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Review

Antifungal proteins: targets, mechanisms and prospective applications

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Abstract. All organisms have evolved several defence systems in order to protect themselves against bacteria, fungi and viruses. Higher organisms have developed a complex network of humoral and cellular responses, called adaptive immunity. A second defence system, innate immunity, was discovered in the early 1980s, consisting of small cationic peptides with a broad antimicrobial spectrum. These proteins act immediately at sites of infection or inflammation. The production of proteins with antimicrobial activity was not limited to higher organisms but was also found in insects, plants and microorganisms. During the last 2 decades a broad range of proteins with very different structural features have been isolated and characterised from differing organisms ranging from bacteria to human beings. Over 500 cationic membrane-acting proteins with antimicrobial and antifungal activities have been identified to date. Apart from these proteins, a very large number of antifungal proteins active on the fungal cell wall, on enzymes of the cell wall synthesis machinery, the plasma membrane and on intracellular targets have been characterised.

Key words. Antifungal protein; chitinase; glucanase; defensin; cell wall; plasma membrane; ribosome-inactivating-protein (RIP).

Introduction

Antifungal proteins have been categorised according to their enzymatic properties (glucanases, chitinases), their structure (e.g. cysteine rich) or their similarity to a known 'type' of protein. Antifungal proteins from plants have been organised into five major groups based on serological and sequence analysis [1]. Such proteins were first detected in tobacco leaves reacting hypersensitively to tobacco mosaic virus and were therefore named pathogenesis-related (PR) proteins [2]. The five groups are: PR-1 (cysteine-rich and small proteins of ~15–17 kDa), PR-2 (β-glucanases), PR-3 (chitinases), PR-4 (chitin-binding proteins), PR-5 (thaumatin-like proteins). Antifungal proteins with similar properties but from species other than plants have also been isolated, and were therefore named 'PR-1/5-like' proteins.

This review will focus on the different cellular target sites of antifungal proteins. Recent data regarding the composition of these structures will be summarised, and molecular models for the antifungal activity at these targets will be presented. Based on these data, possible applications for antifungal proteins will be discussed. General descriptions of antifungal proteins, their structural features, occurrence and regulation can be found elsewhere [3–6]. The target structures which will be discussed in this review range from the outermost part of the fungal cell, which is defined by the cell wall, to the plasma membrane and finally to several intracellular targets. These data should provide the basis for a greater insight into the host selectivity of antifungal proteins, as this specificity is

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most often due to fungal-specific features of the target structures.

The fungal cell wall

The fungal cell wall accounts for ~20–30% of the dry weight of fungal cells [7]. It protects the cell from physical damage and is responsible for its shape. Furthermore, there are several proteins associated with the cell wall which are involved in mating, adherence to substrates, vegetative (in)compatibility and hydrophobic properties of the cell.

The three best-studied *Ascomycotina* species in terms of cell wall composition are currently *Saccharomyces cerevisiae*, *Candida albicans* and *Aspergillus fumigatus*. Smits et al. [8] presented a comprehensive molecular model of the cell wall of *S. cerevisiae*, shown in Figure 1.

The cell wall of *S. cerevisiae* has three classes of carbohydrate polymers: chitin, β1,3-glucan and β1,6-glucan. Furthermore, it contains two classes of glycosylated cell wall proteins (CWPs) which are linked via either a glycosylphosphatidylinositol (GPI) anchor or a Pir (protein with internal repeats) sequence to the cell wall [9, 10]. The β1,3-glucan molecules are held together by hydrogen bridges and form a three-dimensional network which covers the whole cell [8]. Chitin is attached at the interior side of the cell wall, whereas β1,6-glucan and Pir-CWPs are attached on the exterior side. Infrequent linkage of chitin to β1,6-glucan has also been observed.

There is no complete model of the cell wall of filamentous fungi to date, but apart from *Oomycetes*, which lack chitin in their cell walls, all three classes of carbohydrate polymers described in yeast are also present in cell walls of most filamentous fungi. This indicates that the molecular organisation of the hyphal cell wall is similar to that of *S. cerevisiae*. The concentration of chitin in the cell wall of filamentous fungi (~10%) is significantly higher than in yeast (2%). Additionally, Fontaine et al. [11] have shown that β1,4-glucan is covalently linked to β1,3-glucan in *A. fumigatus* cell walls. Many mycelial species also contain a large fraction of alkali-soluble glucans (S-glucans) consisting of α1,3-glucans and α1,4-glucans [12, 13]. S-Glucans account for ~25% of the dry weight of the cell wall of *A. nidulans* [14]. Schoffelmeer et al. [13] showed that *Fusarium* hyphal walls may contain 10% of uronic acids.

The surface properties of most species of the *Ascomycotina*, *Basidiomycotina* and the *Zygomycotina* are largely determined by an outer layer of glycoproteins [15], which are either anchored by GPI or bound in a Pir-like manner. Apart from their functions in anastomosis, hyphal aggregation and vegetative (in)compatibility, these proteins are involved in the adherence of fungal pathogens to their hosts [16, 17] and determine the antigenic properties of the cell. In addition, glycoproteins limit the permeability of the cell wall and therefore play a protective role [18]. The cell wall of aerial hyphae are covered by a special class of proteins, designated as hydrophobins [19].

Carbohydrate polymers of fungal cell walls are mainly synthesised at the plasma membrane [20]. The incorporation of chitin, β1,3-glucan and α1,3-glucan into the cell walls of filamentous fungi seems to be limited to the hyphal tips [21]; thus the composition and organisation of

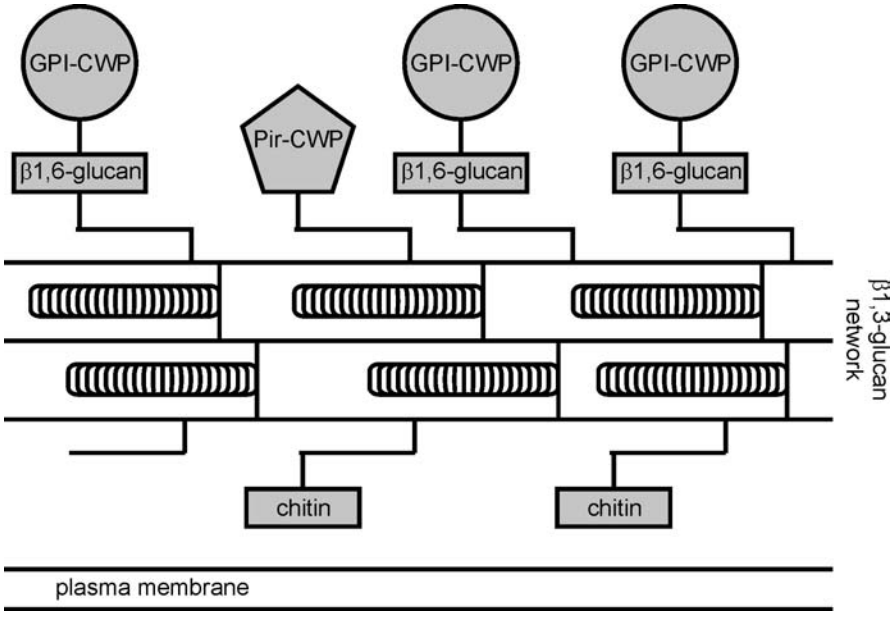

Figure 1. Molecular model of the cell wall of *S. cerevisiae*. [8] GPI, glycosylphosphatidylinositol; CWP, cell wall protein; Pir, protein with internal repeats.

cell walls of apical cells differ from mature cell walls. In contrast, the incorporation of chitin into the lateral walls is delayed until after cytokinesis in *S. cerevisiae* [22].

The unique fungal cell wall with its skeletal layer composed of chitin and β1,3-glucan is a target for a wide range of antifungal proteins. Fungal growth is affected by chitin-binding proteins, chitinases and glucanases. Furthermore, several proteins interfere with fungal chitin- and glucan-synthesis, also leading to a growth inhibitory effect.

Chitin-binding proteins

Chitin-binding proteins which interfere with fungal growth due to their affinity to nascent chitin have been isolated from bacteria [23, 24], crustaceans [25] and plants [e.g. 26–29]. Chitin-binding proteins of plants have been classified as PR-4 proteins. They are subdivided into two classes within this group. Class I PR-4 proteins have an N-terminal chitin-binding domain which is similar to a domain present in hevein, a protein from rubber latex [30], whereas class II PR-4 proteins lack this domain. Chitin-binding proteins from bacteria or crustaceans seem to lack this hevein domain. Figure 2 shows an alignment of the N-terminal amino acid sequence of selected class I chitin-binding proteins.

The hevein domain is highly conserved, comprising six to eight cysteine residues which are all involved in intramolecular disulfide bridges. A reduction of these disulfide bridges resulted in an abolished antifungal activity for some chitin-binding proteins [27, 29]. Additionally, the hevein domain contains one serine and three aromatic residues at conserved positions. Remarkably, the presence of additional hevein domains within one protein does not enhance the antifungal potency of the protein.

Muraki et al. [31] analysed the hevein domain of several chitin-binding proteins, and could show that the aromatic residues within this site are essential for chitin binding. The replacement of aromatic residues resulted in lower affinity to chitin, whereas mutations which cause a replacement of phenolic residues within this domain by a residue with a larger aromatic ring enhanced the affinity to chitin.

The size of chitin-binding proteins from different sources varies from 3.1 kDa up to 20 kDa. The proteins often have a basic pI and are highly resistant to extreme pH and protease treatment [24, 27]. They inhibit fungal growth at concentrations of 1 μg/ml and below, but their antifungal potency is most often species specific and is largely dependent on environmental conditions such as media composition and osmotic strength [27]. As a result, the antifungal activity of chitin-binding proteins is often antagonised by monovalent cations (Na^+). Even stronger antagonisation by divalent cations (Ca^{2+}) has been observed [27, 28].

Although antifungal activity has been demonstrated in vitro, it is still unclear whether all chitin-binding proteins are actually involved in the host defense against fungi and other microorganisms or whether the antifungal action is just a side effect. Tachystatins, chitin-binding proteins from horseshoe crab, may also be involved in wound healing. They recognise chitin exposed at the site of a lesion of the arthropod exoskeleton, thus stimulating and accelerating biosynthesis of chitin at sites of injury [25]. Chitin-binding proteins of *Streptomyces*, which is able to grow on chitin as a sole carbon source, may be more involved in nutrition than in antifungal defense [23, 24]. Moreover, some chitin-binding proteins from sugar beet have been shown to exhibit antifungal activity in vitro, but the detected concentrations in situ are too low for effective antifungal activity [28].

Figure 2. Comparison of the N-terminal amino acid sequence of hevein with hevein-domains occurring in chitin-binding proteins. Identical residues are boxed, and gaps (–) were introduced to maximise homology. Chitin binding domains of the following sequences are shown: hevein (Hevein; accession no. M36986), *Pharbitis nil L* hevein-like protein (Pn-AMP1; [29], tobacco CBP20 protein (Tobacco cbp20; accession no. S72452), potato win-1 protein (WIN 1; accession no. P09761), *Amaranthus caudatus* antimicrobial protein (Ac-AMP2; accession no. X72641), *Beta vulgaris* L. antifungal peptide (IWF4; [28]), *Ginkgo biloba* antifungal peptide (GAFP), Tobacco chitinase (tobacco a11; accession no. X51599), barley chitinase (barley a12; accession no. 629777)

The inhibitory effect on growth that these proteins have is mainly due to their ability to bind to chitin. Since nascent chitin of the hyphal apex, where hyphal growth and therefore cell wall assembly take place, is the most accessible [32], chitin-binding proteins localise along fungal cell walls and accumulate at septa and hyphal tips [24, 29]. This binding often leads to severe morphological changes such as aberrant branching, hyphal swelling, and shorter hyphae [24, 28]. Treatment of *Pichia pastoris* with tachystatins from horseshoe crab resulted in a reduced diameter of the cells [25].

Apart from antifungal activity due to chitin-binding, several of these proteins seemed to have additional modes of action. The chitin-binding protein Pn-AMP1 is able to penetrate the fungal plasma membrane and can be localised intracellularly [29]. Tachystatin C has been shown to form pores within the plasma membrane of treated hyphae [25]. However, the molecular basis of these interactions with the fungal plasma membrane is not known.

Chitinases

The second large group of antifungal proteins which interact with chitin are chitinases (EC 3.2.1.14). In general, these enzymes catalyse the degradation of chitin, and act most often as endochitinases, producing chito-oligosaccharides of 2-6 N-acetylglucosamin units [33]. Chitinases belong to the widespread group of O-glycoside hydrolases which hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a noncarbohydrate moiety. These enzymes are classified in families, based on amino acid sequence similarities. To date, 85 families have been identified, and chitinases are classified in family 18 and family 19 [34]. Their main characteristics are summarised in table 1.

In general, all organisms that contain chitin also produce chitinases, which are presumably required for morphogenesis of cell walls and exoskeleton [21]. Several bacteria species from the genera *Bacillus*, *Pseudomonas* and *Streptomyces* are able to grow on chitin and secrete chitinases in order to use chitin as a sole carbon source [35–37]. Apart from these functions in growth and nutrition, chitinases have also been found to be involved in the host defence against pathogens. Chitinases secreted by plants are classified as PR-3 proteins. According to their amino acid sequence, PR-3 proteins are subdivided into four major classes: class I chitinases contain a chitin-binding hevein-like domain and a highly conserved central region which is separated from the hevein domain by a hinge region. The hevein-domain is identical to that of chitin-binding proteins, shown in figure 2. Class II chitinases are similar to class I chitinases but lack the hevein domain. Class III chitinases share no homology to other chitinases. Class IV chitinases are similar to Class I, but possess major deletions.

The occurrence of chitinases within different plants, insects, fungi and bacteria has been intensively reviewed [36, 38] and will not be discussed here. Most recently, two studies revealed major insights into the catalytic mechanism of family 18 chitinases from plants and bacteria [39, 40].

It seemed quite obvious that the antifungal effect of chitinases was due to the hydrolyses of chitin, which leads to a weakened cell wall and subsequently causes cell lysis. For example, tobacco class I chitinases inhibit the growth of many fungi in vitro by causing cell lysis of hyphal tips [41, 42].

However, the mechanism by which chitinases inhibit fungal growth is not always due to chitinolytic activity. Remarkably, bacterial family 18 chitinases do not have any antifungal activity. Moreover, it is poorly understood whether a difference in the structure or enzymatic activity of chitinases is related to potency of the antifungal activity. Several mutational approaches have been attempted but revealed no general rules for chitinase antifungal activity. A mutant class I chitinase from chestnut seeds, displaying no chitinolytic activity, had as much antifungal activity as the wild-type chitinase [43]. Furthermore, the antifungal activity of a tobacco class I chitinase was three times higher when a chitin-binding domain was present [44]. These results indicate that a large proportion of the antifungal activity of chitinases is due to chitin-binding and not to chitinase activity. In contrast, the chitin-binding domain of a class I chitinase from rye had no antifungal activity at all, whereas the catalytic domain of this chitinase inhibited the growth of a fungal test organism [45]. Andersen et al. [46] used a mutant class II chitinase from barley, without chitinolytic activity, and could show that its antifungal activity was reduced by 85% in comparison with the wild-type chitinase.

Table 1. Characteristics of family 18 and family 19 chitinases.

	Family 18 chitinases	Family 19 chitinases
Occurrence	bacteria, fungi, viruses, animals, class III, class IV plant chitinase	class I, class II, and class IV plant chitinases, *Streptomyces*
Structure of catalytic domain	α/β 8-barrel [173]	α-helical [174]
Hydrolisation of glycosidic bond	retention of anomeric conformation [175]	inversion of anomeric conformation [176]
Hydrolysed linkages	GlcNAc-GlcNAc and GlcNAc-GlcN [176]	GlcNAc-GlcNAc and GlcN-GlcNAc [177]
Sensitivity to inhibitors	sensitive allosamidin [178]	insensitive to allosamidin

Chitinases with a chitin-binding domain (class I + class IV) seemed to have a different antifungal mechanism in comparison with chitinases which lack this domain. When an intact chitin binding domain is present, the majority of the antifungal activity seemed to be due to the binding of the enzyme to chitin.

Glucanases

Glucans are the second major component of the fungal cell wall. Apart from β1,3-glucan, which is the primary glucan within the fungal cell wall, several other linkages such as α1–3, β1-4 and β1-6 have also been detected. Glucanases, which hydrolyse the different glucans, are therefore potent antifungal proteins. They are produced in all organisms. Figure 3 shows an alignment of the region near the active site of glucanases from bacteria, plants and invertebrates.

The active site of glucanases is highly conserved in different organisms. Two Glu residues, present in all shown sequences, are believed to act as catalytic residues responsible for cleaving β1,3- and β1,4-glycosidic bonds.

The most abundant glucanases in the context of host defence are the β1,3-glucanases (EC 3.2.1.39). β1,3-glucanases within plants are referred to as PR-2 proteins and are subdivided into three classes [47]. Class I glucanases are basic proteins of about 33 kDa and are localised in the plant vacuole [48]. They are synthesised as preproproteins and have no enzymatic activity until they are processed [49]. Classes II and III include acidic, extracellular proteins of about 36 kDa. Plant β1,3-glucanases are involved in several physiological and developmental processes, e.g. microsporogenesis [50], fertilisation [51], seed germination [52], fruit ripening [53] and endosperm rupture [54]. Apart from these functions, several studies have shown that class I β1,3-glucanases exhibit antifungal activity in vitro [42]. Class II β1,3-glucanases only showed antifungal activity in vitro when they were applied in combination with chitinases or class I β1,3-glucanases. This synergistic effect was confirmed for other chitinase and β1,3-glucanase combinations in vitro [41]. A combination of class I chitinase and class I β1,3-glucanase was most effective in inhibiting the growth of *Fusarium solani* germlings. Since chitin and β1,3-glucan are synthesised simultaneously in the apex of growing hyphae of filamentous fungi, the effectiveness of a hydrolase may depend on the simultaneous action of another one to hydrolyse mixed chitin-glucan fibres [33].

The antifungal mechanism of glucanases can be divided into direct and indirect activities. The direct antifungal activity was shown to be due to the digestion of the β1,3-glucans in fungal cell walls, which leads to a weakened cell wall and cell lysis. The indirect effect of glucanases, which has also been observed with chitinases, is due to partial digestion of glucans and chitin. The oligosaccharides released have been shown to be perceived by the plant cell as elicitors and induce active defence responses (for review see [55]) or the enhanced production of PR-proteins [56].

Recently, several plant thaumatin-like (TL) proteins have been shown to possess β1,3-glucanase activity [57]. TL proteins from plants are actually classified within the PR-

Figure 3. Alignment of a conserved region from glucanases and glucan recognition proteins. Identical residues within the group of glucan recognition proteins are boxed light grey, whereas overall identical residues are dark grey. Deletions (–) were introduced to maximise homology. Residues corresponding to the active site of bacterial glucosidase are marked with an arrow. The following sequences are shown: (*A*): silkworm *Bombyx mori* β1,3-glucan recognition protein (Bm-GRP, accession no.: BAA92243); silkworm *B. mori* Gram-negative bacteria binding protein (Bm-GNBP, accession no.: L38591); *Manduca sexta* glucan recognition protein (Ms-GRP, accession no.: AF177982); mosquito *Anopheles gambiae* Gram-negative bacteria binding protein (Ag-GNBP, accession no.: AJ001042); (*B*): sea urchin *Strongylocentrotus purpuratus* β1,3-glucanase (Sp-GLCN, accession no.: U49711); *B. circulans* β1,3-glucanase A1 (Bc-GLCNA1, accession no.: P23903); *Thermotoga neapolitana laminarinase* (Tm-LMN, accession no.: CAA88008); *Rhodothermus marinus* endo-β1,3-1,4 glucanase (Rm-GLCN, accession no.: P45789); *Zea mays* xyloglucan endotransglycosylase (Zm-HMLG, accession no.: AAC49012); *Bacillus macerans* endo-1,3-1,4-β-glucanase (Bm-GLUC, accession no.: P23904).

5 group. They show sequence similarities to thaumatin, a sweet tasting protein which was isolated from the katemfe fruit (*Thaumatococcus danielli*) [58]. Several TL proteins which act on fungal membranes [59] or bind to actin [60] have been reported. The discovery of TL proteins with β1,3-glucanase activity may hint at additional antifungal mechanisms which are more widespread between other β-glucanases. As mentioned above, additional antifungal mechanisms have also been found for chitin-binding proteins and chitinases.

Many glucanases from fungi have been characterised. Soil-borne fungi of the genus *Trichoderma* are known to secret chitinases, β1,6-glucanases and β1,3-glucanases [61–63]. Interestingly, in vitro studies revealed that β1,6-glucanase exert an antifungal effect on yeast, but not on filamentous fungi. The lack of antifungal activity against filamentous fungi has been confirmed for a bacterial β1,6-glucanase from *Streptomyces* [64]. However, treatment with β1,6-glucanase derived from *Trichoderma* together with chitinases or β1,3-glucanases resulted in growth inhibition of filamentous fungi [62].

α1,3-glucanases (mutanases) are produced by *A. nidulans* [65], *Trichoderma harzianum* and *Penicillium purpurogenum* [66]. These enzymes hydrolyse mutan, which is a main reserve material within fungi, and is accumulated during vegetative growth as a cell wall component [65]. α1,3-Linked glucans have also been found within the cell wall of several filamentous fungi, but in contrast to the well-characterised β-glucanolytic system, data on the biochemical and antifungal properties of α-glucanases remains sparse. Ait-Lahsen et al. [67] described an exo-α1,3-glucanase from *T. harzianum*, which executes antifungal activity.

Similar to chitin-binding proteins, β1,3-glucan-binding proteins have also been found in the silkworm *Bombyx mori* [68], crustaceans [69] and insects [70]. An alignment of several of these proteins is shown in figure 3. These proteins have similarities to glucanases, but lack enzymatic activity. None of the conserved catalytical residues from glucanases is present within glucan-binding proteins. Similar to the indirect antifungal activity of glucanases, binding of β1,3-glucans to these proteins activates host-specific defense systems. Additional effects, such as aggregation of yeast cells upon β1,3-glucan binding, have also been observed [70].

The fungal membrane

The fungal plasma membrane is the target for the largest group of antifungal and antimicrobial proteins. To date, over 500 naturally occurring proteins have been found [71], which are thought to interact with lipid components of the plasma membrane, leading to pore formation, efflux of essential cellular components and membrane potential changes.

The primary function of the plasma membrane is to define the permeability barrier of cells. Additionally, it serves as the matrix of proteins involved in important functions of the cell such as energy targeting, signal transduction, solute transport, DNA replication, secretion and so on. The lipid bilayer of the fungal plasma membrane is composed of sphingolipids, phospholipids and sterols. These structural components occur in membranes of all living organisms, for which membrane-acting proteins often affect the growth of bacteria as well as fungi. For some proteins, even hemolytic activity has been shown.

The fungal plasma membrane differs from those of higher eucaryotes as regards embedded sterols. The plasma membrane of higher eucaryotes contains cholesterol, whereas the major sterol of the fungal plasma membrane is ergosterol [72], which contributes to ~2% of the fungal dry weight [73]. Tuller et al. [74] reported that depending on growth conditions, 90.5%–97% of total sterol content within the plasma membrane of *S. cerevisiae* is ergosterol. Ergosterol, and the ergosterol pathway, is also the target for most antifungal agents currently used for clinical treatment of fungal infections. Polyene antibiotics such as amphotericin B interact with ergosterol, thus leading to formation of aqueous channels, increased membrane permeability to univalent cations and subsequently to cell death. Ketoconazole, fluconazole or itraconazole as well as allylamines inhibit the ergosterol pathway at different steps, leading to aberrant and toxic steroids in the fungal membrane (for review see [75]). Decreased amounts of ergosterol within the plasma membrane might be responsible for azole resistance of several *Candida albicans* strains [76].

Membrane-acting antifungal and antimicrobial proteins have been shown to interact with phospho- and sphingolipids, which make up the major part of the plasma membrane. Sphingolipids, abundant components of many membranes in eucaryotic cells, comprise about ~30% of the phospholipids or about ~7% of the mass of the *S. cerevisiae* plasma membrane [77]. Most recently, sphingolipid functions in *S. cerevisiae* have been extensively reviewed by Dickenson and Lester [78]. Furthermore, they are discussed as being a receptor for some antifungal, membrane-acting proteins from plants [79].

Phospholipids are classified according to their head groups into negatively charged and zwitterionic phospholipids. As can be seen in table 2, bacterial and fungal membranes contain anionic and most often also zwitterionic phospholipids, whereas the outer leaflet of most higher eucaryotic cells lack anionic phospholipids.

The anionic phospholipid head group is often involved in membrane association of cytoplasmic proteins. For example, protein kinase Cs associates, and is activated by a complex of phosphatidylserine (PS), diacylglycerol, and

Table 2. Phospholipid composition of pro- and eucaryotic cells.[a]

	Ref.	Zwitterionic			Anionic			Others
		PE	PC	PI	PS	PG	CL+lPG	
Gram-positive								
Staphylococcus aureus	[179]	–	–	–	–	57	43	–
S. epidermidis	[179]	–	–	–	–	90	1	9
Bacillus megaterium	[179]	40	–	–	–	40	5	15
B. subtilis	[179]	10	–	–	–	29	47	14
Lactobacillus rhamnosus	[180]	–	–	–	30	59	–	11
Gram-negative								
Escherichia coli	[179]	82	–	–	–	6	12	–
Salmonella typhimorium	[179]	60	–	–	–	33	7	–
Pseudomonas cepacia	[179]	82	–	–	–	18	–	–
Fungi								
Saccharomyces cerevisiae	[74]	25	11	27	32	–	2	3
Candida albicans	[76]	23	29	12	17	–	4	15
Cryptococcus neoformans	[96]	29	51	–	16	–	–	–
Apergillus niger Van Tieghem	[181]	29	51	–	52	–		13
A. niger V35	[182]	58	29	–	1	–	12	–
A. niger UFA_2	[182]	51	21	–	4	–	23	–
Higher eukaryotes								
erythrocyte outer membrane leaflet	[179]	–	62	–	–	–	–	47[b]
erythrocyte inner membrane leaflet	[179]	57	–	–	27	–	–	–

PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; CL, cardiolipin; lPG, lysophosphatidylglycerol.
[a] In percentage of total phospholipid of cytoplasmic membrane.
[b] Zwitterionic sphingomyelin.

Ca^{2+} [80]. Dowhan [81] has made a comprehensive review on the metabolism and function of phospholipids.

Membrane-acting antifungal proteins

A broad range of small, membrane-acting, cationic proteins with antimicrobial and antifungal activity have been isolated from mammals [82], amphibians [83], insects [84], plants [85] and bacteria (table 3). Although membrane-acting proteins are extremely diverse as regards their primary and secondary structure, they share at least two common features, namely a positive net charge under physiological conditions that facilitates interaction with negatively charged microbial surfaces and they assume amphipathic structures which permit incorporation into microbial membranes [71].

Amphipathic structures, which are a prerequisite for the antifungal activity of these proteins, can be assumed from β-sheet proteins as well as from α-helical proteins. Both structural classes are present within membrane-acting proteins. A prominent group of β-sheet proteins are defensins found in mammals, insects and plants [82, 84, 85]. Figure 4 shows a comparison of the amino acid sequences and secondary structures of three representative defensins. The secondary structure of defensins is characterised by β strands which are stabilised by disulfide bridges. An additional structural feature of plant and insect defensins is a cysteine-stabilised α-helix motif, where a Cys-X-X-X-Cys segment of the α-helix is connected by two disulfide bridges to a Cys-X-Cys segment of a β-strand.

Apart from these β-sheet proteins, a very large group of antifungal and antimicrobial membrane-acting proteins with α-helical structures have also been characterised [71]. These linear proteins lack disulfide bridges and only assume their α-helical structure in a lipid environment [86]. One of the best studied peptide families, in terms of their mode of action, are the α-helical magainins, which have been isolated from the granular gland of the skin of the African clawed frog, *Xenopus laevis* [87].

Membrane-acting proteins are most often variously active against a wide range of microorganisms, including Gram-positive and Gram-negative bacteria, fungi and protozoa. When administered to sensitive microorganisms, they exhibit their lytic activity within minutes leading to a rapid loss of intracellular potassium [82, 88]. In vitro experiments with artificial membranes have shown that they interact with vesicles composed of anionic phospholipids, whereas only weak interaction with zwitterionic phospholipids has been detected [86]. In contrast, Kagan et al. [89] reported a lipid-independent formation of ion-per-

Table 3. Representative membrane-active, antifungal proteins from different sources.[a]

Peptide	Source	No. of amino acids	Ref.
Mammalian AFPs			
defensins			
NP-1–2-3 A-3B-4–5	rabbit granulocytes	33–34	[183]
HNP-1 -2 -3	human neutrophils	29–30	[184]
gallinacin-1	chicken	39	[185]
lactoferricin-B	human, bovine	18	[186]
protegrins 1 – 3	human, porcine	16–18	[187]
tracheal AMP	human, bovine	38	[188]
tritrptein	human, porcine	13	[189]
histatin 5	human saliva	24	[190]
Insect and amphibian AFPs			
antifungal peptide	*Sacrophaga peregrina*	67	[191]
cercropins A+B	*Hyalopora cecropia*	37+35	[192]
dermaseptins b+s	*Phyllomedusa sauvagii*	27+34	[193]
drosomycin	*Drosophila melanogaster*	44	[194]
magainin 2	*Xenopus laevis*	23	[87]
thanatin	*Podisus maculiventris*	21	[194]
Bacterial AFPs			
bacillomycin F	*Bacillus subtilis*		[195]
iturin A	*B. subtilis*		[196]
pseudomycin A	*Pseudomanas syringae*		[197]
syringomycin E	*P. syringae*		[198]
syringostatin A	*P. syringae*		[199]
syringotoxin B	*P. syringae*		[199]
Plant AFPs			
Hs-AFP1	*Heuchera sanginea*	54	[114]
Dm-AMP1+2	*Dahlia merkii*	50	[114]
Rs-AFP1+2	*Raphanus sativus*	51	[113]
zeamatin	*Zea mays*	27	[59]

[a] Modified from [3].

meable channels in planar lipid bilayer membranes for defensins. Remarkably, synthetic, enantiomeric peptides composed of all D-amino acids exhibit the same antimicrobial potency as naturally occurring peptides, indicating that chiral molecules are not involved in antimicrobial action [90, 91]. The immediate lytic activity, the interaction with artificial membranes and the lack of chiral recognition, which is often involved in protein-protein interaction, were strong indications that there is an interaction of antimicrobial membrane-acting peptides with the lipid matrix and not with membrane-based proteins.

It is generally assumed that most membrane-acting peptides, especially those with an α-helical structure, have a similar mode of action. Figure 5 illustrates the Shai-Matsuzaki-Huang (SMH) model, which elucidates the antimicrobial activity of these peptides [88, 92, 93]. The first step of the SMH model proposes the interaction of the peptide with the bacterial or fungal plasma membrane. The basis of specificity of most membrane-acting antimicrobial peptides is the lipid composition of the plasma membrane. The amphipathic, positively charged peptides bind with the aid of electrostatic and hydrophobic interaction to bacterial and fungal membranes containing anionic phospholipids, whereas only weak hydrophobic interaction occurs with membranes of higher eucaryotic cells, which are mainly composed of zwitterionic phospholipids [94]. The presence of cholesterol in the target membrane reduces the activity of antimicrobial proteins, due to stabilisation of the lipid bilayer or interactions between cholesterol and the protein [88]. Upon binding to the lipid bilayer, a change in the secondary structure of the protein occurs. For example, magainins, which have little or no well-defined secondary structure in aqueous solu-

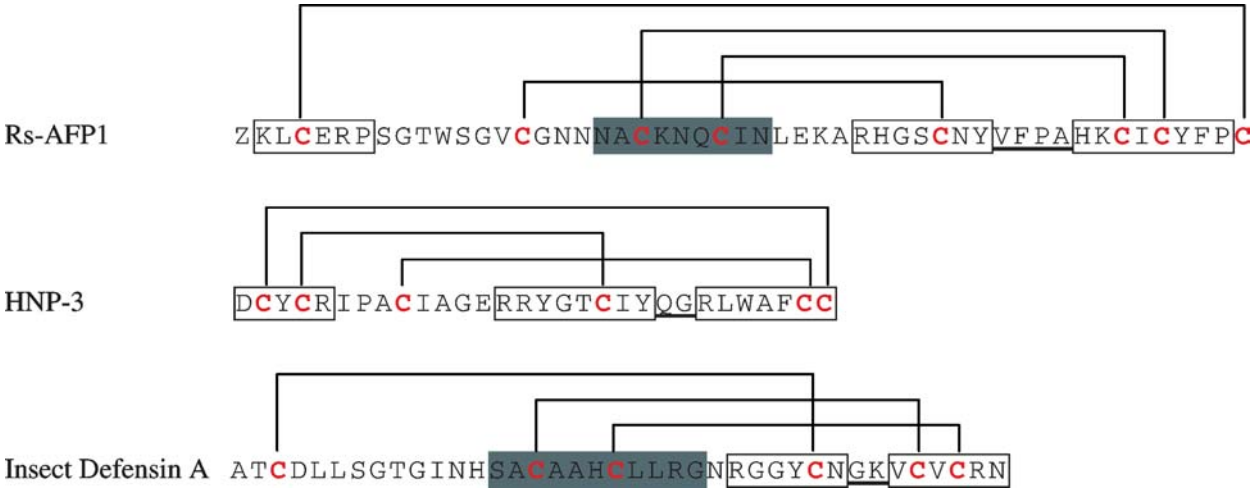

Figure 4. Comparison of the amino acid sequence, the secondary structure, and the disulfide bridges of different defensins (modified from [85]). Boxed sequences represent β-sheet structures, α-helical structures are grey and underlined sequences show β-turns. Cysteine residues are marked in red and disulfide bridges are indicated as lines. The following sequences are shown: plant defensin from *Raphanus sativus* (RS-AFP1; accession no.: AAA69541), human defensin (HNP-3; accession no.: N_005208), insect defensin from *Phormia terranovae* (Insect defensin A; accession no.: CAA81760).

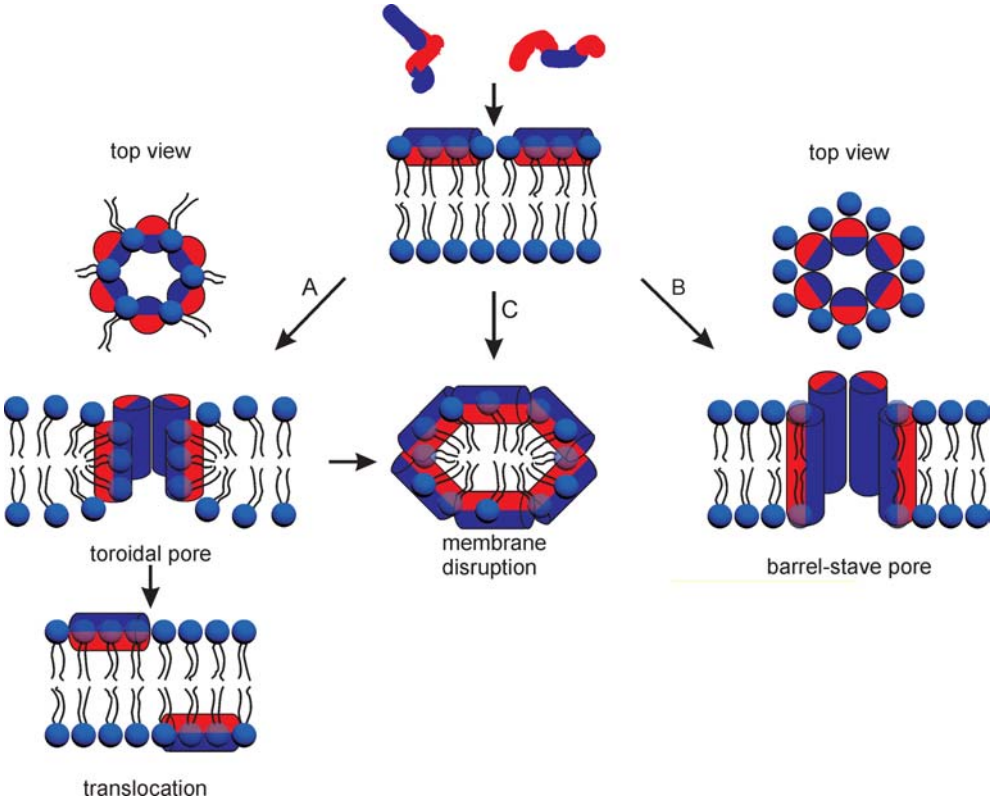

Figure 5. SMH model of membrane permeabilisation (modified from [88]). Upon interaction with membrane phospholipids (blue: hydrophilic head group, black: hydrophobic acyl sidechains), the unstructured antimicrobial peptide adopts an amphipathic, α-helical conformation (blue part: hydrophilic side; red part: hydrophobic part). Depending on the peptide, peptide concentration and lipid composition, a toroidal pore (A) or a barrel-stave pore (B) can be formed. Alternatively, the plasma membrane can be irreversibly disrupted (C). Pore formation can be followed by translocation of the peptide from the outer leaflet to the inner leaflet of the membrane.

tions at neutral pH, assume α-helical structures upon binding to acidic phospholipid bilayers [86–97]. However, diasteriomers of membrane-acting proteins, containing both L- and D-amino acids, have been shown to have lytic activity but an altered secondary structure, indicating that an α-helical structure is not necessary for activity [98, 99]. Two alternative mechanisms have been proposed for the interaction and subsequent insertion of the protein into the lipid bilayer. The barrel-stave model describes how amphipathic α-helices insert into the hydrophobic core of the membrane and form transmembrane pores [100]. It is therefore most likely that membrane binding of proteins which form barrel-stave pores is predominantly due to hydrophobic interactions, irrespective of the phospholipid charge [92].

The occurrence of toroidal pores is explained by the carpet model. In contrast to the barrel-stave model, the protein does not enter the hydrophobic core but is in contact with the phospholipid head group throughout the entire process of membrane permeation [101]. The proteins initially bind to the membrane and cover it in a carpet-like manner. Pore formation only occurs at high protein-to-lipid (P/L) ratios. Ludtke et al. [102] have shown that insertion of magainin 1 helix commences perpendicular to the membrane above a P/L of 1/30. Membrane disruption occurs at P/L values of 1/10. Oriented circular dichroism (OCD) studies revealed two different conformations depending on the protein concentration, expressed as P/L ratio [102, 103]. α-Helical orientation parallel to the lipid bilayer was detected at low P/L values, whereas α-helical orientation perpendicular to the plane of the bilayer was observed at high P/L values. These results led to the proposal of a two-state model for membrane-acting proteins [104]. The protein is in an inactive state ('S' state) at low P/L values. Increasing protein concentrations lead to a change into an active state ('I' state). The threshold value P/L*, at which the transition from the 'S' to 'I' state occurs, depends on the lipid composition of the bilayer. Two different, P/L-dependent OCD spectra, corresponding the 'S' and the 'I' state, have also been found for the β-sheet peptide protegrin [105].

The SMH model of pore formation has gained substantial evidence since Yang et al. [93] were able to directly detect pores formed by the β-sheet membrane-acting protein protegrin and the α-helical magainin by neutron diffraction.

Apart from lipid-induced change in conformation, there is additional evidence that lipid properties are involved in antimicrobial activity of membrane-active proteins. Although negatively charged phospholipids seem to be the predominant target for cationic peptides, membrane curvature is another important factor [106]. Since magainin is much more effective in inducing leakage in liposomes composed of PG than in those composed of PS [107], it has been suggested that magainin can stabilise the formation of a pore formed by phospholipids with a high curvature (large head group) [108]. The initial interaction of membrane-acting proteins with the phospholipid head groups leads to curvature stress. This interaction expands the head group region of the bilayer, leading to membrane thinning. Membrane thickness decreases in proportion to the peptide concentration [109]. However, no correlation between alteration in membrane curvature and lytic properties of a given membrane-acting protein has been found, since proteins that increase positive membrane curvature as well as those that increase negative curvature can be lytic [4]. It has been shown that membranes with intrinsic negative curvature tend to be lysed more readily by peptides which promote additional negative curvature [110]. Membrane pores have been shown to have a finite half-life. Upon disintegration of the pore, a fraction of the magainin molecules translocates across the lipid bilayer. This protein translocation is coupled with phospholipid translocation from the outer to the inner lipid bilayer, termed lipid flip-flop [111]. This protein translocation enables the protein to interact with anionic, intracellular targets, which may contribute to the antifungal activity of the protein. For example, the antifungal protein histatin 5 from human saliva translocates across the plasmamembrane. The antifungal activity of this protein has been shown to be due to an interaction with mitochondria and not due to pore formation [112].

A different mode of action has been proposed for some plant defensins which have been isolated from different plant seeds [113, 114]. Treatment of hyphae of *Neurospora crassa* with these proteins induces a rapid potassium efflux, calcium uptake, alkalinisation of the medium and membrane potential changes. In contrast to proteins which act according to the SMH model, plant defensins do not form pores within artificial membranes [115]. Instead, a specific binding site for a plant defensin on *Neurospora crassa* hyphae has been detected [116]. The binding is specific for some plant defensins, whereas distantly related or structurally unrelated proteins do not bind [117]. Moreover, the binding was shown to be saturable, with similar K_d values for hyphae and microsomal membranes, indicating that the binding site resides on the plasma membrane. In addition, it has been shown that the plant defensin DM-AMP1 from dahlia interacts with a specific sphingolipid of the plasmamembrane of *S. cerevisiae*. *S. cerevisiae* strains that lacked this spingolipid due to a knockout of a gene encoding an enzyme involved in the last step of the synthesis of the sphingolipid were highly resistant to the defensin [79].

Plant defensin-induced membrane permeabilisation was shown to be biphasic. High plant defensin doses led to a strong but cation-sensitive permeabilisation of fungal membranes detectable after 30–60 min, whereas low doses led to a more or less cation-resistant, weak permeabilisation, detectable after 2–4 h [118]. Since membrane permeabilisation occurs within minutes with proteins acting according the SMH model, membrane permeabilisation by plant defensins is assumed only to be a side effect of the antifungal activity. A putative model for the antifungal action of plant defensins might be that binding to a specific receptor entails activation of endogenous signal transduction components, which may affect the activity of endogenous ion channels or ion transporters [116]. Sphingolipids could be possible receptors, since ceramide, which is the basic building block of sphingolipids, has a function as a signalling molecule in response to events that trigger or indicate cellular stress [119]. It is generated when sphingomyelin in the outer leaflet of the plasma membrane is degraded in response to ligand binding to receptors in the plasma membrane [120].

Selective Ca^{2+} uptake through activated ion channels might be a major component of the antifungal action of plant defensins, for it is believed that a gradient in cytosolic Ca^{2+} concentration, generated by tip-localised Ca^{2+} channels, is essential for driving polarised growth of fungal hyphae [121, 122].

Furthermore, the putative receptor is also discussed to be an anchor point, allowing the plant defensin to insert into the membrane and form ion-permeable pores [79].

An additional function has been found for mammalian defensins in the host defence against pathogens. Apart from their direct antifungal activity via pore formation, they also play a role in the activation and recruitment of the cells and machinery of the adaptive immune response. Their additional functions in innate as well as in adaptive immunity have been recently reviewed by Raj and Dentino [123].

Intracellular targets

Antifungal proteins which have entered the cell can interact with a variety of intracellular targets. However, these proteins have to pass both the cell wall and the plasma membrane. Since this internalisation process is accompanied by interacting with the cell wall and the plasmamembrane, it is difficult to distinguish whether the antifungal activity of these proteins is due to cell wall and/or membrane interaction, or to a interaction with intracellular targets. This might be a reason for the very few reports on antifungal proteins interacting with intracellular tar-

gets. However, recent data showed that some antifungal proteins that were initially described as membrane-acting proteins actually exhibit their antifungal activity upon interaction with intracellular targets. For example, treatment of *E. coli* with magainins and cecropins at sublethal concentrations leads to the transcription of stress-related genes [124]. Buforin 2, an antimicrobial and antifungal protein from the stomach tissue of the Asian toad *Bufo bufo gargarizans*, exhibits weak membrane permeabilisation and translocates through the plasmamembrane by a mechanism which is distinct from proteins acting according the SMH model [125, 126]. The antimicrobial activity of buforin 2 is due to DNA and RNA binding [127]. Histatin 5, an antifungal protein from human saliva, has been studied extensively for its candidacidal activity. It has structural features of a membrane-acting protein since it is a small and basic protein that assumes an α-helical structure in nonaqueous solvents [128]. However, the lytic activity of histatin 5 was shown to be a secondary effect, following cell death [129, 130]. Furthermore, a cell wall-localised histatin-binding protein has been identified. This binding protein is also involved in the candidacidal action of the human defensin HNP-1, indicating that both proteins kill fungi via shared pathways [131]. The target of histatin 5 has been shown to be the mitochondrion [132]. Moreover, for toxicity mitochondria must be active, since nonrespirating cells were protected against killing by histatin 5.

Ribosomes

A prominent intracellular target for antifungal proteins are ribosomes. Several ribosome-inactivating proteins (RIPs) with RNA N-glycosidase activity or phosphatase activity have been purified from plants as well as from fungi (table 4).

Three different types of RIPs have been classified. Type-1 RIPs consist of a single polypeptide chain, whereas type-2 RIPs are multimeric proteins with two polypeptide chains, one of which is responsible for the catalytic activity (B chain) and the other (A chain) mediates translocation into the cells via recognition of protein receptors [133]. Type 3 RIPs are similar to type 2, but both domains reside on a single polypeptide [134].

Table 4. Representative ribosome-inactivating proteins with antifungal activity from fungi and plants.

Peptide	Source	Ref.
Fungal RIPs		
α-sarcin	*Aspergillus giganteus*	[138]
gigantin	*A. giganteus*	[200]
mitogillin	*A. restrictus*	[201]
clavin	*A. clavatus*	[202]
c-sarcin	*A. clavatus*	[203]
hypsin	*Hypsizigus marmoreus*	[204]
Plant RIPs		
ricin	*Ricinus communis*	[136]
ME1+2	*Mirabilis expansa*	[205]
RIP1	*Zea mays*	[206]
PAP-H	*Phytolacca americana*	[207]

The ribosome-inactivating activity of RIPs occurs either through RNA N-glycosidase, or -phosphatase activity (fig. 6). Independent of the type of activity, both modifications occur at a highly conserved sequence of the 28S ribosonal RNA (rRNA), the so-called sarcin/ricin loop [135]. Endo et al. [136] have shown that plant RIPs cleave a specific N-C glycosidic bond between an adenine and the ribose of the rRNA. The deadenylated site becomes unstable, and β-elimination occurs after the RNA is treated with acidic aniline, whereby the 3′ end of the rRNA is cleft [136, 137].

The mechanism of fungal RIPs is mostly due to phosphatase activity. For example, α-sarcin, a fungal RIP secreted by the mould *Aspergillus giganteus* [138], has been intensively studied. It is a type 1 RIP and specifically cleaves one phosphodiester bond of the 28S rRNA [139]. The protein has been reported to act as a cyclising ribonuclease [140], and moreover, essential amino acids of the catalytic core have been identified by several mutational approaches [141, 142]. α-Sarcin interacts with negatively charged model membranes [143, 144], and a nine-amino-acid peptide fragment of α-sarcin has been reported to be a membrane-perturbing structure [145]. Membrane insertion has also been shown for the plant type 1 RIP trichosanthin [146]. It can therefore be assumed that internalisation of type-1 RIPs due to membrane interaction may be a common feature of type 1 RIPs.

The ability to reach the intracellular target accounts for the range of activity of a given RIP. In vitro experiments with isolated ribosomes showed that most eucaryotic ribosomes are affected by RIPs. However, the in vivo activity is often limited by the inability of the protein to enter the cell [134].

RIPs also show synergistic effects with other antifungal proteins. For example, Lam and Ng [147] isolated a fungal RIP together with an antifungal protein and could show that the two proteins act synergistically.

Protease inhibitors

Proteases regulate numerous biochemical and physiological processes by controlling protein synthesis and degradation through the hydrolysis of specific amide bonds in polypeptides. They use a metal ion or an aspartic, serine or cysteine residue as a catalyst to activate and direct a water molecule for cleavage of a polypeptide sequence at a specific position [148, 149]. Proteinacious inhibitors of these proteases, which are one of the most abundant

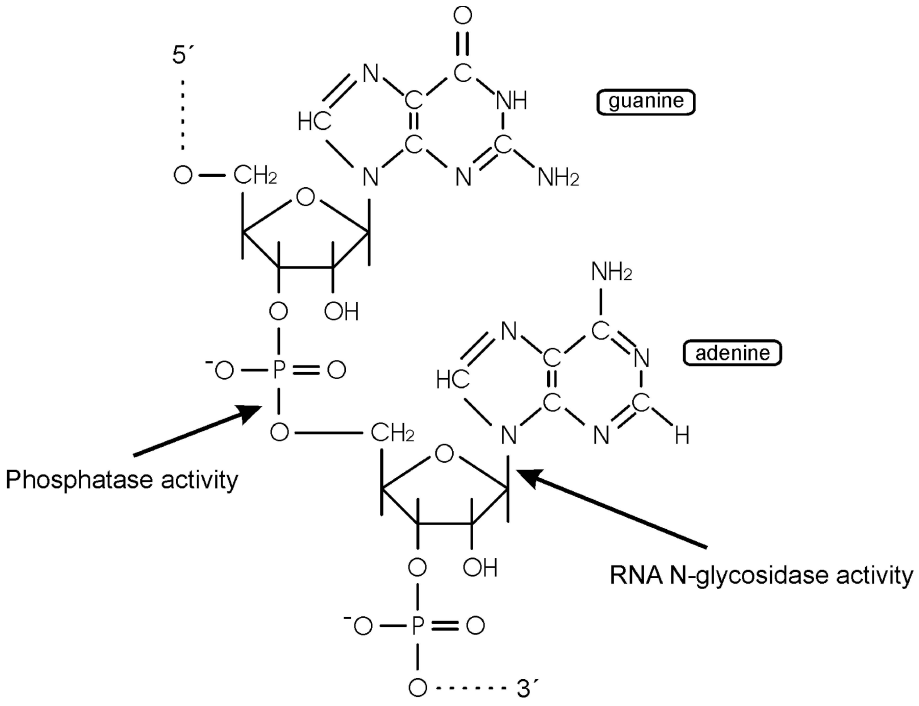

Figure 6. Sites of action of ribosome-inactivating proteins. Ribosome-inactivating proteins either cleave a phosphodiester bond via a phosphatase activity or specifically deadenylate the rRNA via an RNA N-glycosidase activity.

classes of proteins found in plants, animals and microorganisms [150], have emerged as a class of antifungal proteins with potent activity against a wide range of fungi. Antifungal cysteine protease inhibitors (cystatins) have been isolated from many plants [151–153]. Since these inhibitors share similarities in sequence and have several structural features in common, they have been grouped as phytocystatins. Apart from these proteinacious inhibitors, several low molecular peptido-mimetic inhibitors have been reported [149].

Based on the ability to inhibit proteases of insect digestive tracts, which reduces the availability of amino acids, thereby hindering the synthesis of proteins, protease inhibitors have been implicated to possess a defensive role against insects and herbivores [154]. Furthermore, several plant pathogenic fungi, as well as some medically important *Candida* spp. have been shown to secrete proteases [155, 156]. At least plant pathogenicity seems to be related to secreted proteases since protease-deficient mutants had lost the ability to induce lesions in plants [157]. However, the antifungal mechanism of protease inhibitors is not fully understood, and in the case of inhibition of intracellular proteases, there are no data available as to how these proteins reach their intracellular target.

Prospective application of antifungal proteins

Several applications of natural occurring antifungal proteins have been discussed during the last 2 decades. They seem an attractive alternative for chemical food additives, and may also be a new source of clinically useful therapeutics.

Food preservation by use of antimicrobial proteins is not a totally new concept. The use of the antimicrobial protein nisin, which is produced by several *Lactococcus lactis* strains, has been approved by eight European countries. The protein inhibits the growth of a wide range of Gram-positive bacteria [158], and its mode of action is comparable to magainin [159]. It has been shown that an additional plasma membrane-based factor, named Lipid II, is needed for pore formation [160], which might also be a reason for the lack of antifungal activity of nisin.

Another antifungal treatment which involves the use of antifungal proteins is the biological control by antagonistic organisms, such as *Trichoderma* spp., for crop protection [161]. However, one of the most promising tools for crop protection is the use of transgenic plants. Heterologous expression of RIPs, glucanases and chitinases in wheat as well as in tobacco resulted in increased protection of the plants against soilborne fungal pathogens [162, 163]. The transgenic expression of plant defensins led to a protection of vegetative tissue even under field conditions. For example the expression of the alfalfa an-

tifungal protein, a plant defensin from the seeds of *Medicago sativa*, in transgenic potato plants resulted in a robust resistance of these plants to *Verticillium dahliae*, an important fungal plant pathogen [164]. Some plant defensins have been shown to interact with fungal-specific receptor-like structures. Since this interaction is very specific, plant defensins are discussed as being an attractive source for therapeutics [165].

These examples clearly show that food protection could be achieved by the use of antimicrobial and antifungal proteins. However, despite the ubiquitous occurrence of antifungal proteins, none of them is currently utilised, either in food preservation or in treatment of clinically relevant pathogenic fungi, although some proteins are evaluated for pharmaceutical use. For example, heliomycin is currently examined in preclinical tests for antifungal treatment [97].

A main hurdle that has hindered the development of antimicrobial and antifungal proteins as therapeutic agents is the fact that many naturally occurring proteins with antifungal activity in vitro (e.g. magainin) are only effective in vivo at very high doses, often close to the toxic doses of the peptide [97, 166]. One reason for this discrepancy between in vitro and in vivo activity might be the fact that the action of many antifungal proteins, especially membrane-acting proteins, has been shown to be cationsensitive. Sensitivity to high-ionic-strength conditions or a reduced activity under physiological conditions may be a crucial point in the application of antifungal proteins, since many food products have salt concentrations which also would lead to a decreased antifungal activity or even inactivation of the protein. However, plant defensins often retain their antifungal activity, even under high-ionic-strength conditions [118], which would make them ideal candidates for an antifungal treatment of food products.

A prerequisite for any application of antifungal proteins is the lack of effects on the host cells. A common method to demonstrate safety and selectivity is a hemolytic assay. The absence of cytolytic activity to red blood cells is generally accepted as proof that the protein can be regarded as safe [6]. However, this in vitro test often did not reflect physiological conditions since sensitivity to cations, and synergistic effects with other antifungal and antibacterial proteins are often not taken into account. Many antifungal proteins have been shown to act synergistically with other antifungal as well as antimicrobial proteins. Synergistic effects can alter the activity or even the species specificity of a protein. For example, the antifungal protein cecropin B alone has no effect on *Escherichia coli*, but in combination with lysozyme it has been shown to efficiently kill the bacterium [167]. Although synergistic effects could be beneficial for many applications, they also may lead to negative effects, since synergistic interaction with human antifungal and antibacterial proteins might alter the antifungal or antibacterial spectrum.

Another aspect which has to be taken into account for prospective application of antifungal proteins is resistance. The extensive use of classical antibiotics has led to a huge increase of resistant bacteria and fungi. Since the mode of action of antifungal proteins is much more complex, development of resistance against these proteins is probably harder to archive. Nevertheless, it has been shown that fungi are able to adapt to the presence of cell wall-degrading enzymes [168, 169]. Macroconidia of *Fusarium solani* which were exposed to sublethal concentrations of cell wall-degrading enzymes became resistant to much higher concentrations that are lethal to non-induced fungi [169]. Evidence is accumulating that a weakened cell wall activates chitin synthesis and glucan synthase 2 [170, 171]. Furthermore, the expression of the cell wall protein Cwp1p in *S. cerevisiae* which limits the yeast cell wall permeability for nisin is positively influenced [172]. Resistance to membrane-acting proteins has not been reported. However, the initial binding of membrane-acting antifungal proteins to a receptor appears to be a common feature. Therefore, resistance can easily be achieved by slight modifications of the corresponding receptor [79].

Antifungal proteins may be powerful tools in food protection as well as in clinical treatment of pathogens. However, several aspects have to be thoroughly examined prior a possible application. Activity under physiological conditions, resistance, selectivity and synergistic effects are only a few aspects which have to be clarified prior to application of antifungal proteins. In fact, there are other safety considerations such as immunogenicity, or cross-reactions with other host receptors such as neuropeptide and peptide hormone receptors. Therefore, knowledge of the exact mode of action of antifungal proteins is a prerequisite for their application.

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