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MINI-REVIEW

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Small, basic antifungal proteins secreted from filamentous ascomycetes: a comparative study regarding expression, structure, function and potential application

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Abstract Peptides and proteins with antimicrobial activity are produced throughout all kingdoms in nature, from prokaryotes to lower and higher eukaryotes, including fungi, plants, invertebrates and vertebrates. These proteins contribute to an important constitutive or induced defense mechanism of the producer against microorganisms. According to their variety in structure and function, these proteins are classified arbitrarily into groups that are based on their mechanism of action, their structure and their similarity to other known proteins. The present review focuses on a new group of antimicrobial proteins, namely small, basic and cysteine-rich antifungal proteins, which are secreted from filamentous fungi of the group Ascomycetes. These proteins are encoded by orthologous genes and exhibit both similarities and differences concerning their species-specificity, primary structure, protein activity and target sites. The properties of these proteins, their possible mode of action and their potential application for human benefits are discussed in comparison with other already well known antimicrobial proteins.

Introduction

Antimicrobial proteins are produced from the most diverse organisms throughout the phylogenetic tree, either constitutively or upon induction; and they inhibit the growth of bacteria, yeasts, filamentous fungi and viruses. In prokaryotes and lower eukaryotes, antimicrobial proteins might confer an ecological advantage for the producing organism in the competition for nutrients. In higher eukaryotes, these proteins are part of the innate immune system and are the first defense line against invading microorganisms (Baker et al. 1997; Fritig et al. 1998; Lehrer and Ganz 1999; Raj and Dentino 2002).

The incidence of microbial infections in humans is dramatically rising. On the one hand, this is either due to the increasing number of patients suffering from immunosuppressive infections or diseases, e.g., AIDS or leukemia, or due to immunosuppressive therapies (Denning 1991; Dupont et al. 2000; Levitz 1992; Marr et al. 2002; Murphy and Gould 1999). On the other hand, advanced and sophisticated medical treatment prolongs the life of severely affected patients at the cost of an elevated risk for life-threatening microbial infections, even with low-virulence organisms (Hibberd and Rubin 1994; Vanholder and Van Biesen 2002; Walsh and Pizzo 1988). In addition, pathogenic microorganisms which become resistant to conventional drugs are sharply increasing in number, partly because of intrinsic primary resistance or because of secondary resistance developed as a result of long-term antimicrobial therapies (Sefton 2002). Concerning fungal infections, problems in the generation of new drugs are encountered due to the high cellular and physiological similarities between host and invading organism. Fungi comprise about 1.5 million species of which at least 74,000 species are described (Hawksworth 2001) and more than 300 species are reported to be potentially pathogenic or to cause allergy symptoms in man (Gupte et al. 2002). New antimycotics are badly needed and the world market for antifungals is expanding dramatically (Gupte et al. 2002; Pocsi et al. 2001; Steinbach and Stevens 2003). Another aspect is that infectious plant diseases still cause human suffering and enormous economic losses. The world food supply is critical, especially in those regions where an increasing human population encounters decreasing amounts of land available for agriculture. New and cheap strategies are needed to improve the resistance of crops against microbial attack in order to augment agricultural production. For these reasons, drugs have to be found and developed which target structures or functions unique to those microorganisms and which show no or negligible side-effects in the host.

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Antimicrobial proteins from many organisms bear great potential for the treatment or prevention of infections. They are heterogeneous in their origin and in their structural, biochemical and functional properties, which makes their classification difficult and somewhat arbitrary. This review focuses exclusively on small, basic and cysteine-rich antifungal proteins produced by ascomycetes; and their expression, structure, species-specificity and targets are summarized. Although their mechanism of action is mostly not well understood, some possible functions and future biotechnological applications are discussed.

Readers are directed to reviews of other antimicrobial proteins (Broekaert et al. 1995; Dimarcq et al. 1998; Epand and Vogel 1999; Garcia-Olmedo et al. 1998; Kamysz et al. 2003; Nissen-Meyer and Nes 1997; Oren and Shai 1998; Papagianni 2003; Raj and Dentino 2002; Schmitt and Breinig 2002; Selitrennikoff 2001; Tossi et al. 2000; Theis and Stahl 2004).

Primary amino acid sequence and protein structure

The filamentous ascomycetes *Aspergillus giganteus*, *A. niger*, *Penicillium nalgiovense* and *P. chrysogenum* (an imperfect fungus) secrete the homologous antifungal peptides AFP, ANAFP, NAF and PAF, respectively (Geisen 2000; Lee et al. 1999; Marx et al. 1995; Wnendt et al. 1994). In *Trichoderma viride*, a silent and intron-lacking gene was identified with an astonishing 100% nucleotide sequence identity to the *afp* gene of *A. giganteus* (Hao et al. 2000); and only recently the intron requirement for AFP gene expression in *T. viride* was shown (Xu and Gong 2003). Furthermore, examination of all currently available fungal genomic databases identified a *paf* orthologous gene present in the genome of *Gibberella zeae* (EMBL accession no. AACM01000196). The match is to a peptide that has yet to be isolated and characterized. However, the putative gene codes for a protein that exhibits similarities to other antifungal proteins from ascomycetes and that was named *G. zeae* antimicrobial protein A (GAMA; GenBank accession no. BK004091).

AFP, NAF and PAF are synthesized as preproproteins, which contain both a signal sequence for secretion and a prosequence that is removed before or during their release into the supernatant. For ANAFP, only the amino acid (aa) sequence of the mature protein has been identified so far (Lee et al. 1999), but from the close relatedness, it can be assumed that ANAFP is also encoded as a preproprotein. The primary aa sequences of the mature antifungal proteins show 31–42% identity, whereby NAF and PAF are 100% identical. The putative protein GAMA exhibits the highest similarity to PAF/NAF (53.3% identity/73.9% homology; Fig. 1). Although these proteins do not have any significant sequence homology to proteins from other organisms, they share common features with functionally related proteins from plants, invertebrates and amphibians: they are small (51–58 aa, 5.8–6.6 kDa), cysteine-rich and basic, due to the presence of a high amount of arginine and lysine residues (Table 1). No protein modifications have been detected so far.

Numerous proteins with antimicrobial potency carry a prepro-sequence, e.g., the chitin-binding protein from *Streptomyces tendae* (Bormann et al. 1999), the killer toxins from *Saccharomyces cerevisiae* (Schmitt and Breinig 2002) and from *Ustilago maydis* (Tao et al. 1990) and the ribotoxins α-sarcin from *A. giganteus* (Oka et al. 1990; Wnendt et al. 1993) and restrictocin and mitogillin from *A. restrictus* (Fernandez-Luna et al. 1985; Lamy and Davies 1991). From studies with *Rhizopus oryzae* lipase, the prosequence was attributed the role of an "intramolecular chaperone" that is necessary for correct protein-folding into an active form during secretion (Inouye 1991; Takahashi et al. 2001). For ribotoxins, a similar mechanism was suggested to protect the producer from committing suicide during synthesis (Endo et al. 1993; Lamy and Davies 1991; Lamy et al. 1992). In analogy, the prosequence might keep the harmful antifungal proteins in an inactive structural stage as long as they are processed intracellularly, thus conferring immunity to the producing ascomycetes. Interestingly, an inactive long-form AFP (lfAFP) with six extra N-terminal aa has been reported to appear in the broth of cultures of *A. giganteus* at 48–72 h (Martinez-Ruiz et al. 1997; Meyer et al. 2002). The lfAFP disappears as the culture proceeds, indicating an extracellular hydrolyzation to the active AFP. Many secreted proteins in fungi and higher eukaryotes are proteolytically processed during transport by proteases belonging to a subtilisin-like serine protease subfamily, e.g., a *kex2* or *kexB* gene product of *S. cerevisiae* or *A. niger*, respectively (Eisfeld et al. 2002; Jalving et al. 2000;

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                                   ▼                ****
(lf)AFP  1  MKFVSLASLGFALVAALGAVATPVEADSLTAGGLDARDESAVLATYNGKC   50
PAF/NAF  1  MQITTVALF---LFAAMGGVATPIES---VSNDLDARAEAGVLAKYTGKC   44
GAMA     1  MQFSTIIPL---FVAAMGVVATPVNS---PAQELDARGNLFPRLEYWGKC   44
ANAFP    1                                           LSKYGGEC    8

AFP     51  YKKDNICKYKAQSGKTAICKC---YVKKCPRDGAKCEFDSYKGKCYC---- 94
PAF     45  TKSKNECKYKNDAGKDTFIKCPKFDNKKCTKDNNKCTVDTYNNAVDCD--- 92
GAMA    45  TKAENRCKYKNDKGKDVLQNCPKFDNKKCTKDGNSCKWDSASKALTCY--- 92
ANAFP   19  SLEHNTCTYRKD-GKNHVVSCPSAANLRCKTDRHHCEYDDHHKTVDCQTPV 58
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Fig. 1 Alignment of the protein aa sequences of AFP/lfAFP, PAF/NAF, putative GAMA and ANAFP. The aa sequence of the putative protein GAMA (GenBank accession no. BK004091) was deduced from the encoding nucleotide sequence present in the *G. zeae* genome (GenBank accession no. AACM01000196). Identical aa sequences are highlighted in *black*, the signal peptide cleavage site is indicated by an *arrowhead* and the putative consensus sequence for cleavage of the prosequence is *overlined* and marked by four *asterisks*. The first aa of the mature proteins are *underlined*. The six additional N-terminal aa that are present in lfAFP are *in italics*

Table 1 Parameters of the mature protein forms of AFP, ANAFP, NAF, PAF and putative GAMA analyzed by the ProtParam tool (http://www.expasy.org/cgi-bin/protparam)

Protein	Number of aa	Mass (kDa)	Cys residues	Lys/Arg residues	Theoretical pI	Reference
AFP	51	5.8	8	12/1	9.27	Wnendt et al. (1994)
lfAFP	57	6.4	8	12/1	9.05	Martinez-Ruiz et al. (1997)
ANAFP	58	6.6	6	5/3	7.14	Lee et al. (1999)
NAF	55	6.3	6	13/0	8.93	Geisen (2000)
PAF	55	6.3	6	13/0	8.93	Marx et al. (1995)
[a]GAMA	55	6.4	6	12/1	9.10	GenBank accession no. BK004091

[a]Putative parameters

Punt et al. 2003; Seidah and Chretien 1997; Tao et al. 1990). The consensus cleavage site of the processing enzyme was identified to occur after one or two C-terminal basic aa, preferentially after an arginine residue. None of the mold antifungal proteins contain a corresponding cleavage site directly adjacent to the N-terminus of the active mature protein form. However, Martinez-Ruiz et al. (1997) aligned the primary structures at the mature protein–prosequence peptide boundaries of (lf)AFP, ANAFP, PAF and other secreted fungal proteins and identified the consensus sequence XZAR (X=I or L, Z=D or E; Fig. 1), which resembles the Kex2p cleavage sites of various killer toxins from *S. cerevisiae*, *U. maydis* and *Kluyveromyces lactis* (Tao et al. 1990). The XZAR motif might indeed represent a protease cleavage site, e.g., for a Kex2p-homologue or for an alternative endoproteinase; and the antifungal proteins might be further N-terminally trimmed or processed to the active form by another as yet unidentified protease. In the case of lfAFP, these two processing steps would have to be locally and temporally separated and the final processing step would occur by a protease present in the supernatant.

Detailed structural studies revealed that the mature form of the *A. giganteus* AFP folds into five antiparallel β strands which define a small and compact β barrel, stabilized by internal disulfide bridges formed by eight cysteine residues (Campos-Olivas et al. 1995). This folding pattern determines its high stability against protease degradation, high temperature and within a broad pH range (Lacadena et al. 1995). A cationic site (Lys 9, Lys 10, Lys 32) and a spacially adjacent hydrophobic stretch (Val 30, Tyr 29, Tyr 45, Tyr 50) could promote binding to negatively charged residues and to hydrophobic phospholipid moieties in the plasma membrane, respectively, where AFP can induce detrimental effects on the target organism (Campos-Olivas et al. 1995; Lacadena et al. 1995; Nakaya et al. 1990). NMR-based structural analysis of the *P. chrysogenum* PAF is currently in progress (Marx, unpublished data), but it can be assumed that PAF, NAF and ANAFP take an AFP-similar tertiary structure, although only three disulfide bridges can be formed by the six cysteine residues present in these proteins. Interestingly, these cysteine residues (which correspond to aa positions 7, 14, 28, 36, 43 and 54 in the mature PAF protein) are conserved within all fungal proteins, whereas only AFP carries two additional cysteine residues (Fig. 1).

Transcriptional regulation

The expression pattern of the *afp* gene was studied in detail, and some information exists for the expression of the *paf* gene. The 5′-upstream regions of *afp* and *paf* display characteristic features of fungal promoters and carry several putative regulatory elements that might be involved in the transcriptional regulation of both genes in response to environmental signals (Fig. 2A,B; Marx et al. 1995; Meyer et al. 2002). Apart from a TATAA box, two CCAAT consensus sequences for the binding of a HAP-like complex are present in both promoters (Ballance 1986; Brackhage et al. 1999). Induction of *afp* gene expression was observed under alkaline growth conditions (Meyer et al. 2002), which correlates well with the presence of two consensus target sequences (5′-GCCARG) for the wide-domain pH-regulatory PACC transcription factor (Tilburn et al. 1995). In addition, the binding of heterologously expressed *A. nidulans* PACC to respective consensus motifs of the *afp* promoter was proved recently by band-shift experiments (Meyer and Stahl 2002). Despite the occurrence of a CREA-binding site (5′-SYGGRG; Kulmburg et al. 1993), no carbon catabolite repression was observed, which underlines the need for at least two copies of the consensus sequence for CREA-dependent gene regulation (Cubero and Scazzocchio 1994). Five stress elements (STRE, 5′-CCCCT; Toone and Jones 1998), a heat-shock motif (HSE, 5′-NTTCNNGANTTCN; Kobayashi and McEntee 1993) and two GATA factor-binding sites (5′-HGATAR), which are known to mediate response to various environmental stimuli, such as circadian rhythm, iron metabolism or sexual development (Scazzocchio 2000), might be responsible for a stress-related expression pattern (e.g., transcriptional induction by carbon starvation, the presence of excess NaCl or ethanol, heat-shock), whereas no or only minor changes in gene transcription levels were found under N_2-starvation or in the presence of H_2O_2 (Meyer et al. 2002). In addition, phosphate was observed to strongly inhibit AFP production (Meyer and Stahl 2002).

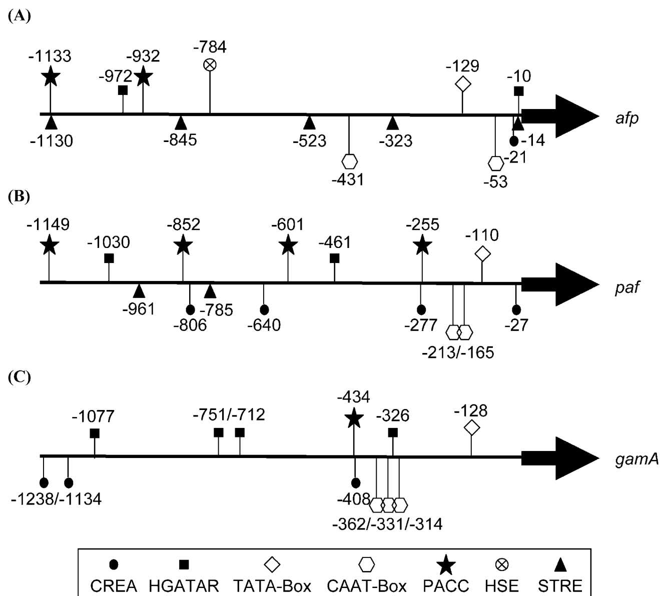

Fig. 2 Comparative analysis of putative binding site positions for fungal transcription factors in the promoter region of the genes *afp* (**A**), *paf* (**B**; GenBank update, accession no. U22944) and putative *gamA* (**C**). The putative binding sites for the carbon catabolite repressor (CREA), the GATA factors (HGATAR), the TATA factor (TATA box), the HAP-like complex (CAAT box), the pH-regulatory factor (PACC), the heat-shock protein (HSE) and the stress-regulatory protein (STRE) are marked by respective symbols listed in the box. Their positions are indicated in base pairs relative to the start codon

The *paf* promoter contains four PACC consensus motifs, but no expression studies concerning pH-dependent gene regulation have been published yet. In contrast to the *afp* promoter, four putative CREA-binding sites are present in the *paf* 5′-upstream region which might mediate gene repression in the presence of glucose (Marx et al. 1995; Vollebregt et al. 1994). Transcription was found to be induced by carbon starvation or limiting glucose concentrations (Vollebregt et al. 1994), or in the presence of secondary carbon sources, e.g., sucrose, starch (Marx et al. 1995) or xylan, but not xylose (Marx, unpublished data). This indicates that not only might carbon catabolite repression be involved, but also transcription induction by specific secondary carbon sources. Two GATA factor-binding sites might play a role in nitrogen metabolite repression (Fig. 2B): glutamine was found to repress and $NaNO_3$ to induce *paf* mRNA synthesis (Marx et al. 1995). In addition, two STRE motifs, but no HSE consensus sequence are present. Indeed, no increase in PAF protein production was found to occur after heat-shock induction (Marx, unpublished data). Moreover, the phosphate concentration in the culture medium did not influence the protein yield of PAF compared with that of AFP (Marx, unpublished data).

The *gaf* promoter displays some characteristic features which are in common with the promoters of *afp* and *paf*, e.g., a TATAA box, three CCAAT boxes, one PACC consensus target site, three CREA consensus motifs and four putative HGATAR sites (Fig. 2C). Neither a STRE nor a HSE motif can be found.

At present, no general pattern can be deduced from the expression studies performed with *afp* and *paf*, except that the maximum mRNA and protein yield is reached during the stationary growth phase after 70–90 h of cultivation (Meyer et al. 2002; Marx, unpublished data). Many organisms produce antimicrobial peptides constitutively or in response to infection or injury (Dimarcq et al. 1998; Ganz 1994; Garcia-Olmedo et al. 1998; Penninckx et al. 1996, 1998; Tossi et al. 2000). Since antifungal activity attributed to the proteins from ascomycetes is the only function identified so far, it can be assumed that their expression is induced or elevated in the presence of other fungi and contributes to an ecological advantage for the producing organisms in the competition for nutrients. From co-cultivation studies of *A. giganteus* with various AFP-sensitive and resistant microorganisms, it became evident that induction of *afp* expression seems to be primarily dependent on the culture conditions, but to a less extent on the presence of co-cultivants (Jacobs 1995; Meyer and Stahl 2003). This is in agreement with the fact that the producing ascomycetes secrete these proteins in pure culture. Therefore, it is more likely that environmental stimuli play a major role in expression induction, which might also include an as yet unidentified cellular signal that is present on living hyphae in response to stress, nutritional supply, cellular density or hyphal age (Meyer and Stahl 2003). Future studies will be necessary to unravel whether these fungal proteins interact with specific cellular receptors and whether they comprise an as yet unidentified function, e.g., cellular signaling.

Protein activity and mode of action

Growth inhibition was determined for various microorganisms and species-specificity differs significantly between these antifungal proteins (Table 2). Major activity was detected for all proteins against members of the filamentous ascomycetes, among them important zoopathogenic and plant-pathogenic fungi, whereas only ANAFP showed growth inhibition of the yeasts *Candida albicans* and *S. cerevisiae* (Kaiserer et al. 2003; Lee et al. 1999; Theis et al. 2003; Vila et al. 2001). Little information is available on the protein activity against basidiomycetes, with the exception of *Rhodotorula mucilaginosa* which showed resistance to AFP and *Trichosporon beigelii* which was sensitive to ANAFP (Lacadena et al. 1995; Lee et al. 1999). No activity against prokaryotes has been reported for any of the antifungal proteins so far. Interestingly, differences in sensitivity exist within members of the same genus, such as within aspergilli, but rarely within strains (Table 2). Although no homology of the primary structure exists between the fungal proteins and those from plants, e.g., defensins or pathogenesis-related proteins, similarities are evident to some extent in their antifungal activity. The members of both protein groups exhibit growth retardation as long as

they are applied in sublethal concentrations; and they become fungicidal with increasing concentrations (Abad et al. 1996; Hejgaard et al. 1991; Kaiserer et al. 2003; Osborn et al. 1995). Plant antimicrobial proteins are divided into morphogenic and non-morphogenic proteins, depending on their ability to evoke a hyperbranched hyphal phenotype in sensitive molds (Osborn et al. 1995; Terras et al. 1992). In analogy, severe changes in morphology are induced by PAF (Kaiserer et al. 2003), whereas no morphological changes are reported for AFP or ANAFP (Lee et al. 1999; Theis et al. 2003). The plant protein activities are neutralized in the presence of minimal concentrations (2–50 mM) of monovalent and divalent cations (Terras et al. 1992, 1993; Osborn et al. 1995). In contrast, a severe decrease in the fungal protein potency can be shown with >20 mM of selected ions ($MgCl_2$ for PAF, KH_2PO_4 for AFP) or in the presence of high ion concentrations (50–100 mM Na_2SO_4 for PAF, KCl or NaCl for AFP), whereby only a limited decrease in the PAF potency was detectable with KCl and there was minimal influence with NaCl (Kaiserer et al. 2003; Theis et al. 2003).

Neutralization of the activity of positively charged proteins from plants and other organisms in the presence of cations was explained by the inhibition of direct protein-binding to negatively charged phospholipid moieties present in the plasma membrane of target organisms (Abad et al. 1996; Cociancich et al. 1993; Hughes et al. 2000; Lee et al. 2002; Osborn et al. 1995; Terras et al. 1993; Thevissen et al. 1996, 1999, 2003). In fact, Lacadena et al. (1995) reported the AFP promoted in vitro aggregation of large unilamellar vesicles of the acidic phospholipid dimyristoylphosphatidylserine, suggesting an interaction of the basic AFP with negatively charged phospholipids. In consequence, AFP could directly evoke plasma membrane perturbations and pore formation (Campos-Olivas et al. 1995; Lacadena et al. 1995). However, from the fact that the antifungal proteins from ascomycetes act species-specifically on fungi only and not on bacteria, a more defined interaction with target organisms has to be favored. Thus, it can be hypothesized that the specific interaction requires a putative receptor in the outer layers of sensitive fungi, as proposed for the action of plant defensins (De Samblanx et al. 1997; Thevissen et al. 1997), and that the binding of the fungal proteins to this molecule is cation-sensitive. Such an interaction might finally result in: (1) induction of plasma membrane destabilization, (2) signal transduction into the cell or (3) internalization of the protein. Consequently, in the latter two cases, detrimental effects would be evoked from inside the target cells.

Recently, detailed studies with AFP and PAF addressed their site of action by indirect immunofluorescence staining and allowed some new insights into their possible targets. AFP accumulated in the outer cell layers (cell wall or plasma membrane) of sensitive fungi, whereas it was internalized by resistant molds, in the latter case indicating a possible utilization of the protein as a nutritional source (Theis et al. 2003). The uptake of the fluorogenic dye

Table 2 Sensitivity of microorganisms to the antifungal proteins AFP, ANAFP, NAF and PAF

Organism	AFP[a,b]	ANAFP[c]	NAF[d]	PAF[e]
Filamentous fungi				
Aspergillus awamorii	++	n.d.	n.d.	n.d.
A. clavatus	−	n.d.	n.d.	n.d.
A. flavus[f]	−	++	+	+
A. fumigatus[f]	n.d.	++	n.d.	++
A. giganteus	+	n.d.	n.d.	+
A. nidulans	+	n.d.	n.d.	++
A. niger[f]	++/−[a]	−	n.d.	++
A. oryzae	−	n.d.	n.d.	n.d.
A. terreus[f]	n.d.	n.d.	n.d.	++
Botrytis cinerea[g]	n.d.	n.d.	n.d.	++
Byssochlamys sp	n.d.	n.d.	+	n.d.
Cochliobolus carbonum[g]	n.d.	n.d.	n.d.	+
Fusarium aquaeductuum[g]	+	n.d.	n.d.	n.d.
F. bubigenum[g]	++	n.d.	n.d.	n.d.
F. culmorum[g]	+	n.d.	n.d.	n.d.
F. equiseti[g]	++	n.d.	n.d.	n.d.
F. lactis[g]	++	n.d.	n.d.	n.d.
F. lini[g]	++	n.d.	n.d.	n.d.
F. moniliforme[f,g]	++	n.d.	n.d.	n.d.
F. oxysporum[f,g]	++	++	n.d.	+
F. poae[g]	+	n.d.	n.d.	n.d.
F. proliferatum[g]	++	n.d.	n.d.	n.d.
F. sambucinum[g]	n.d.	n.d.	n.d.	−
F. solani[f,g]	+	++	+	n.d.
F. sporotrichoides[g]	++	n.d.	n.d.	n.d.
F. vasinfectum[g]	++	n.d.	n.d.	n.d.
Geotrichum candidum[f]	n.d.	n.d.	+	n.d.
Gliocladium roseum[g]	n.d.	n.d.	n.d.	+
Magnaporthe grisea[g]	++	n.d.	n.d.	n.d.
Mucor sp.[f,h]	n.d.	n.d.	+	n.d.
M. circinelloides[f]	n.d.	n.d.	n.d.	−
M. genevensis[f]	n.d.	n.d.	n.d.	−
Neurospora crassa	n.d.	n.d.	n.d.	+
Penicillium chrysogenum	−	n.d.	n.d.	−
P. digitatum	n.d.	n.d.	+	n.d.
P. frequentas	−	n.d.	n.d.	n.d.
P. italicum	n.d.	n.d.	+	n.d.
P. purpurogenum	++	n.d.	n.d.	n.d.
P. roqueforti	n.d.	n.d.	++	n.d.
Phytophthora infestans[g]	++	n.d.	n.d.	n.d.
Trichoderma harzianum	+	n.d.	n.d.	n.d.
T. koningii	++	n.d.	n.d.	++
Yeasts				
Candida albicans[f]	−	++	n.d.	−
Debaryomyces hansenii	n.d.	n.d.	−	n.d.
Endomyces lactis	n.d.	n.d.	−	n.d.
Pichia anomala	n.d.	n.d.	−	n.d.
P. membranaefaciens	−	n.d.	n.d.	n.d.
Rhodotorula mucilaginosa[f,i]	−	n.d.	n.d.	n.d.
Saccharomyces cerevisiae	−	++	n.d.	−
S. exiguus	−	n.d.	n.d.	n.d.
Trichosporon beigelii[f,i]	n.d.	++	n.d.	n.d.

Table 2 (continued)

Organism	AFP[a,b]	ANAFP[c]	NAF[d]	PAF[e]
Zygosaccharomyces rouxii	n.d.	n.d.	––	n.d.
Bacteria				
Bacillus megaterium	–	n.d.	n.d.	n.d.
B. subtilis[f]	–	–	n.d.	–
Escherichia coli[f]	–	–	n.d.	–
Micrococcus luteus	–	n.d.	n.d.	n.d.
Pseudomonas aeruginosa[f]	–	n.d.	n.d.	n.d.
P. fluorescens	–	n.d.	n.d.	n.d.
Salmonella enteriditis[f]	–	n.d.	n.d.	n.d.
Serratia marcescens[f]	–	n.d.	n.d.	n.d.
Staphylococcus aureus[f]	–	n.d.	n.d.	n.d.

++ Highly sensitive, + sensitive, − resistant, *n.d.* not determined
[a]Lacadena et al. (1995)
[b]Theis et al. (2003)
[c]Lee et al. (1999)
[d]Geisen (2000)
[e]Kaiserer et al. (2003)
[f]Opportunistic zoo-pathogenic organism
[g]Potentially plant-pathogenic organism
[h]Not specified by Geisen (2000)
[i]Basidiomycetes

SYTOX-Green within minutes in AFP-treated sensitive molds points to a fast membrane permeabilization and strengthens the former assumption of an interaction of AFP with the plasma membrane (Theis et al. 2003). In contrast, PAF was found to localize intracellularly in sensitive fungi and no specific interaction of the protein with resistant fungi could be detected (Oberparleiter et al. 2003). The internalization of PAF was dependent on active metabolism, availability of ATP and intact actin microfilaments. All these factors are prerequisites for a receptor-mediated endocytotic uptake mechanism. As reported by Kaiserer et al. (2003), the detrimental effects of PAF on sensitive fungi are multifactorial, including morphological changes, metabolic inactivation, membrane perturbation and intracellular oxidative stress. It is conceivable that PAF activity is launched from inside the cell. In this respect, numerous protein toxins are known which are delivered to their site of action by endocytosis (Falnes and Sandvig 2000), e.g., fungal and plant ribotoxins (Olmo et al. 2001; Sandvig et al. 2002), or bacterial toxins (Abrami et al. 2003; Montesano et al. 1982; Sandvig et al. 2002; Skretting et al. 1999). For the *Saccharomyces cerevisiae* killer toxins KP1 and KP28, two different mechanisms of action were reported, respectively (Schmitt and Breinig 2002). Both proteins bind to receptors present in the cell wall and the cytoplasmic membrane. Whereas KP1 directly acts from outside the cell and disrupts cytoplasmic membrane function by ion channel formation (Ahmed et al. 1999; Breinig et al. 2002; De la Pena et al. 1981; Martinac et al. 1990), KP28 enters the cell by endocytosis in order to reach its final target, the nucleus (Eisfeld et al. 2002). However, the retrograde transport of the yeast killer toxin KP28, or the bacterial Cholera toxin, requires the 4-aa motif HDEL/KDEL at their C-termini to act as intracellular targeting signal (Schmitt and Breinig 2002). This targeting signal is absent in PAF and neither plant ricin (Lamb et al. 1985), Anthrax toxin (Leppla 1982) nor Shiga toxin (Kozlov et al. 1987; Strockbine et al. 1988) possess this consensus sequence, although they are efficiently transported via subcellular organelles to the cytosol (Abrami et al. 2003; Bradley and Young 2003; Sandvig et al. 2002). The internalization of α-sarcin via endocytosis gives rise to new aspects in the mode of action of cationic antimicrobial proteins that differs from the hitherto existing concept of plasma membrane perturbation and pore formation as the primary function of basic antimicrobial proteins (Gasset et al. 1994; Olmo et al. 2001).

Apart from the interaction with cytoplasmic membranes, mitochondria and nucleic acids were reported to be possible intracellular targets for antimicrobial proteins. Human histatin 5 and amphibian magainins target the mitochondrion and dissipate the mitochondrial transmembrane potential (Helmerhorst et al. 1999; Westerhoff et al. 1989). KP28 induces lethal events by inhibiting DNA synthesis and arresting the cell cycle in target cells (Riffer et al. 2002; Schmitt et al. 1996). In contrast, α-sarcin inhibits protein biosynthesis by specifically cleaving one phosphodiester bond of the 23–28S rRNA (Schindler and Davies 1977; Wool et al. 1992). Martinez Del Pozo et al. (2002) reported in vitro binding of the *A. giganteus* AFP to DNA via a putative oligonucleotide/oligosaccharide-binding (OB-fold) structural motif. However, the nucleic acid-binding capacity of the *A. giganteus* antifungal protein has not yet been linked with its predominant localization to the extracellular layers of sensitive molds (Theis et al. 2003). Concerning PAF, no DNA charge neutralization could be detected in electrophoretic mobility shifts (Marx, unpublished data) and the cytoplasmic distribution of the PAF-specific immunofluorescent signal excludes a compartmentation of PAF, e.g., to the nucleus (Oberparleiter et al. 2003). However, an interaction of PAF with ribonucleic acids in the cytoplasm might be possible and has to be further investigated.

Potential applications

None of the small and basic antifungal proteins from ascomycetes have been therapeutically or biotechnologically applied to date, although they exhibit strong potential for future exploitation. AFP, ANAFP, NAF and PAF are secreted in high abundance into the supernatant of easily fermentable molds for which the growth conditions could be optimized for large-scale fermentation, to gain maximum protein yields. In addition, the extracellular accumulation of the proteins in the culture broth facilitates protein purification and avoids costly purification from

cellular extracts. The PAF-secreting mold *Penicillium chrysogenum* is used in biotechnology as the main producer of β-lactam antibiotics and is therefore "generally recognized as safe" by the United States Food and Drug Administration. Besides, the AFP-resistance of *P. chrysogenum* and its applicability for heterologous gene expression offers the possibility of using this mold for recombinant antifungal protein production (Graessle et al. 1997).

The high stability of these proteins at high temperatures, against protease cleavage and within a wide pH range could make them suitable for use in medical treatments, plant protection or food preservation (Lacadena et al. 1995; Oberparleiter et al. 2003; Marx, unpublished data). On account of the diverse species-specificities of AFP, ANAFP and PAF/NAF, a combination of these proteins might provide a wide-range antimycotic drug. The proteins could be useful for oral and/or intravenous administration or topical application, provided that they do not exhibit any detrimental effects on mammalian cells. To date, no negative effects of PAF on human NT2 cells have been observed (Marx, unpublished data), but protein activity has to be further tested on other mammalian cell types and in an animal model.

Systemic production of the antifungal proteins in crop plants should be taken into account as a novel strategy to engineer biological control of fungal pathogens, as shown for AFP and other antimicrobial proteins (Kinal et al. 1995; Oldach et al. 2001; Osusky et al. 2000; Park et al. 1996). Finally, they could complement or substitute for chemical preservatives in food, as suggested for NAF (Geisen 2000). For instance, the lantibiotic nisin, a member of bacterial antimicrobial polypeptides (bacteriocins), is successfully used as a food preservative (Epand and Vogel 1999). Once the active sites of these proteins are determined, the activity and the specificity range could be enhanced by site-directed mutagenesis and/or by the development of synthetic derivatives, in order to design new compounds with pharmaceutical value (Epand et al. 2003; Hoover et al. 2003; Schaaper et al. 2001).

Further, the internalization of PAF by sensitive fungi makes this protein a useful marker for studying endocytotic mechanisms in filamentous fungi whose occurrence is still a matter of controversial debate (Fischer-Parton et al. 2000; Torralba and Heath 2002; Yamashita and May 1998).

Filamentous fungi have an important value in our life. Apart from the fact that they may have a negative impact on our health, science and biotechnology would be unthinkable without these organisms. Numerous fungal products are exploited for our benefit and it is conceivable that a lot of as yet unidentified useful compounds await discovery. As such, the small, basic and cysteine-rich antifungal proteins from ascomycetes bear great potential for utilization. The science of these proteins is still in its infancy—compared with the knowledge available for other well studied antimicrobial proteins—and much remains to be learned about which of their structural features are important for antifungal activity and species-specificity. This information holds a key position for the elucidation of their mode of action and will be required for their potential use in pharmacology and/or agriculture.

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