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Review

The *Penicillium chrysogenum* antifungal protein PAF, a promising tool for the development of new antifungal therapies and fungal cell biology studies

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Abstract. In recent years the interest in antimicrobial proteins and peptides and their mode of action has been rapidly increasing due to their potential to prevent and combat microbial infections in all areas of life. A detailed knowledge about the function of such proteins is the most important requirement to consider them for future application. Our research in recent years has been focused on the low molecular weight, cysteine-rich and cationic antifungal protein PAF from *Penicillium chrysogenum*, which inhibits the growth of opportunistic zoo-pathogens including *Aspergillus fumigatus,* numerous plant-pathogenic fungi and the model organism *Aspergillus nidulans.* So far, the experimental results indicate that PAF elicits hyperpolarization of the plasma membrane and the activation of ion channels, followed by an increase in reactive oxygen species in the cell and the induction of an apoptosis-like phenotype. Detailed knowledge about the molecular mechanism of action of antifungal proteins such as PAF contributes to the development of new antimicrobial strategies that are urgently needed.

Keywords. Antifungal protein, *Penicillium chrysogenum*, growth inhibition, endocytosis, heterotrimeric G-protein signaling, plasma membrane hyperpolarization, oxidative stress, programmed cell death.

Introduction

Among the estimated 1.5 million fungal species worldwide, only the diminutive number of ~300 species have been described to date to have pathogenic potential or to be causative agents of allergies [1, 2]. However, the number of patients with a compromised health status due to fungal contact has sharply increased during the last decades. The main reasons for this increase are: (i) the development of secondary resistance of microorganisms to conventional drugs as a result of long-term antimicrobial therapies, (ii) a rising number of patients suffering from immunosuppressive infections or diseases, such as AIDS or leukemia [3–8], and (iii) a considerable increase in immunosuppressive therapies prolonging the life of patients with a severely compromised state of health at the cost of an elevated risk for life-threatening fungal infections [9–11]. In contrast to antibiotics, only a limited number of antimycotics are available to date. New antifungal drugs are difficult to generate because the host, *e.g.* vertebrates and plants, and the invading fungal organisms share similar cellular and

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physiological characteristics. Thus, only few fungal targets have been considered for antimycotic drug therapies to date, for example the presence of a cell wall (echinocandins), the lipid composition and the ergosterol content of the fungal plasma membrane (polyenes, azoles, allylamines, morpholines), and the ability to metabolically activate drugs that interfere with DNA and RNA synthesis (nucleoside analogues) [2].

Apart from the severe health threat for humans and animals, plant diseases caused by fungal infections are a reason for crop damage and economic losses. New and cheap strategies are needed to combat fungal attack. For these reasons, novel antimycotics that target unique structures or functions of fungi and that lack severe side effects for the infected host are urgently needed [2, 12, 13].

One approach to encounter these problems is to make use of small, basic, cysteine-rich proteins with antimicrobial activity, which are produced by the most diverse organisms throughout all kingdoms. The number of proteins with recognized antimicrobial activity is rapidly increasing. The diversity of these proteins is reflected in their mode of action (only few examples given, transmembrane pore formation, inhibition of cell wall synthesis, interference with nucleic acids and their synthesis, inhibition of protein synthesis, interference with cell cycle control) and their species specificity. These proteins are able to inhibit the growth of bacteria, yeasts and filamentous fungi, can hinder viral infections or even exhibit tumor cell cytotoxicity. Due to their vast variety, at present the classification of antimicrobial proteins is mainly based on their secondary structure (for reviews refer to [14–21]). Whereas in prokaryotes and lower eukaryotes these proteins might confer an ecological advantage for the producing organism, in higher eukaryotes they are part of the innate immune system and represent the first defense against invading microorganisms [22–25].

One promising antifungal protein is PAF (*Penicillium chrysogenum* antifungal protein), which is produced as a preproprotein by the imperfect filamentous ascomycete *P. chrysogenum.* PAF is a cysteine- and lysine-rich protein of 55 amino acids (aa) with a molecular mass of 6.25 kDa. The abundantly secreted protein can be easily purified from the supernatant by ion exchange chromatography owing to its cationic character [26] and exhibits cytotoxic activity towards a variety of filamentous fungi *in vitro*, including important zoo- and plant-pathogenic fungi [27, 28]. The fact that various mammalian cells remain unaffected and no production of proinflammatory cytokines is induced [29] renders PAF a promising candidate for the development of new antifungal therapies. In addition to its biotechnological importance, PAF and similar antimicrobial peptides can also be considered valuable tools to study those aspects of fungal cell biology that have been little investigated so far, such as protein uptake mechanisms and apoptosis.

This review focuses on an antifungal protein originating from a filamentous ascomycete and points out parallels to and differences from other antimicrobial proteins and their mechanisms of action.

Structure-function analysis of PAF

So far only three other ascomycetes have been reported to secrete antifungal proteins, which are closely related to PAF: *A. giganteus* (AFP), *A. niger* (NAF) and *P. nalgiovense* (NAF) [30–32]. The availability of the whole genome sequence of an increasing number of microorganisms facilitates the search for more genes that encode potentially antifungal proteins. By *in silico* analysis of all currently available fungal genomic databases, a new *paf* orthologous gene could be identified in the genome of the plant pathogenic ascomycete *Gibberella zeae* [33]. Although PAF shows no sequence homology with antimicrobial proteins of organisms other than ascomycetes, it exhibits structural similarities: 13 lysine residues result in a net positive charge (pI 8.9), and six cysteine residues are involved in disulfide bond formation. These primary structural motifs are likely to contribute to a compact tertiary structure that mediates high stability against environmental impact, as our latest results propose (unpublished data).

PAF is synthesized as a precursor protein: a signal sequence of 18 aa residues for protein secretion and a prosequence of 19 aa are located N-terminally; the latter is removed before or during the release of mature PAF (55 aa) into the culture medium [26]. There are various examples of similarly processed antimicrobial proteins, *e.g.* the antifungal protein AFP [32] and the ribotoxin α-sarcin [34, 35] from *Aspergillus giganteus*, killer toxins from *Saccharomyces cerevisiae* [36] and *Ustilago maydis* [37] and the chitin-binding protein of *Streptomyces tendae* [38]. In general, prosequences play an important role as "intramolecular chaperones" by ensuring the interaction of the unfolded protein with the transport machinery of the cell and preventing protein activity before secretion. Only after further protein processing, when the prosequence is cleaved off, does the mature protein adopt its correct tertiary structure and gain its final activity [39].

Indeed, such a protective function of the prosequence could be shown for PAF as well. The effects of secreted PAF as well as of intracellularly retained pro-PAF and

mature PAF were investigated by generating transgenic strains of the genetic model organism *A. nidulans* that expressed various N-terminally truncated forms of the PAF protein [40]. It was demonstrated that expression of prepro-PAF resulted in secretion of the functional recombinant protein and dramatic impairment of growth of the producing strain, accompanied by severe changes in hyphal morphology and cellular ultrastructure. In fact, comparable changes were observed upon external administration of purified, native PAF to wild-type (wt) *A. nidulans* [27, 41]. In contrast, the intracellular expression of recombinant PAF containing the pro-sequence had no detrimental effects. The phenotype of the pro-PAF-producing transgenic strain resembled the wt strain. Thus, the inactivity of the protein and its cytoplasmic localization emphasize the role of the prosequence in preventing premature activity of PAF. Finally, the intracellular expression of mature recombinant PAF in the cytoplasm resulted in relatively mild effects on the growth and morphology of the fungus. These results suggest that either inproper folding accounts for the reduced protein activity of the mature PAF form or that PAF activity is dependent on its passage through the outer layers (plasma membrane and/or cell wall).

Protein activity and specificity

The use of PAF as a novel agent for the development of a new antimicrobial therapy necessitates detailed knowledge about its species specificity as well as its mechanism of action in the target organisms. Using a microdilution method described previously [42], several filamentous ascomycetes, among them important zoo-pathogenic and plant-pathogenic fungi including *A. fumigatus*, *A. niger* and *Botrytis cinerea*, could be detected as most sensitive organisms, whereas no activity of PAF was observed against prokaryotes or yeasts [27, 28, 33, 43] (Table 1). All sensitive fungal strains tested reacted at any time point in a dose-dependent manner [27]. With the most sensitive species, growth retardation was observed at sublethal concentrations, whereas increasing PAF concentrations had fungicidal effects. The minimal inhibitory concentrations (MICs) of PAF were found within micromolar concentrations (Table 1), comparable to those of general antimycotics that have been tested under the same assay conditions; for example, for *A. fumigatus* the MICs were 4 μM for amphotericin B and caspofungin, 6 μM for itraconazole and 12 μM for voriconazole.

Conidia are the most sensitive cellular structures: for example, 8 μM PAF inhibits efficient germination of *A. niger* conidia and prevents colony formation. Removal of PAF does not cure this fungicidal effect. In contrast, fungistatic properties can be observed with hyphae: when 8 μM PAF are added to a 24-h liquid culture of *A. niger*, hyphal growth is delayed but does not cease in the presence of the antifungal protein.

Table 1. The antifungal activity of the *P. chrysogenum* protein PAF.

Organism	IC_{50}	MIC
B. cinerea[a]	0.03	0.3
A. flavus	3	>16
A. fumigatus	1.6	16
A. nidulans	0.8	8
A. niger	0.16	3

Protein concentrations (μM) required for 50% growth inhibition (IC_{50}) and the minimal inhibitory concentration (MIC) were determined after 30–40 h incubation in complete medium by dose-response curves as described [27].

[a] The values were determined after 72 h incubation.

Growth retardation at sublethal concentrations is associated with a severe change in morphology, which becomes apparent as crippled hyphae with atypical branching. This hyperbranched phenotype is accompanied by the burst of a few hyphae at their tips [27] and closely resembles the effects of "morphogenic" antimicrobial proteins such as certain plant defensins [21, 44, 45]. PAF toxicity is strongly influenced by the ionic strength of the culture medium. The PAF-induced effects can be partially neutralized in the presence of magnesium ions (>20 mM) and potassium ions (>80 mM). In contrast, sodium ions had no significant effect at the concentrations tested (20–100 mM) [27]. In plants and other organisms, *e.g.* bacteria and insects, the cation-dependent neutralization of the positively charged antimicrobial proteins was explained by the inhibition of their direct binding to negatively charged phospholipid moieties present in the plasma membrane of target organisms [42, 45–52], which may indeed be the case for numerous proteins with general antimicrobial activity. However, the exclusive specificity of PAF towards filamentous fungi and not towards bacteria or yeasts strongly supports the hypothesis of a distinct mode of action for PAF, involving specific targets or increased target accessibility in filamentous fungi, as proposed for the action of certain plant defensins [21, 53, 54].

Localization of PAF

The identification of the localization of toxic proteins in target cells could provide important information

about their mode of action. Several studies have addressed this question with fluorescence-labeled proteins. Internalization has been shown for numerous antimicrobial toxins, and intracellular targets that interfere with DNA/RNA condensation, protein synthesis or chaperone-assisted protein folding have been identified [16]. For the antifungal defensin 1 of *Pisum sativum*, colocalization of the fluorescence-labeled protein with the nucleus was shown by fluorescence microscopy, which parallels with its reported interaction with cyclin F and cell cycle inhibition in *Neurospora crassa* [55]. The signal of the fluorescence-labeled antifungal protein AFP from *A. giganteus* – a protein homologous to PAF – was found to accumulate at the extracellular layers of sensitive fungi but to be internalized by resistant fungi [56]. In another report, the fluorescence signal colocalized with nuclei [57], which seems to be supported by the reported *in vitro* DNA/RNA-binding capacities of AFP through an oligonucleotide/oligosaccharide-binding (OB fold) structural motif [58]. This OB fold structure could, however, also account for the *in vitro* chitin-binding properties of AFP observed by Hagen et al. [59] and may explain the observation of the localization of AFP at the outer cellular layers of sensitive microorganisms.

Indirect immunofluorescence staining (IIF) represents a reliable method of choice, provided that specific antibodies are available. Using a specific polyclonal antiserum, internalization of PAF was shown in sensitive fungi (*e.g. A. niger*, *A. fumigatus*, *A. nidulans*), whereas no specific interaction of the protein was evident with resistant fungi (*e.g. A. terreus*, *Mucor sp.*) [60]. This signal pattern contrasts in part to that reported for the AFP protein. The distribution of the PAF-specific fluorescence signal clearly shows cytoplasmic localization, and no co-localization with fluorescence-labeled nuclei could be verified (unpublished data). Furthermore, no *in vitro* nucleotide-binding activities with PAF were reported [33]. Instead, the internalization of PAF was dependent on a functional protein structure, the active metabolism of the test organism, the availability of ATP and intact actin microfilaments. All these prerequisites substantiate an endocytosis-like mechanism [60]. Indeed, endocytosis has been suggested for the delivery of various protein toxins to their target site [61], *e.g.* the α-sarcin from *A. giganteus* [62], the plant toxin ricin [63], the human neutrophil protein defensin [64] and various bacterial toxins [65–67].

Mode of action

Many antimicrobial proteins exert their function by their pore-forming activity and permeabilization of the plasma membrane [42, 49–51, 68–70]. Detailed studies with PAF excluded this hypothesis and allowed important insights into a clearly different mode of action.

Heterotrimeric G-protein signaling. It was shown in *S. cerevisiae* and *A. nidulans* that antifungal proteins interfere with G-protein signaling, although the data are controversial [47, 71, 72]. The role that G-protein signaling plays in mediating the toxicity of antifungal proteins seems to strongly depend on the antifungal proteins tested.

Using a transgenic *A. nidulans* strain that carries a mutation in the *fadA* (*fadA*^{G203R}) gene, the G-protein-coupled activity of PAF was confirmed [73]. The *fadA* gene encodes the heterotrimeric G-protein α-subunit FadA. The exchange of a glycine residue by an arginine at position 203 (*fadA*^{G203R}) results in inhibition of the dissociation of the Gα subunit from Gβγ [74]. Proliferation assays revealed reduced sensitivity of the *A. nidulans fadA*^{G203R} mutant strain to PAF [73], indicating that PAF toxicity requires active heterotrimeric G-protein signaling. This finding resembles that of Coca et al. [71], who proved resistance of a *fadA*^{G203R} *A. nidulans* mutant strain towards the antifungal protein osmotin from plants. In analogy to other toxins, it is possible that PAF interacts with one of the G-protein subunits directly [75, 76] or that PAF interferes indirectly with G-protein signal transduction. Alternatively, mutations in components of the G-protein signaling pathway, as in *fadA*^{G203R}, might affect the target accessibility for PAF and hence account for the observed PAF-resistance.

Plasma membrane polarization. One major effect of many antimicrobial proteins is the destabilization of the plasma membrane by pore formation, which leads to ion leakage [14]. A significant elevation in the potassium concentration in the supernatant of PAF-treated *A. nidulans* hyphae was detected, which could not be explained by the leakage of burst hyphal tips under the applied experimental conditions [27]. Measurements of the membrane potential revealed hyperpolarization of the fungal membranes at the hyphal tips immediately after PAF exposure [41]. In our opinion, the hyperpolarization event excludes an unspecific ion leakage of the membrane, which would lead to depolarization of the plasma membrane; instead, it supports the assumption of the induction of selective potassium permeability. The perturbation of the intracellular ion homeostasis

might account for the observed changes in the morphology of PAF-treated sensitive fungi. This mechanism resembles that of certain plant defensins [47] but diverges from the reported mode of action of antimicrobial proteins from bacteria, insects and humans, which form pores with little species specificity in the membranes of target organisms and lead to cell leakage [51, 69, 70].

Induction of intracellular ROS. Macromolecules, such as lipids, proteins and nucleic acids, as well as organelles, especially mitochondria, are challenged by reactive oxygen species (ROS) in organisms with aerobic metabolism. Adaptive responses prevent major damage in healthy cells; however, the accumulation of ROS in response to specific stimuli can have a severe impact on cells, resulting in the oxidation of biopolymers and, consequently, in the destruction of cellular membranes and organelles. In this respect, mitochondria are not only the main ROS source but are also especially prone to oxidative damage, and their ROS-induced disintegration is known as "mitoptosis" [77–80]. Importantly, the generation of elevated levels of ROS represents a major elicitor of programmed cell death (PCD) in both lower and higher eukaryotes [81–87].

Indeed, the exposure of sensitive *Aspergillus* strains such as *A. niger*, *A. fumigatus*, *A. nidulans* and transgenic *A. nidulans* mutants (expressing recombinant PAF) to PAF induced the generation of ROS [27, 40, 73]. In contrast, no such effects could be detected in insensitive fungi (*e.g. A. terreus*, *Mucor sp.*). Oxidative damage of cell organelles in PAF-treated hyphae was illustrated by aberrant ultrastructural morphology of the mitochondria in PAF-treated *A. nidulans* cells and in the transgenic PAF-expressing *A. nidulans* strains [40, 73]. Transmission electron microscopy revealed that mitochondria of PAF-affected cells were less well defined and often displayed discontinuous or missing outer membranes compared to the organelles in the cytoplasm of control hyphae.

Induction of an apoptosis-like phenotype. When the ROS burden of the cell reaches a critical level, basic physiological functions are impaired and PCD might occur. The onset of PCD leads to a distinct phenotype showing phosphatidylserine (PS) externalization, membrane blebbing, increased vacuolization, DNA and nuclear fragmentation, and apoptotic body formation. Although apoptosis was thought to be confined to multicellular higher eukaryotes in the past, there is an increasing body of evidence that prokaryotes and lower eukaryotes such as yeasts and filamentous fungi also show characteristics of apoptosis-like cell death [84, 85, 88–93].

The potential of PAF to induce apoptosis in sensitive fungi was tested with *A. nidulans* protoplasts, either left untreated or exposed to PAF, using an array of cytological assays. The increased exposure of PS on the surface of PAF-treated protoplasts and the appearance of DNA strand breaks were shown by Annexin V staining and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL), respectively. Ultrastructural analysis by transmission electron microscopy revealed the loss of cell wall granularity and architecture, shrinkage of the cytoplasm, detachment of the plasma membrane from the cell wall and the formation of microvesicle-like structures between the cell wall and the cell membrane, whereby the latter two events resembled membrane blebbing of apoptotic cells [94–97]. Furthermore, the nuclear membrane disappeared and large vacuoles became apparent. With progressing cell injury, no distinct cytoplasmic organelles were detectable, and the whole cell had disintegrated. Thus PAF seems to induce a strictly regulated cell death. This observation further underlines that a specific interaction of the protein with the target organism is highly probable.

Hypothesis of the mechanism of action of the antifungal protein PAF. Since no direct interacting molecule has been identified so far, we can only speculate on the first interaction event of PAF with sensitive target fungi (Fig. 1). The cell wall represents the first characteristic structure that could act as a selective barrier, mediating the access of antifungal proteins to sensitive fungi depending on its composition. *In vitro* chitin-binding properties for the PAF-homologous AFP protein from *A. giganteus* have been reported [59, 98], although this has not been shown *in vivo* so far.

Several studies propose the interaction of antifungal proteins with components of the plasma membrane [46, 53, 99, 100]. Indeed, many antimicrobial proteins contain cationic and/or hydrophobic motifs that could promote interaction with the plasma membrane and its permeabilization. For example, structural analysis of the *A. giganteus* AFP allows the assumption that the presence of such sites promotes the binding and insertion of the antifungal protein into the plasma membrane of sensitive fungi [101]. The plasma membrane composition differs significantly between bacteria, plants, fungi and mammals. Excellent reviews on this topic have been written [102, 103]. Whereas bacterial membranes contain more phospholipids, phosphoglycerolipids, glycoglycerolipids, sphingolipids and sterols are major components of cellular membranes in eukaryotes. As for plant and insect defensins, yeast sphingolipids, such as manno-

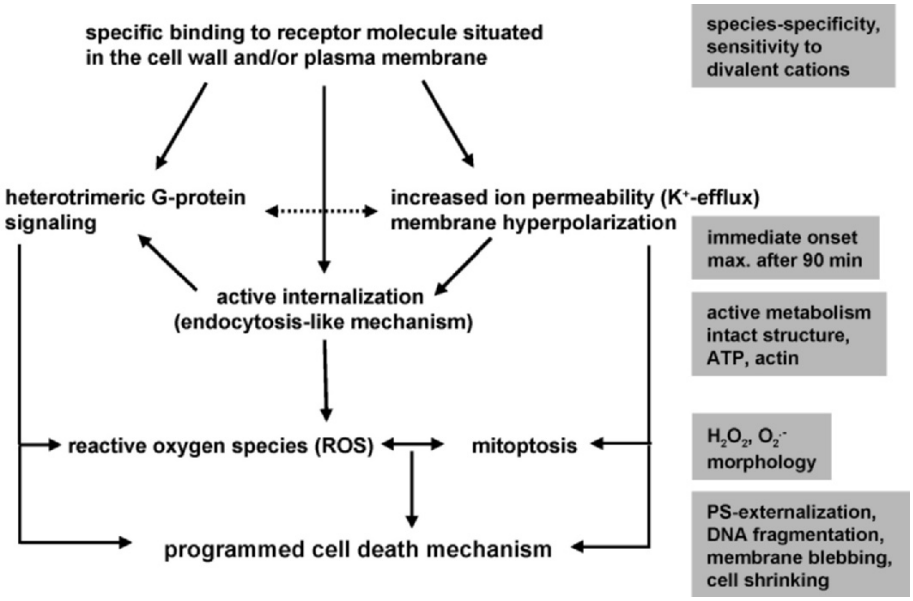

Figure 1. Schematic diagram for the hypothetical mechanism of action of the antifungal protein PAF (*Penicillium chrysogenum* antifungal protein). Species specificity of the PAF protein may be determined by specific molecules expressed on the outer layer of the organism (*e.g.* cell wall and/or plasma membrane). The interaction of PAF with the target organism immediately elicits hyperpolarization of the membrane and increased ion permeability. This effect could involve heterotrimeric G-protein signaling (dotted arrow), which induces the elevation of reactive oxygen species (ROS). This increase in ROS leads to the oxidation of biomolecules and the disintegration of cellular structures (*e.g.* mitochondria – mitoptosis), which in turn might further aggravate the ROS burden of the cell and induce a programmed cell death phenotype in sensitive fungi. The cytotoxicity of PAF is accompanied by its active internalization into the target cell.

syldiinositolphosphorylceramide, were found to interact with the defensin DmAMP1 from dahlia [100]. The yeast sphingolipid glucosylceramide (GlcCer) interacts with both the defensin RsAFP2 from radish and the insect defensin heliomycin, although distinct motifs of GlcCer seem to be involved in the latter interaction [46]. Furthermore, analysis of *N. crassa* mutants with increased resistance towards plant defensins revealed differences in the GlcCer structure and significantly elevated levels of steryl glycosides [104]. This indicates that interaction of antimicrobial proteins with the plasma membrane of target organisms is not only determined by the presence of a single kind of plasma membrane component; lipid composition, molar distribution and structure of the components determine the species-specific interaction with target fungi. Interestingly, the binding of antimicrobial proteins to the cell membrane need not inevitably result in its disruption by pore formation as generally proposed. Thevissen et al. [102] presented a model in which plant defensins bind to sphingolipid rafts in the plasma membrane and subsequently change the membrane permeability for specific ions. Alternatively, interference with signal transduction by lipid rafts could occur. Fantini et al. [105] reported the existence of glycosphingolipid-enriched microdomains on mammalian cells that are closely associated with G-regulatory proteins and function as docking sites for microbial toxins from where they activate G-protein signaling. It is conceivable that such domains also exist on fungal cells. So far no interaction of PAF with any of these components of the cell layers – cell wall or plasma membrane – could be verified.

Nevertheless, PAF elicits an immediate response at the plasma membrane, evoking hyperpolarization of the membrane near the hyphal tips and an increase in potassium ion permeability. Both events could result from PAF-mediated heterotrimeric G-protein signaling, which is known to regulate, among many other effects, the levels of second messengers, such as cAMP, or ion channels [106]. Thus, activation of heterotrimeric G-protein signaling could be one of the early events that is induced by PAF and that could account for further effects, such as ROS formation and the induction of PCD [107–110]. Increased ROS formation could impair mitochondrial integrity, as shown by transmission electron microscopy [41], aggravate the ROS burden of the cell and thus contribute to the induction of the apoptosis-like phenotype observed with PAF sensitivity [41]. Induction of PCD has been shown to occur in yeast exposed to antifungal proteins that are structurally unrelated to PAF, *e.g.* the plant osmotin [16, 111], the yeast killer toxins [112] and recently the amphibian dermaseptin [113]; the report by Leiter et al. has demonstrated for the first time an apoptosis-like phenotype in a filamentous ascomycete as a result of the action of a cationic, cysteine-rich antifungal protein, PAF [41].

Active internalization seems to be closely related to PAF cytotoxicity. However, the question of whether the uptake of PAF is receptor-mediated must still be intensively studied. In case that internalization is a prerequisite for its cytotoxicity, the existence of intracellular interaction molecules has to be assumed. The elucidation of the mechanisms of action of antimicrobial proteins in more detail deserves further intensive studies, *e.g.* by identifying and characterizing resistant or hypersensitive fungal mutants as documented for several plant defensins [114–116]. Compared to the vast number of well-characterized *S. cerevisiae* mutants, the number of mutants available from filamentous fungi (*A. nidulans*, *A. niger*, *A. fumigatus*, etc.) is rather restricted, which is one factor that has hampered the progress in elucidating the cell biology of these organisms. Importantly, antifungal proteins in general represent a valuable tool to increase our knowledge in certain aspects of the cell biology of filamentous fungi including endocytosis, heterotrimeric G-protein signal transduction, ROS production and PCD.

Important aspects for potential future applications

High stability *in vivo*, low production costs and negligible side effects are the most important criteria for considering antimicrobial proteins for future pharmaceutical applications. The fact that PAF evokes perturbation of the plasma membrane might pose a severe problem for its potential application in mammalian cells. Therefore, the effects of PAF on various primary excitable and non-excitable mammalian cells were investigated *in vitro* [29]. PAF shows no cytotoxic effects on human endothelial cells and fails to activate voltage-gated potassium channels of neurons, skeletal muscle fibers and astrocytes from rat. Furthermore, neither the hyperpolarization-activated non-specific cationic current nor L-type calcium current in cells of semi-thin slices of the rat cochlear nucleus is affected by PAF, as shown by electrophysiological experiments. The latter observation clearly contrasts the effects of other small antimicrobial proteins such as the KP4 killer toxin from *U. maydis* and the seed defensin MsDef1 from *Medicago sativa* that block L-type calcium channels in mammalian cells [117, 118]. Finally, PAF has no erythrocytelysing activity and does not stimulate human peripheral activated monocytes to increase the production of the pro-inflammatory cytokines IL-6, IL-8 and TNF-α [29], whereby the lack of any detrimental effect on cultured mammalian cells is not attributed to serum inactivation. The homologous antifungal protein AFP from *A. giganteus* showed a comparable lack of activity against primary mammalian cells *in vitro*, which supports the promises of members of this new group of antifungals as potential future therapeutics [119].

Similar to the resistance of primary mammalian cells towards PAF, no cytotoxicity of PAF on the human teratocarcinoma cell line NT2 could be detected (unpublished data), which stands in contrast to reports of the antitumor activity of certain human defensins [18]. However, this topic deserves further detailed investigations.

In a most recent study, it was demonstrated that PAF acts synergistically with the statins lovastatin, rosuvastatin and atorvastatin, which are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and exhibit potent antifungal activity [43]. This fact renders the investigation of the interactions between antimicrobial proteins and other types of antifungal compounds very promising and supports the assumption that these proteins can be regarded as potential candidates in future antifungal drug research and combinatorial therapies.

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