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Review

The mode of antifungal action of plant, insect and human defensins

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Abstract. Defensins are small (~5 kDa), basic, cysteine-rich antimicrobial peptides that fulfill an important role in the innate immunity of their host by combating pathogenic invading micro-organisms. Defensins can inhibit the growth or virulence of micro-organisms directly or can do so indirectly by enhancing the host's immune system. Because of their wide distribution in nature, defensins are believed to be ancient molecules with a common ancestor that arose more than a billion years ago. This review summarizes current knowledge concerning the mode of antifungal action of plant, insect and human defensins.

Keywords. Mode of antifungal action, defensin, plant, insect, human.

Introduction

Every living organism, whether animal, plant or even microbe, is constantly confronted with attacks by various kinds of pathogens. Despite this threat, illness remains the exception. This illustrates that all these organisms must have evolved rather efficient mechanisms to defend themselves against pathogen attack. The most sophisticated of these mechanisms deploys antibodies and killer cells to recognize and eliminate specific invaders and possesses immunological memory and true self versus non-self discrimination. These adaptive immune responses are only elaborated in a small subset of living species, the higher vertebrates [reviewed in ref 1]. Innate immunity, on the other hand, is a much more widespread, ancient defense strategy involving, among other responses, the production of antimicrobial peptides (AMPs). The latter lack the antigen-recognition specificity of antibodies. However, being simply produced by transcription and translation of a single gene, antimicrobial peptides can be delivered relatively rapidly after infection with a limited input of energy and biomass, and can efficiently repel pathogenic invaders [reviewed in ref 2]. While it is increasingly recognized that AMPs may also function as modulators of the innate and adaptive immune response in higher organisms, their primary role is believed to lie in the killing of invading pathogenic organisms [reviewed in refs 3–7].

An intriguing class of AMPs comprises the defensins. They have been found in invertebrate [reviewed in ref 8], and vertebrate animals (α-, β- and θ-defensins) [reviewed in refs 4, 9, 10], as well as in plants [reviewed in refs 11–13]. Defensins are small (~5 kDa), basic, cysteine-rich peptides. The global fold comprises an antiparallel β sheet and an α helix and is stabilized by disulfide bridges into a compact shape, as in the case for plant, insect and mammalian β–defensins [reviewed in refs 4, 8–10, 13] (Fig. 1). Mammalian α-defensins lack the α helix and mammalian θ defensins are cyclic α-defensin-derived peptides [reviewed in

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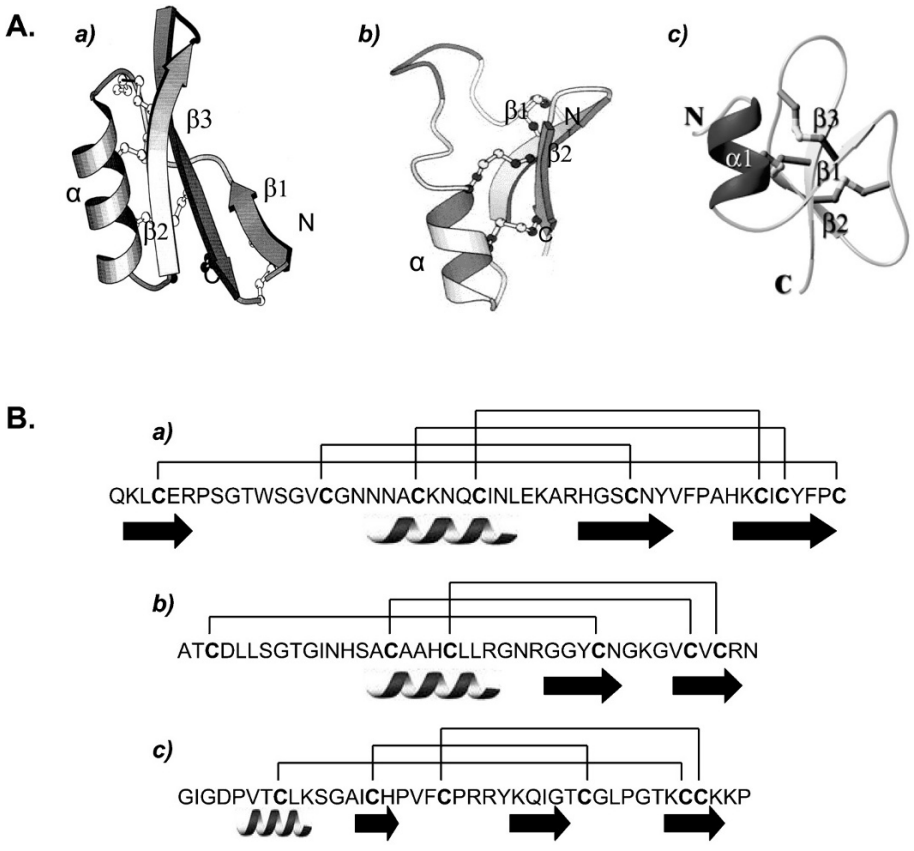

Figure 1. (*A*) Three-dimensional structures from RsAFP1 (a) [adapted from ref 90], insect defensin A (b) [adapted from ref 91] and HBD2 (c) [adapted from ref 4]. (*B*) Amino acid sequence from RsAFP1 (a), insect defensin A (b) and HBD2 (c). Connecting lines between cysteine residues represent disulfide bonds. Symbols represent positions of the α helices (spiral) and β strands (arrows).

refs 4, 9, 10]. It has been postulated that all defensins evolved from a single precursor [14]. This hypothesis arose from the observation that, based on sequence homologies, there is a closer relationship between vertebrate β-defensins and insect defensins than between vertebrate α- and β-defensins [14]. Recently, Mygind et al. [15] isolated the first defensin from a fungus, i.e. plectasin from the saprophyte *Pseudoplectania nigrella*, displaying antimicrobial activity. The identification of this defensin in a lower eukaryote further demonstrates the widespread distribution of the defensin class of peptides over different eukaryotic kingdoms and suggests that ancestral defensin genes existed even before fungal and insect lineages diverged (i.e. over a billion years ago) [10]. Apart from the structural homologies between defensins from different eukaryotic kingdoms, there also seems to exist functional homology among them. For example, overexpression of either insect [16, 17] or mammalian defensins [18, 19] in plants can contribute to an increased plant resistance to fungal diseases, comparable to that obtained by overexpression of plant defensins [20–26]. Although much progress has been made in recent years, the complete molecular basis of the mode of antimicrobial action for most of these defensins still needs to be unraveled. It would be interesting to explore whether defensins from different eukaryotic kingsdoms also display similarities regarding their mode of antimicrobial action.

In contrast to the better documented antibacterial properties of defensins [reviewed in refs 4, 8–10, 13], this review provides an overview of their less documented antifungal properties and mode of antifungal action. For reasons of structural homology and available current knowledge, this review focuses only on plant, insect and human β-defensins. For information about the structure, biological activity and role, expression pattern and antibacterial mode of action of plant defensins, and of defensins from invertebrate and vertebrate animals, the interested reader is also referred to some other excellent reports [e.g. 4, 8–10, 13, 27].

Plant defensins

Plant defensins exhibit antifungal activity against a broad range of phytopathogenic fungi and can also inhibit human fungal pathogens (such as *Candida albicans*) at low concentrations (1–100 μg/ml) [28, 29]. So far, only a few plant defensins have been shown to possess antibacterial activity [30–33]. Based on their effects on the growth and morphology of the test fungus *Fusarium culmorum*, two groups of defensins

could originally be distinguished [33]. The 'morphogenic' plant defensins cause reduced hyphal elongation with a concomitant increase in hyphal branching, whereas the 'non-morphogenic' plant defensins reduce the rate of hyphal elongation, but do not induce marked morphological distortions [33]. Note however that the morphogenic/non-morphogenic character of these defensins can depend on the test fungus and test medium [33]. Therefore, to date, plant defensins are divided into two major classes according to the structure of their precursor proteins predicted from cDNA clones [34]. In the first and largest class, the precursor protein is composed of an endoplasmic reticulum (ER) signal sequence and a mature defensin domain. The second class of plant defensins is produced as larger precursors with both an ER signal sequence and a C-terminal prodomain [34]. The exact function of this prodomain is not yet known [34].

The antifungal activity of plant defensins is generally found to be reduced in the presence of monovalent and especially divalent cations in the medium [33, 35, 36]. The antagonizing effect of cations and salt in the medium on antimicrobial activity is also a common observation for other small, basic, antimicrobial proteins, including insect [37] and mammalian defensins [38, 39, 40]. The antagonistic effect of cations on the antifungal activity of plant defensins strongly depends on the test fungus, indicating that electrostatic interactions probably alter the target site on the fungal membrane rather than the conformation of the plant defensin itself [41].

In contrast to mammalian and insect defensins, plant defensins have never been shown to induce ion-permeable pores in artificial membranes composed of phospholipids, nor to change their electrical properties. This demonstrates that a direct interaction between plant defensins and plasma membrane phospholipids is unlikely [42, 43]. The precise mode of action of plant defensins is still not completely unraveled, and for most plant defensins molecular components involved in signaling and putative intracellular targets remain unknown. It is only recently that different research groups were able to reveal a part of the molecular basis for the antifungal inhibitory activity of some of plant defensins, i.e. for defensins from dahlia [33], radish [35], coral bells [33], pea [44] and alfalfa [22].

DmAMP1 from dahlia (*Dahlia merckii*), RsAFP2 from radish (*Raphanus sativus*) and HsAFP1 from coral bells (*Heuchera sanguinea*)

The first characteristics of the mode of antifungal action of plant defensins were obtained for the seed defensins DmAMP1 from dahlia (*D. merckii*) [33], RsAFP2 from radish (*R. sativus*) [35] and HsAFP1 from coral bells (*H. sanguinea*) [33]. DmAMP1 and RsAFP2 were found to inhibit fungal and yeast growth by inducing an array of relatively rapid responses (starting at 1 to 10 min after peptide addition) in fungal cells, including increased K^+ efflux, Ca^{2+} uptake, alkalinization of the medium and membrane potential changes [42]. Membrane permeabilization determined by measuring α-aminoisobutyric acid leakage out of hyphae of sensitive fungi was only observed at 100 μg/ml DmAMP1 or RsAFP2 (about 10 times their IC_{100}, which is the concentration causing 100 % inhibition of growth) [42]. Subsequent studies using SYTOX green revealed that the membrane permeabilization induced by DmAMP1, RsAFP2 or HsAFP1 was either cation sensitive and detectable after 30–60 min at high plant defensin doses (50–200 μg/ml, aspecific permeabilization), or weak and cation resistant and only detectable after 2–4 h at lower concentrations (0.5–5 μg/ml), which correlates with the concentrations required for growth inhibition [45]. The relatively long lag phase before the specific permeabilization by plant defensins occurs suggests that the induced permeabilization by plant defensins is rather a secondary effect of their antifungal acitivity and probably not the cause of the observed plant defensin-induced ion fluxes [46]. The observation that DmAMP1, RsAFP2 and HsAFP1 can exert their antifungal action independently of the ion concentration favored the hypothesis of the existence of specific binding sites on the fungal envelope for these plant defensins rather than an aspecific, mere electrostatic interaction [45].

The existence of high-affinity binding sites for DmAMP1 on fungal cells and membrane fractions was demonstrated using radiolabeled DmAMP1 [47]. Using a genetic complementation approach, the binding site for DmAMP1 was identified as mannosyldiinositolphosphoryl-ceramide [$M(IP)_2C$], an acid complex sphingolipid (Fig. 2A) [48]. Consequently, we showed that yeast mutants deficient in the $M(IP)_2C$ biosynthesis genes *IPT1* and *SKN1* are resistant to DmAMP1 [48, 49]. Moreover, enzyme-linked immunosorbent assay (ELISA)-based binding studies revealed that DmAMP1 interacts directly with sphingolipids isolated from *Saccharomyces cerevisiae* [50]. Sphingolipids represent one of the three major types of lipids found in eukaryotic membranes, along with sterols and phosphoglycerolipids. It has become increasingly evident, however, that sphingolipids, beside their structural role, are also important signaling molecules in cell regulation, cell growth and cell stress responses [51, 52]. Subsequently, we demonstrated that RsAFP2 interacts with the neutral sphingolipid class of glucosylceramides (GlcCer) (Fig. 2B) in fungal membranes [29]. Accordingly, we

showed that yeast mutants deficient in the GlcCer biosynthesis gene *GCS1* are resistant to RsAFP2 [29]. Moreover, the intrinsic RsAFP2 resistance of *S. cerevisiae* and *Candida glabrata* could now be explained by the natural lack of the RsAFP2 target GlcCer in their membranes [29, 35, 53–55]. Interestingly, using an ELISA-based binding assay, we found that RsAFP2 interacts with GlcCer, isolated from *P. pastoris*, but not with GlcCer from soybean or human GlcCer [29]. This finding may explain the earlier observed non-toxicity of RsAFP2 for plant or human cells [20, 35]. Since in fungal membranes sphingolipids are co-localized with ergosterol in so-called 'lipid-rafts' that are highly enriched in glycolsylphosphatidylinositol (GPI)-anchored membrane proteins [56], a possible role of these rafts in DmAMP1 and RsAFP2 antifungal action was further investigated. It was shown that the defensin-sphingolipid interaction was enhanced in the presence of equimolar concentrations of ergosterol in the case of DmAMP1, but not for RsAFP2 [29, 50]. Finally, using radiolabeled HsAFP1, HsAFP1 was also found to interact with specific, high-affinity binding sites on fungal hyphae and microsomal membranes [57]. However, these HsAFP1-binding sites have not yet been identified. HsAFP1 is unlikely to interact with $M(IP)_2C$ or GlcCer, since yeast deletion mutants defective in biosynthesis of GlcCer or $M(IP)_2C$ have wild-type sensitivity to HsAFP1 [B. P. A Cammue and K. Thevissen, unpublished data].

Recently, we demonstrated that RsAFP2 induces endogenous reactive oxygen species (ROS) in *C. albicans*, but not in an RsAFP2-resistant Δ*gcs1* deletion mutant [58]. Moreover, we showed that the antioxidant ascorbic acid abolishes both RsAFP2-induced ROS and RsAFP2 antifungal action, pointing to a causative link between RsAFP2 antifungal activity and RsAFP2-induced ROS. This may suggest that RsAFP2 induces an intracellular signaling pathway leading to membrane permeabilization upon initial interaction with GlcCer, rather than merely causing membrane permeabilization by directly inserting into the plasma membrane upon its interaction with GlcCer [58]. Whether DmAMP1 or HsAFP1 induce ROS in susceptible yeast is currently not known. ROS are produced as byproducts of aerobic respiration and may damage proteins, lipids and DNA, resulting in loss of viability [59]. In mammalian cells, an increase in membrane ceramide content can induce ROS production in mitochondria [60]. Whether RsAFP2 induces ROS production through degradation of GlcCer resulting in elevated ceramide levels needs to be investigated further. Since endogenous ROS induction is a typical phenotypic characteristic of apoptosis or programmed cell death in yeast [61], RsAFP2 may induce yeast apoptosis.

Until now, it is unclear whether DmAMP1 and RsAFP2 are taken up by fungal cells upon sphingolipid interaction, or whether they stay outside and modulate cellular processes leading to fungal cell death (e.g. ROS production, apoptosis) via their sphingolipid interaction. As described below, for the pea defensin Psd1, cellular uptake has now been demonstrated [62]. However, it is not known whether Psd1 interacts with sphingolipids before cellular entry.

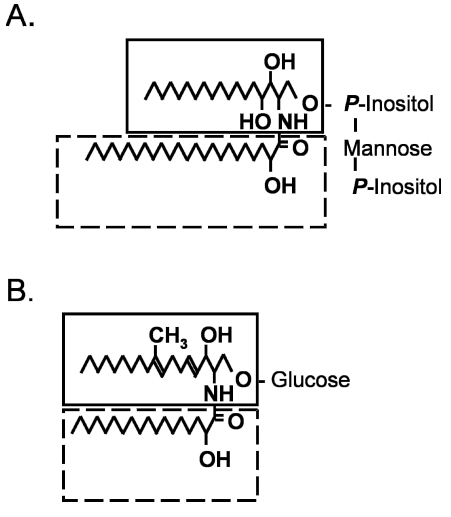

Figure 2. Structure of sphingolipids $M(IP)_2C$ (*A*) and GlcCer (*B*) [adapted from ref 29]. They are composed of a sphingoid base (boxed with full lines) that is linked to an (un)saturated fatty acid (boxed with dashed lines), together forming the ceramide moiety. The ceramides are further decorated with a polar head group to form the sphingolipids.

Psd1 from pea (*Pisum sativum*)

A yeast two-hybrid screening and successive glutathione-S-transferase (GST) pull-down assay was used to identify direct protein-protein interactions between pea defensin Psd1 [44] and proteins from the fungus *Neurospora crassa* [62]. In this way, cyclin F, related to cell cycle control, was found to be an interaction partner for Psd1. Subsequent fluorescence microscopy analysis of FITC-labeled Psd1 and DAPI-stained fungal nuclei showed the co-localization of Psd1 and the nucleus. Analysis of the DNA content of *N. crassa* conidia using flow cytometry showed a temporal increase of conidial DNA content in the presence of Psd1 without subsequent completion of cell division. The latter suggests that Psd1 affects the normal progression of the cell cycle. Because of the highly conserved cell cycle machinery between eukaryotic uni- and multicellular organisms, the developing retina of neonatal rats was used as a model to observe the interkinetic nuclear migration during proliferation of an organized tissue from the S phase

toward the M phase of the cell cycle in the presence of Psd1. In this way it was demonstrated that Psd1 can regulate interkinetic nuclear migration in retinal neuroblasts [62]. Whether Psd1 is toxic to mammalian cells remains to be determined.

MsDef1 from alfalfa *(Medicago sativa)* and MtDef2 from barrel medic (*M. truncatula*)

MsDef1 is a broad-spectrum antifungal defensin from alfalfa (*M. sativa*) seed, previously referred to as AlfAFP [22]. MsDef1 inhibits hyphal elongation of the fungal pathogen *F. graminearum*, thereby causing a hyperbranching phenotype [63]. Spelbrink et al. (2004) [63] demonstrated that MsDef1 blocks the L-type Ca^{2+} channel in mammalian cells. Structurally similar barrel medic (*M. truncatula*) MtDef2 [63] and radish RsAFP2 defensins failed to block this L-type Ca^{2+} channel. It was suggested that MsDef1 likely also targets Ca^{2+} channels in susceptible fungi since Ca^{2+} is a ubiquitous signaling molecule in fungi, regulating e.g. cAMP levels [64], bud formation [65] and hyphal elongation [66]. Hyphal elongation is a process that is controlled by a gradient in cytosolic Ca^{2+} generated by hyphal tip-localized Ca^{2+} channels [67]. The disruption of this hyphal tip Ca^{2+} gradient is known to cause hyperbranching [66]. Other support for the hypothesis that MsDef1 causes hyphal growth defects by blocking fungal Ca^{2+} channels came from the fact that EGTA and lanthanum, known Ca^{2+} channel blockers, also inhibit the growth of *N. crassa* spores and induce hyperbranching [63]. Mutational analyses led to the hypothesis that MsDef1 binds to the extracellular side of the Ca^{2+} channel in a manner similar to a virally encoded and structurally unrelated antifungal toxin KP4 from the fungus *Ustilago maydis* [63].

Recently, it was shown that a Δ*gcs1* deletion mutant of *F. graminearum*, which is devoid of GlcCer, is resistant to both MsDef1 and RsAFP2 [68]. This observation may suggest that MsDef1, like RsAFP2, targets GlcCer in membranes of susceptible yeast.

To determine the signaling cascades that are modulated upon defensin treatment, Ramamoorthy et al. [69] screened 4800 insertional mutants of *F. graminearum* for hypersensitivity toward MsDef1. Seven hypersensitive mutants were identified of which two mutants (*esd1* and *esd2*) were chosen for further analysis. *esd1* and *esd2* were shown to have insertions in Fg*STE11* and *MGV1*, respectively. FgSte11p and Mgv1p are part of the Gpmk1 and Mgv1 mitogen-activated protein kinase (MAPK) signaling pathways, respectively, which regulate multiple developmental processes related to cell wall integrity, sexual reproduction and pathogenicity [70, 71]. To confirm that the MsDef1 hypersensitivity of *esd1* and *esd2* was indeed due to their insertions in Fg*STE11* and *MGV1*, deletion mutants ΔFg*ste11* and Δ*mgv1* were generated. It was observed that these also were hypersensitive toward MsDef1. Moreover, MsDef1 hypersensitivity of deletion mutant Δ*gpmk1* was demonstrated. Gpmk1p functions downstream from FgSte11p in the Gpmk1 MAPK signaling pathway. Both Gpmk1p and Mgv1p are protein kinases that activate transcription factors in the MAPK signaling pathways. Furthermore, it was demonstrated that Gpmk1p and Mgv1p show a rapid increase in phosphorylation in response to MsDef1. Interestingly, the Δ*gpmk1* and Δ*mgv1* deletion mutants were also shown to exhibit hypersensitivity to the related barrel medic defensin MtDef2, which lacks antifungal activity against *F. graminearum*, and to the radish defensin RsAFP2.

Insect defensins

Most insect defensins identified to date have antibacterial activity with particular efficacy against Gram-positive bacteria, which are inhibited at low concentrations (1–100 μg/ml). Gram-negative bacteria, yeast and filamentous fungi are less sensitive to insect defensins [reviewed in ref 8]. Indeed only few antifungal defensins have been reported to date: termicin from the termite *Pseudacanthotermes spiniger* [72], and the defensin-like peptides drosomycin from the fruitfly *Drosophila melanogaster* [73], heliomicin from the tobacco budworm *Heliothis virescens* [74], and gallerimycin from the greater wax moth *Galleria mellonella* larvae [75]. The relatively low number of antifungal insect defensins reported in the literature is probably not a reflection of the lack of insect antifungal peptides but rather a lack of deliberate search for such molecules [8]. Below, information about the antifungal mode of action of termicin, drosomycin and heliomicin is reviewed. To our knowledge there is no information about the antifungal mode of action of gallerimycin available in the literature.

Termicin from the termite *P. spiniger*

Lamberty et al. [72] showed that 400 μg/ml termicin from the termite *P. spiniger* induces morphologic distortions of hyphae of *Aspergillus fumigatus*. Moreover, it was demonstrated that termicin at this concentration perforates the *A. fumigatus* hyphal cell wall with occasionally local leakage of cytosolic material. Remarkably, termicin can not inhibit spores of *A. fumigatus* at this concentration [72]. No further information regarding its molecular mode of antifungal action is currently available.

Drosomycin from the fruitfly *D. melanogaster*

Fehlbaum et al. (1994) [73] demonstrated that high concentrations of drosomycin (800 μg/ml) from the fruitfly *D. melanogaster* completely inhibit germination of spores of susceptible fungi. Lower concentrations of drosomycin cause delayed growth and abnormal morphology of hyphae. Moreover, it was shown that more than 50 % of the hyphae of *Botrytis cinerea* treated with drosomycin at a concentration of 10 μg/ml extruded cytoplasmic material along the hyphae, indicating that the protein causes partial lysis of susceptible fungi [73]. No further information regarding its molecular mode of antifungal action is currently available.

Heliomicin from the tobacco budworm *H. virescens*

Inspired by the similarity of the three-dimensional structures and biological activies between heliomicin from the tobacco budworm *H. virescens* and radish defensin RsAFP2, we investigated the possible involvement of the RsAFP2 sphingolipid interaction partner glucosylceramide (GlcCer) in heliomicin-induced fungal growth inhibition [29]. Similar to the observations for RsAFP2, growth of *C. albicans* and *P. pastoris* is inhibited by heliomicin (10 μg/ml), whereas deletion mutants in the GlcCer biosynthesis gene *GCS1* of both yeast strains are at least 20-fold more resistant. Furthermore, heliomicin was shown to interact directly and in a dose-dependent manner with GlcCer isolated from *P. pastoris* in an ELISA-based binding assay [29]. The finding that heliomicin, in contrast to RsAFP2, interacts with both fungal and plant GlcCer points, however, towards different GlcCer-interacting characteristics for RsAFP2 and heliomicin. This is also reflected by the observation that interaction of heliomicin with fungal GlcCer could not be competed for by RsAFP2 and vice versa, indicating that heliomicin and RsAFP2 probably interact with different structural motifs of fungal GlcCer [29].

The similarity of their three-dimensional structures suggests a close relationship between plant and insect defensins. The fact that both plant and insect defensins can target similar structures, glucosylceramides, in the fungal plasma membrane supports the hypothesis that defensins from plants and insects have evolved from a single precursor [29]. However, the mode of action of the structurally related human β-defensins (see below) seems not to depend on GlcCer or $M(IP)_2C$ in fungal membranes. Indeed, yeast mutants devoid of these sphingolipids were shown to be as sensitive toward human β-defensin 2 as the corresponding wild-type strains [B. P. A. Cammue and K. Thevissen, unpublished data].

Human defensins

Mammalian defensins are subdivided into three classes, α-, β- and θ-defensins. Alpha- and β-defensins differ in their cysteine spacing, disulfide bond connectivity and gene organization. Moreover α-defensins lack an α helix [reviewed in refs 9, 10, 76]. Theta-defensins are cyclic, α-defensin-derived peptides [77] and only exist in several species of Old World monkeys and in orangutans but not in humans or New World primates [78]. Since β-defensins are structurally most closely related to plant and insect defensins, we will review here the mode of antifungal action of human β-defensins (HBDs). Most information is available about HBD1, HBD2 and HBD3. Human β-defensins have the capacity to kill and/or inactivate bacteria and fungi *in vitro* at low concentrations (1–100 μg/ml) [reviewed in refs 4, 9, 10, 79]. A further effect of HBD2 and HBD3 is the inhibition of HIV1 replication *in vitro* [80, 81]. Apart from inhibiting pathogens through direct contact with the microorganisms, HBDs have the capacity to protect their host indirectly by triggering the innate and adaptive immune responses [reviewed in refs 4, 9, 10].

Human β-defensin 1 (HBD1)

HBD1 can inhibit various *Candida* species such as *C. albicans*, *C. krusei* and *C. parapsilosis* and demonstrates better killing activity against *C. glabrata* than do HBD2 and HBD3, even though it kills only 25–50 % of cells [82]. Furthermore, it has been demonstrated that HBD1 is able to inhibit *C. glabrata* adherence to epithelial cells *in vitro* [82].

Human β-defensin 2 (HBD2)

HBD2 possesses activity against *C. albicans*, *C. krusei* and *C. parapsilosis*, but is inactive against *C. glabrata* [82, 83]. However, as for HBD1, *C. glabrata in vitro* adherence to epithelial cells was inhibited by HBD2 [82].

Confocal microscopy analysis of *C. albicans* after incubation with HBD2 revealed dramatic changes in the *C. albicans* cell envelope [82]. Indeed, HBD2 treatment resulted in thinning and dissolution of the cell walls and possible cell lysis [82]. The latter was expected from the observed extrusion of cytoplasmic debris [82]. However, it has to be noted that in the latter study, HBD2 concentrations were used that exceeded the IC_{50} (concentration causing 50 % inhibition of growth) by more than 10-fold, thereby probably producing robust cellular effects that are not likely to occur at physiological peptide concentrations. Indeed, Vylkova et al. [40] demonstrated that HBD2 does not cause gross membrane damage in *C. albicans* cells when treated with IC_{90} (concentration

causing 90 % inhibition of growth) HBD2 concentrations for 90 min.

In the same study, it was shown that killing of *C. albicans* cells by HBD2 causes ATP release, and is salt sensitive and energy dependent [40]. Whether the salt sensitivity of HBD2 candidacidal action is due to altered attachment to the *Candida* cell surface or to other downstream events in the mechanism of killing remains to be elucidated. Evidence for the energy dependency of HBD2 candidacidal action came from the fact that pre-treatment of *C. albicans* cells with the metabolic inhibitor sodium azide resulted in a significant decrease of both ATP release and sensitivity of cells to HBD2 [40]. Furthermore, it was shown that HBD2 exhibits reduced killing of Δ*ssa1* and Δ*ssa2 C. albicans* deletion mutants compared to the wild type, showing that the cell surface heat shock proteins Ssa1p and Ssa2p are required for HBD2 candidacidal activity [84]. However, resistance of certain *C. glabrata* strains to HBD2 seems not to be the result of loss of expression of these Ssa proteins. It was noticed that the candidacidal action of HBD2 shares several similarities with that of the human salivary antifungal peptide histatin 5 (Hst5) in terms of salt sensitivity, ion selectivity and energy requirements. Moreover, it was shown that Hst5 binds with Ssa1p and Ssa2p on the fungal cell wall and that this is necessary for subsequent intracellular translocation [85]. Whether HBD2 also interacts physically with Ssa1p or Ssa2p and subsequently crosses the surface of yeast cells should be investigated in the future. HBD2 candidacidal action has, however, also unique pathways compared to that of Hst5, since, in contrast to Hst5 action, it does not rely on the potassium transporter Trk1p [40].

Human β-defensin 3 (HBD3)

Like HBD2, HBD3 possesses activity against *C. albicans*, *C. krusei* and *C. parapsilosis*, but is inactive against *C. glabrata* [82, 83]. However, HBD3 can inhibit *C. glabrata* adherence to epithelial cells *in vitro* [82].

Vylkova et al. [40] showed that, in contrast to its salt-resistant antibacterial activity [86], the activity of HBD3 against *C. albicans* is salt sensitive (as measured in 20–100 mM sodium phosphate buffer), suggesting differences in the HBD3 bactericidal and candidacidal mechanisms of action. In contrast to HBD2, HBD3 candidacidal action was, however, not inhibited by Ca^{2+} or Mg^{2+}. It was furthermore shown that HBD3, like HBD2, causes ATP release in *C. albicans* cells. Pre-treatment of *Candida* cells with sodium azide decreased the ATP release together with the sensitivity of the cells to HBD3, pointing to an energy-dependent candidacidal mechanism of action. However, killing by HBD3 was partially restored in the presence of sodium azide at higher concentrations of HBD3 (≥ 4 μg/ml), showing energy-independent mechanisms at higher HBD3 doses [40]. Like HBD2, HBD3 requires Ssa1p and Ssa2p for its antifungal activity, although a different killing profile of Δ*ssa1* or Δ*ssa2 C. albicans* deletion mutants was observed for HBD3 as compared to HBD2: Δ*ssa1* or Δ*ssa2* deletion mutants showed a much more pronounced resistance to HBD3 than to HBD2. In conclusion, despite similarities in their biological activities, the difference in cation sensitivity and the dissimilar killing profiles of the Δ*ssa1* and Δ*ssa2* deletion mutants by HBD2 and HBD3 point to differences in their mechanisms of action against *C. albicans* [84].

Conclusions

Defensins from humans, insects and plants fulfill an important role in the immunity of their host, combating pathogenic invading micro-organisms. Table 1 summarizes current knowledge concerning the mode of antifungal action of plant, insect and human β-defensins. Apparently there are no clear similarities in the mode of antifungal action among these defensins. However, sphingolipid GlcCer in fungal membranes seems to play a central role in the antifungal action mechanism of some defensins. Indeed, it was demonstrated that both the insect defensin-like peptide heliomicin and the radish defensin RsAFP2 interact with GlcCer on the fungal plasma membrane [29]. These data indicate that structurally homologous antifungal peptides, present in species from plants and insects, can interact with the same target in the fungal plasma membrane and as such support the hypothesis that defensins could have evolved from a single precursor [29]. Furthermore, it was recently observed that an *F. graminearum* mutant devoid of GlcCer is resistant to both RsAFP2 and alfalfa defensin MsDef1, suggesting that MsDef1 targets GlcCer as well [68]. Of the defensins described in the text, only for the pea defensin Psd1 has it been demonstrated that it is internalized in the fungal cell, where it then affects the normal progression of the cell cycle [62]. Rather than being taken up by fungal cells, it is possible that the other defensins stay outside the cell and induce fungal cell death via modulating intracellular signaling cascades after their interaction with their target on the fungal evelope (e.g. sphingolipids). An example of the induction of such an intracellular signaling cascade is the induction of ROS generation by RsAFP2 [58]. An excess of ROS can lead to apoptotic cell death in yeast [61]. On the other hand, it was demonstrated that upon treatment with

Table 1. Summary of current knowledge concerning the mode of antifungal action of plant, insect and human β-defensins.

		Interaction partners on the fungal envelope	Cellular uptake	Intracellular signaling cascades a) defensin action b) fungal tolerance	Mechanisms of cell death
Plant	DmAMP1 *dahlia*	Sphingolipid $M(IP)_2C$ [48, 49, 50]	nd	a) nd b) nd	K^+ efflux [42] Ca^{2+} uptake [42] Alkalinization of the medium [42] Membrane potential changes [42] Membrane permeabilization [42, 45]
	RsAFP2 *radish*	Sphingolipid GlcCer [29]	nd	a) Induction of ROS accumulation [58] b) MAPK signaling pathways [69]	K^+ efflux [42] Ca^{2+} uptake [42] Alkalinization of the medium [42] Membrane potential changes [42] Membrane permeabilization [42, 45]
	Psd1 *pea*	nd	Yes, colocalizes with the nucleus [62]	a) nd b) nd	Interaction with cyclin F [62] Affects cell division completion [62]
	MsDef1 *alfalfa*	Sphingolipid GlcCer (probably) [68]	nd	a) nd b) MAPK signaling pathways [69]	Blocks mammalian Ca^{2+} channel [63]
Insect	Termicin *termite*	nd	nd	a) nd b) nd	Perforation of the hyphal cell wall [72] Leakage of cytosolic material [72]
	Drosomycin *fruitfly*	nd	nd	a) nd b) nd	Inhibition of spore germination [73] Leakage of cytosolic material [73]
	Heliomicin *tobacco budworm*	Sphingolipid GlcCer [29]	nd	a) nd b) nd	nd
Human	HBD2 HBD3	nd	nd	a) nd b) nd	ATP release [40] Energy dependent [40] Reduced killing of Δ*ssa* mutants [84]

nd: not determined.

various plant defensins, intracellular signaling cascades are modulated that are involved in tolerance mechanisms [69]. Indeed, *F. graminearum* mutants targeted in MAPK signaling pathways were shown to be hypersensitive toward RsAFP2, alfalfa defensin MsDef1 and barrel medic defensin MtDef2 [69]. A repeating theme in the mode of antifungal action of the discussed defensins is the permeabilization of the fungal cell wall and/or membrane [42, 45, 72, 73]. However, further research is necessary to reveal whether this permeabilization is a primary action or rather a secondary effect of fungal cell death. Future research may reveal more similarities in the mode of antifungal action of defensins of different eukaryotic kingdoms.

Unraveling the mode of antifungal action of antimicrobial peptides like defensins will be of great interest in the search for new antifungal therapeutics. Control of fungal pathogens constitutes a challenging problem in medicine. Indeed, during the past 20 years, the incidence of fungal infections in humans has risen considerably [reviewed in refs 87, 88]. The most common causes of these infections are *Candida* spp. and filamentous fungi such as *Aspergillus* spp. [reviewed in refs 87, 88]. Since there are no fungal vaccines currently licensed, the only clinical resource to combat fungal infections is the use of antifungal therapeutics (antimycotics) [reviewed in ref 89]. Among the currently used antimycotics some offer only a limited activity spectrum, are available only in intravenous formulations, favor resistance development, show harmful drug-drug interactions, or are associated with serious side effects such as high cytotoxicity [reviewed in ref 89]. Therefore, the search for new antifungal compounds with a novel mode of action is imperative. Unraveling the mode of action will be crucial in the rational design of novel antifungal peptide variants with enhanced activities or altered pathogen target specificities compared to the current antimycotics [reviewed in ref 89]. Therapies based on antifungal peptides, like defensins, could be promising alternative antimycotic treatments. In this

respect, succesful pre-clinical studies that address the *in vivo* performance of defensins in animal models against fungal infections have been reported, i.e. studies regarding the insect defensin-like AMP heliomicin and the plant defensin RsAFP2 [reviewed in ref 55].

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