* (FS 210901), *Gliocladium roseum* (GR 210901), *Mucor circinelloides* (MC 080801), *Mucor genevensis* (MG 080801), *Neurospora crassa* (CBS 404.59), *P. chrysogenum* wild-type (wt, PC 67), *P. chrysogenum* strain Q176 (ATTC 10002), *Trichoderma koningii* (TC 060901), *Candida albicans* (CBS 5982), *Saccharomyces cerevisiae* (AH 109, Clontech), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* TG2. All strains were obtained from the culture collections of FGSC, ATTC, CBS, or from the Institute of Microbiology, Division of Systematics, Taxonomy and Evolutionary Biology (University of Innsbruck, Austria).

All fungi were grown in complete medium (CM) (0.2% peptone, 0.1% yeast extract, 0.1% NZ-amine A, 2% glucose, 0.05% KCl, 0.04% $MgSO_4 \cdot 7H_2O$, 0.15% KH_2PO_4, pH 6.5), except for *C. albicans* and *S. cerevisiae* cells which were cultivated in YPD (2% peptone, 1% yeast extract, 2% glucose, pH 5.8). Bacteria were grown in LB (1% yeast extract, 0.5% peptone, 1% NaCl, pH 7.0). *B. cinerea*, *C. carbonum*, *F. oxysporum, F. sambucinum*, *G. roseum*, *M. circinelloides, M. genevensis*, *N. crassa,* and *P. chrysogenum* were incubated at 25 °C, all other microorganisms were grown at 30 °C.

Purification of PAF

PAF was isolated from the supernatant of *P. chrysogenum*, strain Q176. The supernatant of a 72-h culture was cleared by centrifugation for 30 min at 10,000×*g* and 4 °C, and ultrafiltrated through a YM-30 membrane (Millipore, Bedford, Mass., USA) using an 8.200 Amicon stirring cell (Millipore). The flow-through was loaded on a CM-sepharose CL-6B column (Amersham, Uppsala, Sweden), equilibrated in 10 mM Na-phosphate buffer, pH 6.6, 25 mM NaCl, 0.15 mM EDTA. The protein was eluted by a continuous NaCl-gradient (0.5–1 M) and PAF-containing fractions were checked by Coomassie or silver staining after SDS-PAGE (15% polyacrylamide, Novex, San Diego, Calif., USA). PAF-containing fractions were pooled, dialyzed against equilibration buffer, concentrated in Centriprep YM-3 filter devices (Millipore) and sterilized by filtration through Millex-GV filters (0.22-µm, Millipore).

Antifungal activity assay

The growth rate of PAF-treated fungi was checked by microscopy and monitored by measuring the optical density at 620 nm (OD_{620}) with a microtiter plate reader 340 ATC (SLT Lab. Instruments, Groeding, Austria) according to the protocols of Broekaert et al. (1990) and Ludwig and Boller (1990). In brief, 1×10^3–1×10^4 conidia/ml were incubated in 96-well flat-bottom microplates (Greiner, Kremsmuenster, Austria) with 0.5–50 µg PAF/ml in the appropriate growth medium in a total volume of 200 µl/well. Plates were incubated first at the optimum temperature of the tested strain for 24 h and then shifted to 22 °C to avoid rapid overgrowth of untreated controls. The optical density was determined periodically. Duplicate cultures were used in each experiment.

Unless otherwise stated, all subsequently described experiments were conducted with at least three different concentrations of PAF, ranging from 1–50 µg/ml. *A. niger* served as the positive control for a PAF-sensitive organism, and *A. terreus* as the PAF-insensitive control. For testing germinated hyphae, 1×10^4 conidia/ml were incubated until the OD_{620} reached 0.1. PAF was added to the wells and the OD_{620} was determined periodically. The effect of 0.02–0.1 M salt (KCl, NaCl, $MgCl_2$, Na_2SO_4) and 0.15–1.5 M sugar (sucrose, sorbitol) on the antifungal activity of PAF was determined on conidia in CM. Growth of fungal hyphae was monitored as described above. To test the germination efficiency, 1×10^5 conidia were incubated with 50 µg PAF/ml in a total volume of 0.5 ml CM in 1.5-ml Eppendorf tubes for 24 h at 22 °C, pelleted for 15 min at 10,000×*g*, and washed twice with culture medium. The conidia were resuspended in CM, counted, and 1×10^2 conidia were plated onto solid CM in four replicates. Untreated conidia were used as control. After 24 h, the colony number was determined. For all experiments, conidia, cells, or hyphae treated with equilibration buffer instead of PAF were used as controls. The conidial germination rate was ≥80%. All experiments were conducted at least twice.

Staining with fluorescent dyes

To assess the metabolic activity of PAF-treated hyphae, viability staining with the membrane-permeable nucleic acid stain FUN-1 (Molecular Probes, Eugene, Ore., USA) was carried out according to the method of Lass-Florl et al. (2001) and Millard et al. (1997). The conidia of the tested organisms were incubated with PAF for 24 h in 8-well LAB-TEK chamber slides (Nunc, Naperville, Ill., USA) as described for the 96-well microplate assay. The supernatant was removed and the fungi were washed with 10 mM HEPES (pH 7.5) before staining the hyphae with 100 µl FUN-1 (5 µM in HEPES), according to the manufacturer's instructions for 30 min at 30 °C. To visualize the exit of nuclei from PAF-treated hyphal tips, double-stranded DNA was fluorescently labeled by washing the fungi in PBS before incubating in 100 µl of the dye Hoechst 33258 (1.6 µM in PBS; Molecular Probes, Eugene) for 30 min at 22 °C. To detect the formation of peroxides such as H_2O_2, PAF-treated hyphae were incubated in 100 µl dichlorodihydrofluorescein diacetate (H_2DCFDA, 10 µM in PBS; Molecular Probes) as described by Ezaki et al. (2000). In all fluorescence staining experiments, controls for intact hyphae were established by staining *A. niger* that had been cultivated without PAF or the PAF-treated insensitive strain *A. terreus*.

Microscopy

PAF-treated fungi were directly examined and photographed in the wells of the microtitre plates with a Leitz Labovert microscope (Vienna, Austria), equipped with a 35-mm camera. For visualization of Hoechst-33258-stained samples, fluorescence microscopy was carried out with a Zeiss Axioplan fluorescence microscope (Jena, Germany) equipped with a 50-W mercury arc lamp. The microscope was fitted with a 35-mm camera and an electronic light meter and shutter (Zeiss, Jena, Germany). For fluorescence microscopy of FUN-1 and H_2DCFDA-stained samples, a Zeiss 510 confocal laser scanning microscope (Jena, Germany), equipped with a 63×/1.4-numerical aperture objective lens, was used. Stacks of images were taken through a *z*-axis of the fungal cells at intervals of 0.3 µm, and three-dimensional projections of the *z*-axis series were processed by use of the LSM 510 software. Filter sets were used according to the manufacturer´s instructions (Molecular Probes).

K^+ efflux measurements

A. nidulans conidia (5×10^7) were incubated in 10 ml CM at 30 °C for 16 h. The supernatant was removed, the hyphae were washed three times in double-distilled water and resuspended in 2% (w/v) glucose and 16 mM glutamine. The effect of PAF concentrations of 1 µg/ml and 10 µg/ml was tested. Hyphae treated with nystatin (10 µg/mg wet weight) were used as a positive control, untreated hyphae as negative control. After incubation for 80 min at 22 °C, the mycelium was removed by centrifugation and the potassium concentration was measured in the supernatant by flame atom absorption spectrophotometry at 766.5 nm (FAAS 2380, Perkin Elmer,

Shelton, Conn., USA). The potassium concentration was corrected by subtracting the background that resulted from the potassium concentration determined in the testing solution. The data from two experiments with triplicates were analyzed.

Statistical analysis

All statistics were done with the Microsoft Excel program and statistical significance was calculated by Student's *t* test.

Results

Induction of growth inhibition and morphological changes

Seventeen filamentous fungi were tested for hyphal growth inhibition in the presence of PAF. Twelve strains were found to be sensitive, whereas five strains remained unaffected, such as *A. terreus*, *F. sambucinum*, *M. circinelloides*, *M. genevensis,* and *P. chrysogenum Q176* (Table 1).

Table 1 Growth of filamentous fungi, treated with 50 µg PAF/ml. Percent growth was calculated from the percent changes in OD_{620} of PAF-treated fungi compared to untreated controls (=100%). Values are expressed as mean±SEM (*n*=3)

Organism	Growth (%)	Sensitivity[a]
Aspergillus flavus	31.2±6.6	+
Aspergillus fumigatus	5.4±0.2	+++
Aspergillus giganteus	10.5±2.1	++
Aspergillus nidulans	6.4±2.2	+++
Aspergillus niger	0.5±0.1	+++
Aspergillus terreus	100±9.2	–
Botrytis cinerea	4.2±3.1	+++
Cochliobolus carbonum	10.1±0.79	++
Fusarium oxysporum	54.9±3.4	+
Fusarium sambucinum	92±5.1	–
Gliocladium roseum	42.2±3.1	+
Mucor circinelloides	99±6.5	–
Mucor genevensis	97±3.2	–
Neurospora crassa	12.9±0.5	++
Penicillium chrysogenum Q176	81.5±1.9	–
Penicillium chrysogenum (wild-type)	34.2±5.3	+
Trichoderma koningii	8.7±2.9	+++

[a]*Growth reduction: +++ ≥90%, ++ 70–90%, + 30–70%, – 0–30%*

Very little correlation of growth inhibition within the members of the same genus was detectable, e.g. *A. terreus* was insensitive to PAF compared to the other five *Aspergillus* species, whereby *A. niger* showed the highest and *A. flavus* only moderate growth inhibition. No effects of PAF could be detected on yeasts (*S. cerevisiae, C. albicans*) or on bacteria (*E. coli, B. subtilis*) (data not shown).

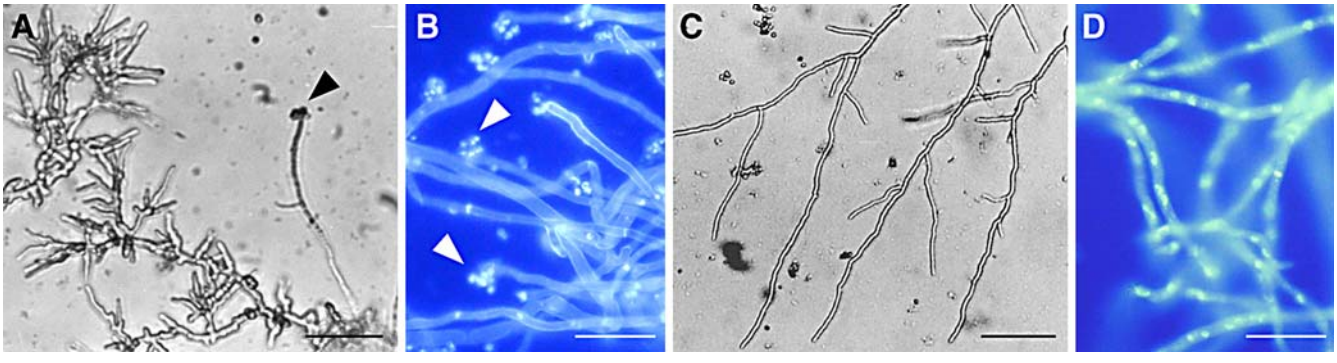
All sensitive fungal strains reacted at any point in time in a dose-dependent manner, showing increasing growth inhibition with increasing PAF concentrations (data not shown). PAF activity could be detected on conidia as well as on hyphae. Less than 1% of *A. niger* conidia that were treated with 50 µg PAF/ml formed colonies on agar plates (0.25±0.4%; $p<0.0001$ versus control), whereas the controls formed colonies to 91.8±8.3%. The extension of *A. niger* hyphae that had germinated in the absence of PAF and that were subsequently treated with the antifungal protein was effectively inhibited. The growth rates resembled those obtained when PAF was added directly to conidia (data not shown).

Hyphae of all sensitive fungal species exhibited severe changes in morphology when cultivated in PAF-containing liquid medium compared to untreated controls. Affected hyphae swelled and formed very short hyphae with multiple branches leading to a distorted appearance. Hyphae with reduced thickness, a fragmentation of the cytoplasm, and an accumulation of nuclei at broken hyphal tips were detected. The aberrant morphology is shown in Fig. 1 with *A. nidulans* as representative example.

The influence of salts and sugars on the activity of PAF

The growth inhibition and morphological changes induced by PAF were significantly counteracted with increasing concentrations of $MgCl_2$ and Na_2SO_4, and less influenced by KCl. The most prominent effect was reached with 0.1 M salt in the presence of 5 µg PAF/ml. In contrast, NaCl increased fungal growth in the presence of PAF only by a maximum of 10±1% (Fig. 2). Salt-related effects on PAF

Fig. 1 *Aspergillus nidulans* cultivated for 24 h in complete medium (CM) containing 10 µg PAF/ml (**A, B**), or no PAF (**C, D**). **B**, **D** Nuclei are stained with Hoechst 33258. *Arrows* Accumulation of nuclei at hyphal tips. *Scale bars* 50 µm (**A, C**), 20 µm (**B**, **D**)



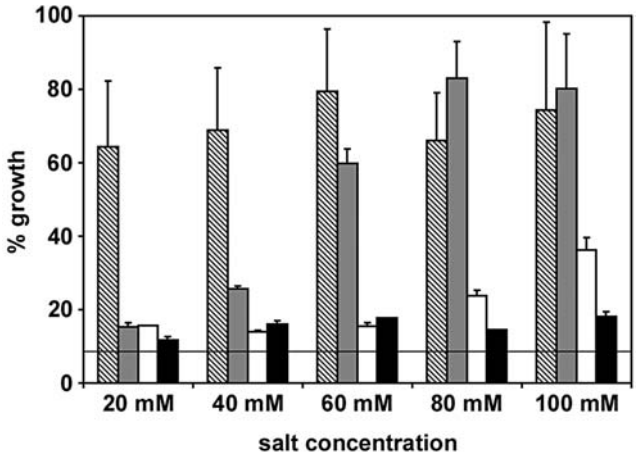


Fig. 2 Effect of $MgCl_2$ (*hatched*), Na_2SO_4 (*gray*), KCl (*white*) and NaCl (*black*) on the activity of 5 µg PAF/ml. *Bars* Growth values (%) calculated from percent changes in OD_{620} of PAF-treated *Aspergillus niger* at the corresponding salt concentrations compared to PAF-untreated controls (=100%). *Line* Percent growth of PAF-treated fungi in the absence of salts (7.5±2.6%). Results are expressed as mean±SEM (*n*=4)

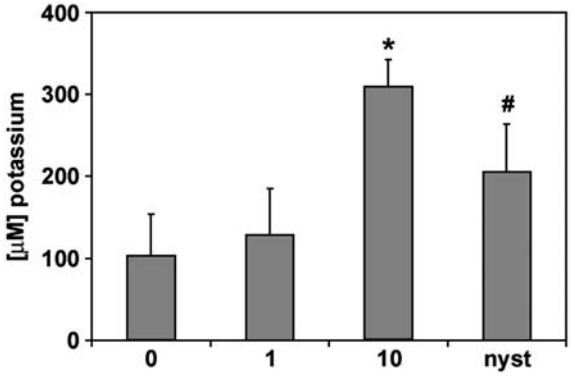


Fig. 3 Determination of K^+ efflux in the supernatant of *A. nidulans* by flame atom absorption spectrophotometry (FAAS) after the addition of 1 µg PAF/ml (*1*), 10 µg PAF/ml (*10*), and 10 µg nystatin/mg wet weight (*nyst*) for 80 min; untreated hyphae (*0*). Data are expressed as mean±SEM (*n*=3); **p*<0.0005, # *p*<0.006 versus control, respectively

activity were less pronounced with increasing PAF concentrations (data not shown).

PAF activity was also investigated under hypotonic, isotonic, and hypertonic growth conditions in order to elucidate a putative role of a weak cell wall in growth inhibition. Sorbitol and sucrose had no stabilizing effect on PAF-treated cells (data not shown).

Plasma membrane permeabilization

Potassium efflux measurements were used to study the effect of PAF on the integrity of the plasma membrane. With the highly sensitive test organism *A. niger*, an unspecific increase of extracellular potassium ions due to broken hyphal tips could not be excluded under the assay conditions. Therefore, the slightly less sensitive *A. nidulans* was used for determination of K^+ efflux (Fig. 3). After an incubation of 80 min, hyphal tips were still intact. The background potassium concentration in the potassium efflux solution was 35±1 µM without hyphae and 138±5 µM with hyphae. Therefore, incubation of hyphae without PAF increased the extracellular potassium concentration by 103±50 µM. Additional potassium efflux was observed with 1 µg PAF/ml (26±56 µM) and 10 µg PAF/ml (209±33 µM), (p<0.08 for 1 µg PAF/ml, p<0.0005 for 10 µg PAF/ml versus control, respectively). Nystatin, which was used as a control for plasma membrane permeabilization, induced a potassium efflux of 104±56 µM K^+ (p<0.006 for nystatin versus control).

Oxidative stress and metabolic inactivation

Assaying of oxidative activity by H_2DCFDA revealed the presence of intracellular reactive oxygen intermediates in PAF-treated *A. niger*. The fluorescence signal was most intensive in hyphae treated with 50 µg PAF/ml (Fig. 4A), and resembled the signal induced with 10 µg nystatin/mg hyphal wet weight (results not shown). In contrast, radical formation was detected neither in untreated hyphae

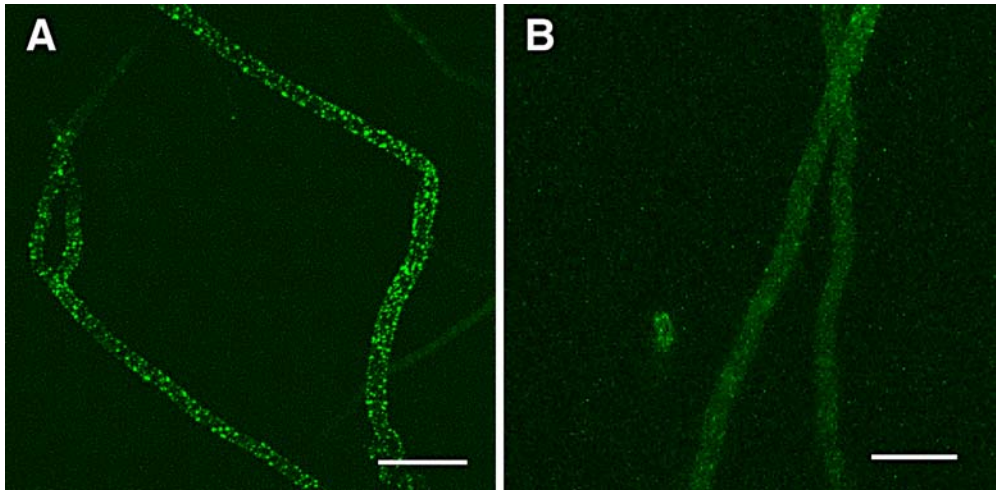


Fig. 4A, B Detection of intracellular reactive oxygen species by fluorescence staining of *A. niger* hyphae with H_2DCFDA. **A** Hyphae were treated with 50 µg PAF/ml for 90 min. **B** Untreated control. *Scale bars* 10 µm

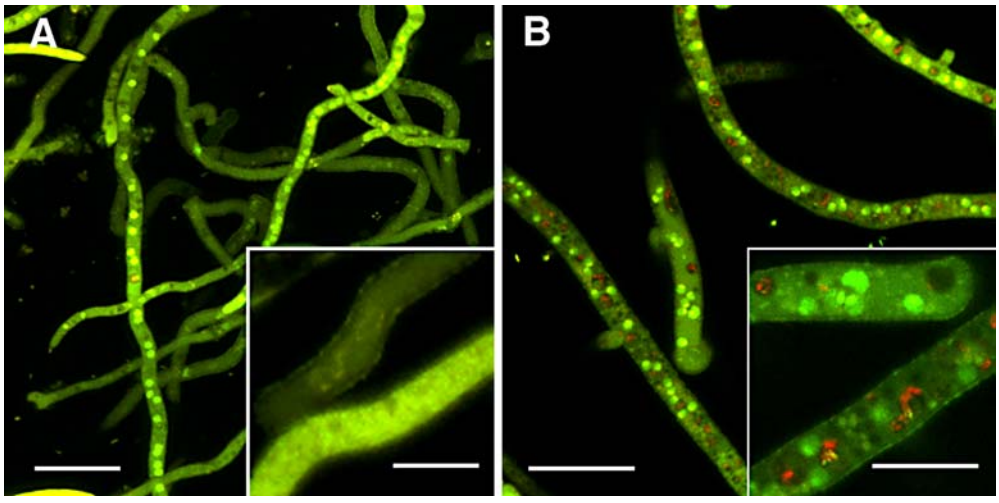


Fig. 5A, B Viability staining of *A. niger* hyphae with FUN-1 dye. **A** The hyphae were treated with 10 µg PAF/ml for 24 h. **B** Untreated control. *Scale bars* 20 µm (**A**), 10 µm (**B**), and 5 µm (*insets*)

(Fig. 4B) nor in PAF-treated insensitive *A. terreus* (results not shown).

Reduced cellular metabolism was detectable in PAF-treated hyphae by viability staining with the dye FUN-1 (Fig. 5A). The *A. niger* hyphae fluoresced bright yellow-green and the number of metabolically inactive hyphae augmented with increasing concentrations of PAF. In contrast, untreated *A. niger* as well as PAF-treated *A. terreus* developed an intravacuolar red fluorescence which indicated metabolic activity (Fig. 5B).

Discussion

Our results demonstrate that the antifungal activity of PAF seems to affect mainly filamentous fungi, although PAF is not universally effective. Differences in susceptibility towards PAF exist within species of the same genus. Remarkably, the specificity of PAF differs from that of the homologous proteins AFP, from *A. giganteus*, and ANAFP, from *A. niger*; the growth of *A. nidulans* was only slightly affected by AFP (Theis et al. 2003), and ANAFP inhibited the proliferation of *C. albicans* and *S. cerevisiae* (Lee et al. 1999). The antifungal protein NAF from *P. nalgiovense* is identical to PAF, but was found to be active against *Mucor*. The methodology of testing antifungal activity might account for this difference, since Geisen (2000) did not purify NAF, but determined the inhibition spectrum according to the inhibition zone around a *P. nalgiovense* colony plug on agar plates. Thus other growth inhibitory proteins might have been present. Finally, Geisen (2000) did not specify the *Mucor* species, which might have been different from the PAF-insensitive *Mucor* strains tested in our study.

PAF inhibited the germination of conidia and the growth of germinated hyphae, which implies that conidia, or germinating conidia, and hyphae contain the same target structures necessary for PAF activity. In contrast to AFP of *A. giganteus*, which did not cause changes in morphology (Theis et al. 2003), an influence of PAF on apical growth was evident in the present study. Similar effects were reported for the antifungal protein AFP1 from *Streptomyces tendae* and for the class of "morphogenic" plant defensins (Bormann et al. 1999; Osborn et al. 1995; Terras et al. 1992). Thus, factors that establish apical growth, e.g. Ca^{2+} fluxes, cell wall synthesis, the transport of cell-wall-material-containing vesicles by the cytoskeleton, and actin polymerization, might represent targets for PAF (Bormann et al. 1999; Levina et al. 1995). The effects of PAF on other filamentous growing organisms, such as filamentous bacteria or filamentous forms of dimorphic fungi, will be the subject of further investigations and should provide additional information regarding this hypothesis. The accumulation of nuclei at broken tips of PAF-affected organisms indeed points to a severe disturbance of cell wall synthesis. Nevertheless, the disruption of the cell wall and destabilization of osmotic turgor can be excluded as primary effects of the protein, because isotonic growth conditions did not inhibit PAF activity.

So far, the mechanism of action of antifungal proteins remains a matter of debate. For plant thionins, membrane permeabilization resulting from binding of the cationic protein to the negatively charged membrane surface and subsequent pore-formation has been discussed (Thevissen et al. 1996). In this respect, small amounts of mono- and divalent cations (up to 50 mM) were shown to severely decrease the potency of antifungal plant proteins, possibly by stabilizing membrane phospholipid structures (Abad et al. 1996; Osborn et al. 1995; Terras et al. 1993; Thevissen et al. 1999). Recently, AFP of *A. giganteus* was found to bind to the membrane or cell wall of sensitive fungi, and it was shown that protein activity was severely reduced with 0.1 M KCl or NaCl (Theis et al. 2003). Although our studies also revealed protective effects of $MgCl_2$ and Na_2SO_4, only a limited decrease of the potency of PAF by KCl and a minimal influence by NaCl were detected. However, the species specificity of PAF cannot be satisfactorily explained by a cation-sensitive protein-membrane interaction alone. Instead, we favor the idea of a more specific interaction of PAF with its target organisms, e.g. an interaction with a putative receptor, as proposed for plant de-

fensins (De Samblanx et al. 1997; Thevissen et al. 1997). Thus, $MgCl_2$, Na_2SO_4, and to some extent KCl, would compete with PAF for an ionic interaction with the putative target. However, this hypothesis still has to be proved.

An effect of PAF on the plasma membrane was demonstrated by a significant potassium efflux. The detection of relatively high concentrations of potassium with untreated hyphae could be either due to a passive or glutamine-induced efflux (Slayman and Slayman 1968), or to a contamination with growth medium, which contained at least 6 mM potassium. However, discrimination between a primary or secondary effect of PAF on the integrity of the plasma membrane is not possible, so far. Membrane permeabilization, for instance, might also result from the generation of reactive oxygen species, which were found intracellularly after PAF treatment. Oxidative radicals are known to disintegrate the phospholipid residues of membranes by peroxidation, e.g. in response to the polyene antimycotic drugs nystatin and amphotericin B (Moore et al. 2000). In addition, the elevated potassium efflux induced by 10 µg PAF/ml (1.6 µM) compared to that by 8.6 µM nystatin suggests that PAF permeabilizes also intracellular membranes, in contrast to nystatin which attacks predominantly the plasma membrane (Roos et al. 1997). Finally, the significant loss of K^+-ions into the supernatant occurred at a PAF concentration that was well above the protein amount necessary to induce growth inhibition and morphological changes, i.e. 1 µg PAF/ml. This clearly points to additional, possibly intracellular effects of PAF apart from the formation of transmembrane pores and K^+-efflux.

Our studies clearly indicate that some differences exist in the mode of action of PAF compared to that of *A. giganteus* AFP and of other antifungal proteins. Presently, we do not know, whether the reported detrimental effects of PAF originate from, or cause membrane permeabilization and changes in morphology, but our results clearly point to the induction of multifactorial effects by PAF. The identification of the site of action of PAF will provide valuable information on the function of this protein. Studies addressing the localization of PAF in sensitive target organisms are currently in progress.

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