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Small Cysteine-Rich Antifungal Proteins from Radish: Their Role in Host Defense

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Radish seeds have previously been shown to contain two homologous, 5-kD cysteine-rich proteins designated *Raphanus sativus*–antifungal protein 1 (Rs-AFP1) and Rs-AFP2, both of which exhibit potent antifungal activity in vitro. We now demonstrate that these proteins are located in the cell wall and occur predominantly in the outer cell layers lining different seed organs. Moreover, Rs-AFPs are preferentially released during seed germination after disruption of the seed coat. The amount of released proteins is sufficient to create a microenvironment around the seed in which fungal growth is suppressed. Both the cDNAs and the intron-containing genomic regions encoding the Rs-AFP preproteins were cloned. Transcripts (0.55 kb) hybridizing with an Rs-AFP1 cDNA–derived probe were present in near-mature and mature seeds. Such transcripts as well as the corresponding proteins were barely detectable in healthy uninfected leaves but accumulated systemically at high levels after localized fungal infection. The induced leaf proteins (designated Rs-AFP3 and Rs-AFP4) were purified and shown to be homologous to seed Rs-AFPs and to exert similar antifungal activity in vitro. A chimeric Rs-AFP2 gene under the control of the constitutive cauliflower mosaic virus 35S promoter conferred enhanced resistance to the foliar pathogen *Alternaria longipes* in transgenic tobacco. The term "plant defensins" is proposed to denote these defense-related proteins.

INTRODUCTION

In vegetative plant tissues, a series of dynamic defense mechanisms can be triggered upon wounding or perception of microorganisms. Newly formed carbohydrate material can be deposited in the cell wall in response to penetration attempts by fungal hyphae (Aist, 1976), and preexisting cell wall proteins can be oxidatively cross-linked upon wounding and elicitor treatment (Bradley et al., 1992). Both responses result in an induced fortification of the cell wall. Another strategy followed by plants to thwart invaders is based on the localized production of antimicrobial low molecular weight secondary metabolites known as phytoalexins (Van Etten et al., 1989; Maher et al., 1994). Furthermore, the synthesis of many presumed defense-related proteins is induced when plants are confronted with pathogens (Linthorst, 1991). Among these proteins are the pathogenesis-related (PR) proteins. Members of five different PR protein families have been shown to possess antifungal activity in vitro (Mauch et al., 1988; Woloshuk et al., 1991; Hejgaard et al., 1992; Niderman et al., 1993; Sela-Buurlage et al., 1993; Ponstein et al., 1994), and some PR proteins confer enhanced resistance to fungal diseases when expressed in transgenic plants (Broglie et al., 1991; but see Neuhaus et al., 1991; Alexander et al., 1993; Yoshikawa et al., 1993; Zhu et al., 1994). Many stress signals are able to induce the expression of defense-related proteins not only locally (e.g., in the vicinity of the infection site) but also in distant, nonstressed leaves. This phenomenon is known as systemic acquired resistance (Ross, 1961; Tuzun et al., 1989; Ward et al., 1991; Uknes et al., 1992). Candidate signal molecules involved in the immunization of a plant against subsequent infection are salicylic acid (Malamy et al., 1990; Métraux et al., 1990; Gaffney et al., 1993; Delaney et al., 1994) and methyl jasmonate (Farmer and Ryan, 1990; Farmer et al., 1992; Xu et al., 1994). Finally, some incompatible interactions between pathogens and plants can trigger localized necrosis of cells (Dixon and Lamb, 1990). This hypersensitive response can most probably be explained by "gene-for-gene" incompatibility (Ellingboe, 1981). A well-studied example of such a mechanism is the interaction between different tomato lines and different *Cladosporium fulvum* races. In the plant, *C. fulvum* produces race-specific elicitors (3-kD cysteine-rich peptides) that trigger the hypersensitive response only in tomato plants with the matching resistance gene (de Wit et

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al., 1992). The occurrence of the hypersensitive response is mediated by reactive oxygen species produced early in the plant–pathogen interaction (Levine et al., 1994).

Thus, plant defense mechanisms have been studied very intensively in vegetative tissues. However, little is known about the strategies used by seeds to survive and germinate in substrates densely populated with microorganisms. A particularly vulnerable stage occurs at early germination when the seed coat, which forms an effective physical barrier against microbes, is disrupted and the young seedling becomes exposed to the soil.

We have recently characterized a novel family of 5-kD cysteine-rich antifungal proteins (AFPs) from seeds of radish (Terras et al., 1992b) and four other crucifers, including Arabidopsis (Terras et al., 1993). Radish (*Raphanus sativus*) seeds contain almost equal amounts of two isoforms, Rs-AFP1 and Rs-AFP2, that exert antifungal activity against a broad spectrum of plant pathogenic filamentous fungi by causing hyperbranching and growth reduction of the hyphal tips. These proteins have little or no effect on bacteria and cultured human cells (Terras et al., 1992b). Using radish seed as a model system, we studied the release of Rs-AFPs during germination and the significance of this phenomenon with respect to seedling protection against fungal pathogens. In addition, the expression of Rs-AFPs or Rs-AFP–like proteins was examined in nonstressed and stressed leaves. To validate the presumed role of Rs-AFPs in host defense, Rs-AFP2 was constitutively expressed in transgenic tobacco plants that were subsequently analyzed for resistance to a foliar fungal pathogen.

RESULTS

Release of Rs-AFPs from Germinating Seeds

To study the release of antifungal compounds during germination of radish seeds, we developed a bioassay in which seeds were allowed to germinate on a medium supporting growth of a fungal colony. When the edges of the expanding colony approached the germinating seed, a growth inhibition halo appeared around the seed, as shown in Figure 1A. The growth inhibition effect could be mimicked by applying 1 μg of either purified Rs-AFP1 or Rs-AFP2 to a well in the agar medium (shown for Rs-AFP1 in Figure 1A). Addition of the endoprotease Pronase E to the medium resulted in the abolition of the inhibition zones caused by the germinating seed as well as by Rs-AFP1 (Figure 1B). Likewise, autoclaved seed or autoclaved Rs-AFP1 lost their inhibitory capacities (Figure 1C). When seed germination was prevented by addition of the plant hormone abscisic acid to the medium, no growth inhibition halo was observed around an intact seed. Under these conditions, however, seeds with a mechanically applied incision in their seed coat were still capable of releasing their antifungal components (Figure 1D). Thus, radish seed release a heat-sensitive proteinaceous antifungal compound only after disruption of their seed coat, either by germination or by mechanical incision. Of the previously purified radish seed proteins with antifungal properties, including Rs-AFPs, 2S albumins (Terras et al., 1992b), and a nonspecific lipid transfer protein (Terras et al., 1992a), only Rs-AFPs could restrict growth of fungal colonies in the agar diffusion bioassay at amounts <20 μg (data not shown). These results suggest that Rs-AFPs are the predominant proteinaceous antifungal compounds released from germinating radish seed. In the assay shown in Figure 1, the fungus *Pyrenophora tritici-repentis* was used because this fungus grows very evenly. Similar results (data not shown) were obtained when using other fungi, for example, *Fusarium culmorum* and *Pyricularia oryzae*.

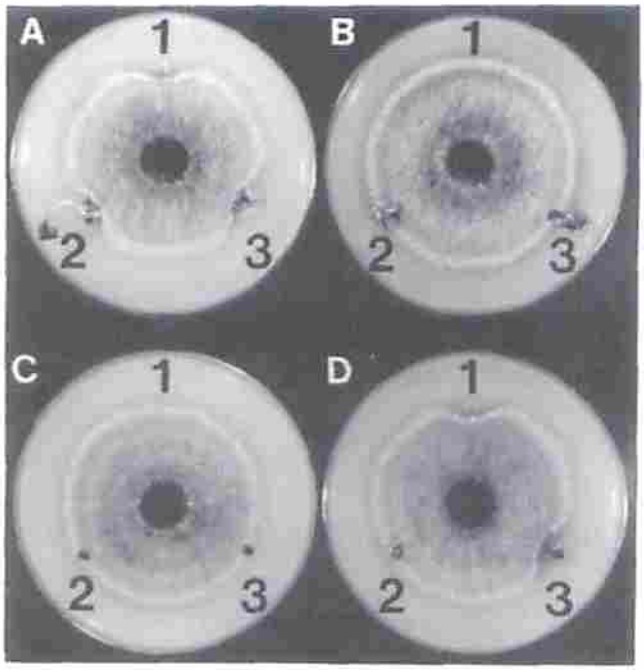

Figure 1. Release of Antifungal Compounds by Germinating Radish Seeds.

One microgram of purified Rs-AFP1 was applied at the positions indicated by the number 1. Radish seeds at the positions 2 had an intact seed coat, whereas seeds at positions 3 had an incised seed coat (along half of the seed periphery). The fungus *P. tritici-repentis* was used in this assay.

(A) Assay plates containing five cereal agar.

(B) Assay plates containing five cereal agar supplemented with 50 μg/mL Pronase E.

(C) Assay plates as given in **(A)**. The Rs-AFP1 solution and the seeds were autoclaved.

(D) Assay plates containing five cereal agar supplemented with 100 μM abscisic acid.

To verify and quantify the presumed Rs-AFP release from radish seeds, seeds with a mechanically applied incision in their seed coats were imbibed in water, after which the imbibition solution was analyzed by gel electrophoresis. Proteins released from a single imbibing seed were loaded on two

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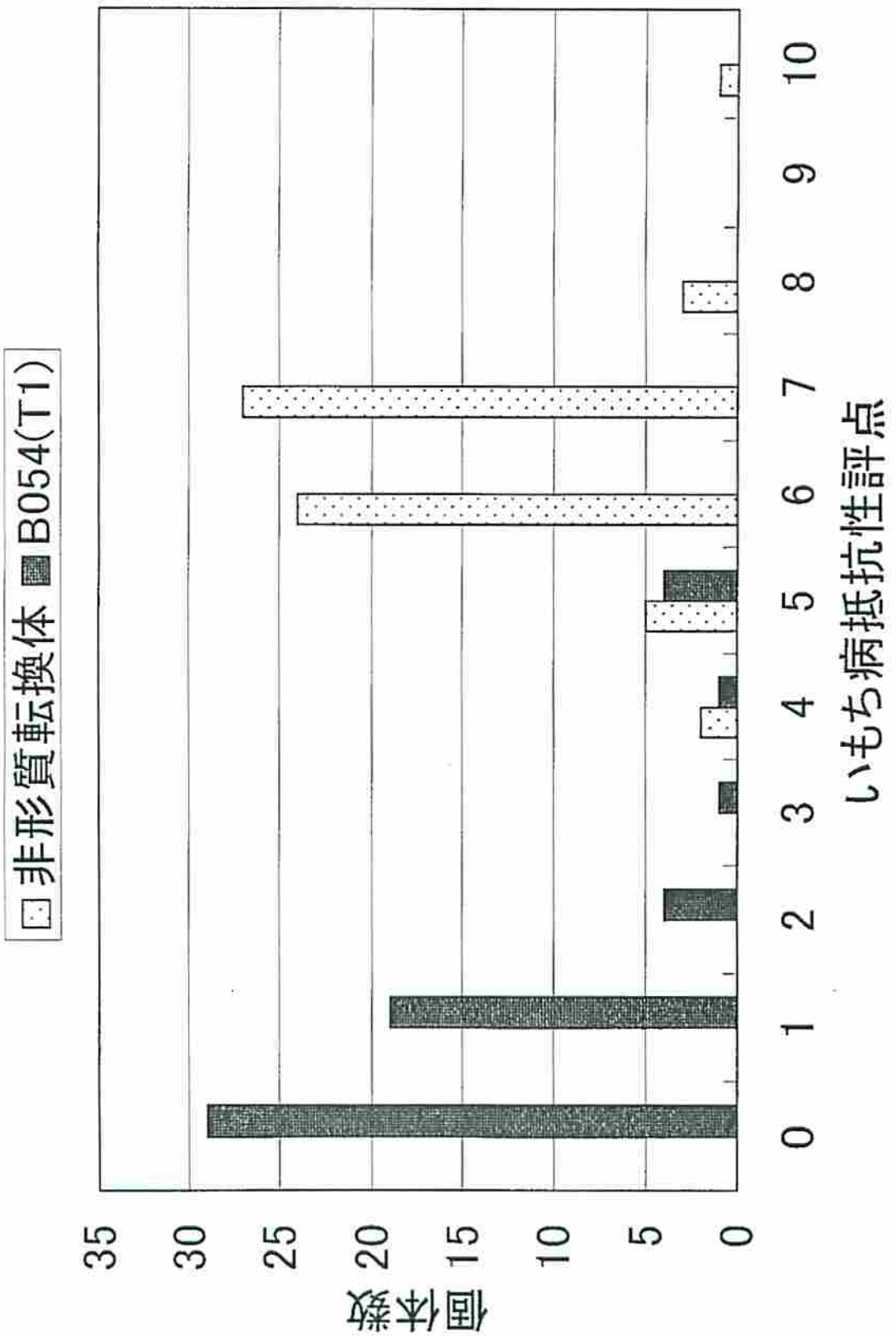
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review article

Antimicrobial peptides of multicellular organisms

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Multicellular organisms live, by and large, harmoniously with microbes. The cornea of the eye of an animal is almost always free of signs of infection. The insect flourishes without lymphocytes or antibodies. A plant seed germinates successfully in the midst of soil microbes. How is this accomplished? Both animals and plants possess potent, broad-spectrum antimicrobial peptides, which they use to fend off a wide range of microbes, including bacteria, fungi, viruses and protozoa. What sorts of molecules are they? How are they employed by animals in their defence? As our need for new antibiotics becomes more pressing, could we design anti-infective drugs based on the design principles these molecules teach us?

Antimicrobial peptides are evolutionarily ancient weapons. Their widespread distribution throughout the animal and plant kingdoms suggests that antimicrobial peptides have served a fundamental role in the successful evolution of complex multicellular organisms. Despite their ancient lineage, antimicrobial peptides have remained effective defensive weapons, confounding the general belief that bacteria, fungi and viruses can and will develop resistance to any conceivable substance. Antimicrobial peptides target a previously under-appreciated 'microbial Achilles heel', a design feature of the microbial cellular membrane that distinguishes broad species of microbes from multicellular plants and animals. The insights provided by this large body of research have spawned considerable commercial effort to create new classes of anti-infective therapeutics.

A diversity of peptides

The diversity of antimicrobial peptides discovered is so great that it is difficult to categorize them except broadly on the basis of their secondary structure. (An online catalogue of all reported molecules, now about 500, can be found at http://www.bbcm.univ.trieste.it/~tossi/antimic.html). The fundamental structural principle underlying all classes is the ability of the molecule to adopt a shape in which clusters of hydrophobic and cationic amino acids are spatially organized in discrete sectors of the molecule ('amphipathic' design) (Fig. 1). Linear peptides, such as the silk moth's cecropin[1] and the African clawed frog's magainin[2], adopt this organization only when they enter a membrane, whereupon they assume an amphipathic α-helical secondary structure[3]. Frog species of the genus *Rana* modify this design by adding a single loop formed by a disulphide bond at the carboxy end[4]. Peptides such as bactenecin[5] and defensins[6] use a relatively rigid anti-parallel β-sheet constrained by disulphide bonds as the framework, around which segregated patches of cationic and hydrophobic residues are organized. A large family of linear peptides characterized by a predominance of one or two amino acids, such as the tryptophan-rich indolicidin of the cow neutrophil[7] and the proline–arginine-rich PR39 (ref. 8) of the pig neutrophil, segregate hydrophobic and hydrophilic side chains around an extended peptide scaffold in the setting of the membrane (Table 1). Most multicellular organisms express a cocktail comprising multiple peptides from several of these structural classes within their 'defensive' tissues.

All antimicrobial peptides are derived from larger precursors, including a signal sequence. Post-translational modifications include proteolytic processing, and in some cases glycosylation[9], carboxy-terminal amidation and amino-acid isomerization (reviewed in ref. 4), and halogenation[10]. A rather complex modification involves the cyclization of two short peptides leading to the fully circular θ-defensin isolated from neutrophils of *Rhesus* (macaque) monkeys[11]. Some peptides are derived by proteolysis from larger proteins, such as buforin II from histone 2A (ref. 12) and lactoferricin from lactoferrin[13].

The diversity of sequences is such that the same peptide sequence is rarely recovered from two different species of animal, even those closely related, be they insects, frogs or mammals (exceptions include peptides cleaved from highly conserved proteins, such as buforin II). However, both within the antimicrobial peptides from a single species, and even between certain classes of different peptides from diverse species (reviewed in ref. 4), significant conservation of amino-acid sequences can be recognized in the preproregion of the precursor molecules; the design suggests that constraints exist on the sequences involved in the translation, secretion or intracellular

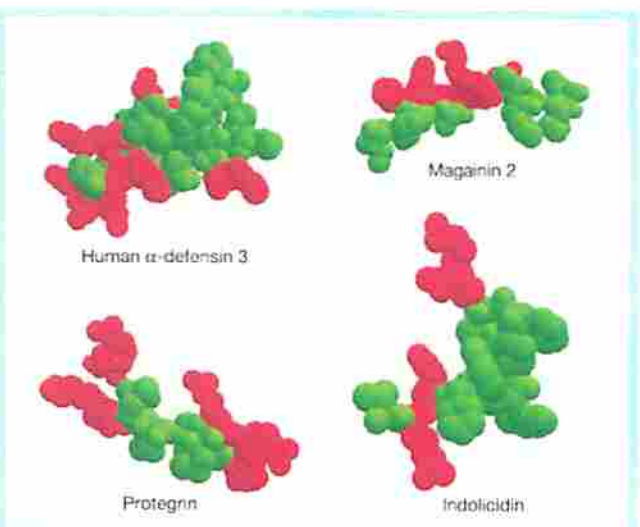

Figure 1 Clustering of cationic and hydrophobic amino acids into distinct domains in several antimicrobial peptides of different structural classes. This 'amphipathic' design is evident in many, but not all, antimicrobial peptides. Red, basic (positively charged) amino acids; green, hydrophobic ('oily') amino acids. Other amino acids are not shown. Magainin is depicted in its α-helical configuration.

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trafficking of this class of membrane-disruptive peptide. This feature is dramatically illustrated by the cathelicidins (reviewed in ref. 14).

Why does diversity arise? Because single mutations can dramatically alter the biological activity of each peptide, the diversity probably reflects the species' adaptation to the unique microbial environments that characterize the niche occupied, including the microbes associated with acceptable food sources (reviewed in refs 4 and 15). It seems reasonable to speculate that an individual could find itself in the midst of microbes against which the peptides of its species were ineffective; although the individual might suffer, the species itself could survive through emergence of individuals expressing beneficial mutations. Adaptive immunity, through its plasticity, permits a species to empower individuals to explore new environments and avail themselves of new food sources. However, compared with the equipment of the innate system, such as antimicrobial peptides, the effectors of adaptive immunity are more costly to maintain and slower to respond to assault[15].

With respect to the diversity created in the synthetic laboratory, almost all active molecules are composed of hydrophilic, hydrophobic and cationic amino acids arranged in a molecule that can organize into an amphipathic structure (reviewed in ref. 16). Natural peptides composed of all D-amino acids, in place of L-amino acids, typically retain full antibiotic potency while exhibiting expected resistance to enzymatic proteolysis[16]. Short linear or cyclic amphiphillic peptides that contain both L- and D-amino acids can be generated with various degrees of selectivity and antimicrobial potency[17,18]. Recently, protease-resistant antimicrobial peptides composed of □-amino acids have been constructed[19,20].

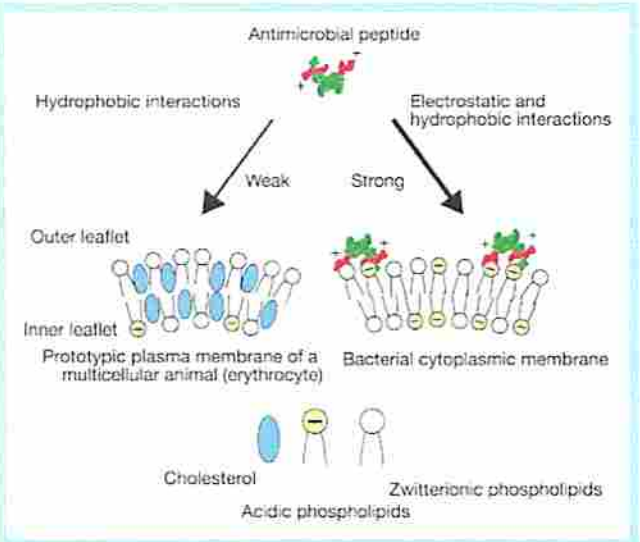

Figure 2 The membrane target of antimicrobial peptides of multicellular organisms and the basis of specificity. (Modified from ref. 21.)

Mechanism

Antimicrobial peptides have targeted a surprising but clearly fundamental difference in the design of the membranes of microbes and multicellular animals, best understood for bacterial targets. Bacterial membranes are organized in such a way that the outermost leaflet of the bilayer, the surface exposed to the outer world, is heavily populated by lipids with negatively charged phospholipid headgroups. In contrast, the outer leaflet of the membranes of plants and animals is composed principally of lipids with no net charge; most of the lipids with negatively charged headgroups are segregated into the inner leaflet, facing the cytoplasm (Fig. 2) (reviewed in ref. 21). A model that explains the activity of most antimicrobial peptides is the Shai–Matsuzaki–Huang (SMH) model (refs 21–23; Fig. 3). The model proposes the interaction of

Representative peptides		Origin	Tissue*
□-helical			
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAKa	Silk moth	E, BC, H
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	Frog	E
Pexiganan	GIGKFLKKAKKFGKAFVKILKKa	Synthetic	
Dermaseptin 1	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ	Frog	E
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Human	E, BC
Buforin II	TRSSRAGLQFPVGRVHRLLRK	Vertebrate	E
One disulphide bond			
Bactenecin 1	RLCRIVVIRVCR	Cow	BC
Thanatin	GSKKPVPIIYCNRRTGKCQRM	Insect	BC
Brevinin 1T	VNPILGVLPKVCLITKKC	*Rana* frogs	E
Ranalexin	FLGGLIKIVPAMICAVTKKC	*Rana* frogs	E
Ranateurin 1	SMLSVLKNLGKVGLGFVACKINKQC	*Rana* frogs	E
Esculentin 1	GIFSKLGRKKIKNLLISGLKNVGKEVGMDVVRTGIDIAGCKIKGEC	*Rana* frogs	E
Two disulphide bonds			
Tachyplesin	RWC$_1$FRVC$_2$YRGIC$_2$YRKC$_1$Ra	Horseshoe crab	BC
Androctonin	RSVC$_1$RQIKIC$_2$RRRGGC$_2$YYKC$_1$TNRPY	Scorpion	H
Protegrin 1	RGGRLC$_1$YC$_2$RRRFC$_2$VC$_1$VGRa	Pig	BC
Three disulphide bonds			
□-defensin (HNP3)	DC$_1$YC$_2$RIPAC$_3$IAGERRYGTC$_2$IYQGRLWAFC$_2$C$_1$	Human	BC, E
□-defensin (TAP)	NPVSC$_1$VRNKGIC$_2$VPIRC$_2$PGSMKQIGTC$_2$VGRAVKC$_1$C$_2$RKK	Cow	E, BC
□-defensin	GFC$_1$RC$_2$LC$_2$RRGVC$_2$RC$_1$IC$_1$TR	Monkey	BC
Defensin (sapecinA)	ATC$_1$DLLSGTGINHSAC$_2$AAHC$_3$LLRGNRGGYC$_2$NGKAVC$_3$VC$_1$RN	Insect	E, BC, H
Thionin (crambin)	TTC$_1$C$_2$PSIVARSNFNVC$_2$RIPGTPEAIC$_3$ATYTGC$_3$IIPGATC$_1$PGDYAN	Plant	E
Four disulphide bonds			
Defensin	QKLC$_1$QRPSGTWSGVC$_2$GNNNAC$_3$KNQC$_4$IRLEKARHGSC$_2$NYVFPAHC$_3$IC$_4$VFPC$_1$	Radish	Seeds, E
Drosomycin	DC$_1$LSGRYKGPC$_2$AVWDNETC$_3$RRVC$_4$KEEGRSSGHC$_2$SPSLKC$_3$WC$_4$EGC$_1$	*Drosophila*	H
Hepcidin	DTHFPIC$_1$IFC$_2$C$_3$GC$_4$C$_1$HRSKC$_2$GMC$_3$C$_4$KT	Human	Liver
Linear, not □-helical			
Bac 5	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGRPFPa	Cow	BC
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFPa	Pig	BC
Indolicidin	ILPWKWPWWPWRRa	Cow	BC
Apidaecin	GNNRPVYIPQPRPPHPRI	Honeybee	H
Pyrrhocoricin	VDKGSYLPRPTPPRPIYNRN	Insect	H
Histatin 5	DSHAKRHHGYKRKFHEKHHSHRGY	Human	Saliva

Cysteines paired in disulphide linkages are noted by common numerical subscripts. C-terminal amides are noted by *a*. In □-defensin, the first and last residues are joined in a peptide bond.
*BC, blood cell; H, haemolymph; E, epithelial tissue.

the peptide with the membrane, followed by displacement of lipids, alteration of membrane structure, and in certain cases entry of the peptide into the interior of the target cell. The presence of cholesterol in the target membrane in general reduces the activity of antimicrobial peptides, due either to stabilization of the lipid bilayer or to interactions between cholesterol and the peptide[21]. Similarly, it is believed that increasing ionic strength, which in general reduces the activity of most antimicrobial peptides, does so in part by weakening the electrostatic charge interactions required for the initial interaction.

In general, peptides operating by the SMH mechanism kill microbes at micromolar concentrations. In contrast, the peptide nisin, a 14-amino-acid amphipathic molecule produced by *Lactococci*, operates at nanomolar concentrations. Nisin binds with high affinity to Lipid II, the fatty acyl proteoglycan anchor in the bacterial membrane, from which it subsequently diffuses into the surrounding membrane[24]. Certain plant defensins use a similar strategy[25].

How do antimicrobial peptides actually kill microbes? Many hypotheses have been presented, which include: fatal depolarization of the normally energized bacterial membrane[26]; the creation of physical holes that cause cellular contents to leak out[22]; the activation of deadly processes such as induction of hydrolases that degrade the cell wall[27]; the scrambling of the usual distribution of lipids between the leaflets of the bilayer, resulting in disturbance of membrane functions[24]; and the damaging of critical intracellular targets after internalization of the peptide, as suggested by the example of the peptide pyrrhocoricin[28].

Resistance

Unlike conventional antibiotics such as penicillin, which microbes readily circumvent, acquisition of resistance by a sensitive microbial strain against antimicrobial peptides is surprisingly improbable. Resistant species of genera such as *Morganella* and *Serratia* express an outer membrane that lacks the appropriate density of acidic lipids to provide peptide-binding sites. Other resistant species, such as *Porphyromonas gingivalis*, secrete digestive proteases that destroy peptides. Published studies of 'acquired resistance' against antimicrobial peptides, by and large, have identified genes that, when disrupted, make sensitive organisms more susceptible to a particular antimicrobial peptide; indeed, these genes usually appear to have a role in virulence.

A recent report of a survey of thousands of clinical isolates against the synthetic magainin analogue pexiganan illustrates the general picture that has emerged over the past decade regarding the issues of resistance[29]. Bacterial species exhibit a wide range of susceptibilities, with some, such as anaerobes in the case of pexiganan, among the most sensitive. The basis for the different susceptibilities of bacterial and fungal species against particular peptides remains unexplained. Attempts at inducing pexiganan resistance in *Escherichia coli* and *Staphylococcus aureus* by chemical mutagenesis have been unsuccessful. As expected, no evidence of cross-resistance between pexiganan and any antibiotic in clinical use has been documented.

Gram-negative bacteria possess an outer membrane composed of lipopolysaccharide (LPS), which is held together by magnesium and calcium ions that bridge negatively charged phosphosugars. Addition of cationic peptides results in displacement of metal, damaging the outer membrane, and facilitates entry of additional molecules from the exterior (as reviewed in ref. 30). Peptides, having gained access to the periplasmic space, can now integrate into the cytoplasmic membrane. In many species of Gram-negative bacteria, the charge on the outer membrane is modulated by the PhoPQ regulon, a two-component system that uses a sensor (PhoQ) and an intracellular effector (PhoP)[31]. The PhoP/PhoQ regulon affects antimicrobial peptide sensitivity through modulation of the PmrA regulon, which controls a bank of genes that mediate decoration of the outer membrane with the positively charged moieties ethanolamine and 4-aminoarabinose[32].

Why have microbes not been more successful in resisting the

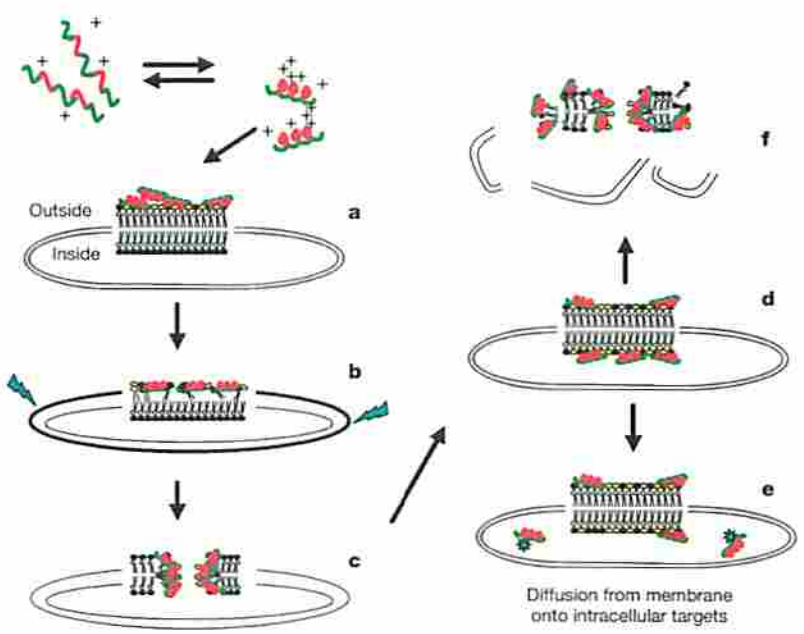

Figure 3 The Shai–Matsuzaki–Huang model of the mechanism of action of an antimicrobial peptide. An α-helical peptide is depicted. **a**, Carpeting of the outer leaflet with peptides. **b**, Integration of the peptide into the membrane and thinning of the outer leaflet. The surface area of the outer leaflet expands relative to the inner leaflet, resulting in strain within the bilayer (jagged arrows). **c**, Phase transition and 'wormhole' formation. Transient pores form at this stage. **d**, Transport of lipids and peptides into the inner leaflet. **e**, Diffusion of peptides onto intracellular targets (in some cases). **f**, Collapse of the membrane into fragments and physical disruption of the target cell's membrane. Lipids with yellow headgroups are acidic, or negatively charged. Lipids with black headgroups have no net charge.