Eukaryotic antibiotic peptides: not only a membrane business

Until recently, eukaryotic antibiotic peptides were considered a curious evolutionary relic, an element of innate immunity that appeared insignificant when compared with the diversity and potency of antigen-specific responses in mammals. However, insights obtained during the past few years are changing this view. Recently noted features of antimicrobial peptides include their omnipresence in plants and animals, their distribution in diverse anatomical locations, and the recognition of diseases produced by their natural or artificial absence or malfunction. The interest is also fuelled by increasing microbial resistance to classical antibiotics and the hope that antimicrobial peptides will emerge as prototypes for new classes of therapeutic antibiotics.

To date, more than 400 sequences of natural antibiotic peptides or their modified synthetic analogues have been reported, and at least three pharmaceutical companies have peptides in clinical trials. The purpose of the workshop held at the Juan March Foundation (8–10 February 1999, Madrid, Spain) was to exchange information in the diverse field of plant and animal antimicrobial peptides.

Evolutionary aspects of antibiotic peptides

Ancestors of antibiotic peptides in eukaryotes have been found among primitive eukaryotes that feature bactericidal activity. Hans Boman (Karolinska Institute, Stockholm, Sweden) presented data on a cecropin-like fragment located at the N-terminal end of the L1 ribosomal protein from *Helicobacter pylori*. A range of bacteria, but not *Helicobacter*, was susceptible to the synthetic peptide at micromolar concentrations. Boman proposed that *Helicobacter* might use this fragment as a weapon against competing bacteria.

Antibiotic peptides provide a good example of structural evolutionary convergence. Secondary structures of drosomycin, as well as insect defensins [Charles Hetru, Centre Nationale de la Recherche Scientifique (CNRS) and University Louis Pasteur, Strasbourg, France] and plant defensins (Bruno Cammue, Catholic University of Leuven, Belgium) share the CSαβ domain, a structural motif that stabilizes the α-helix by intra-chain disulfide bonds to the β-strands. Despite their structural similarity, their pathogen specificity differs: whereas plant defensins and drosomycin are fungicidal, insect defensins are not and are most active against Gram-positive bacteria. This difference is thought to be related to the presence of an additional sequence at the N-terminus of plant defensin and drosomycin, because the extension of a lepidopteran defensin N-terminus becomes fungicidal. Renato Gennaro (University of Trieste, Italy) discussed the cathelicidin family of antibacterial peptides whose N-terminal proregion is conserved despite the high divergence in sequence and activities among the different C-terminal mature peptides. The function of this proregion, that would account for the selective conservation of the ~100 amino acid cathelin domain, remains a mystery.

Two related peptide families in mammals, the α- and β-defensins, differ in their internal disulfide pattern as well as their tissue location. Tomas Ganz (University of California, Los Angeles, CA, USA) proposed an evolutionary tree based on the position of the individual genes inside the defensin cluster on the human chromosome 8p23, on their distribution among mammals, snakes and birds, and on the sequence homologies previously reported by Charles Bevins (Cleveland Clinic Foundation, OH, USA). In this scheme, β-defensins are the oldest member, from which intestinal Paneth cell α-defensins evolved. In turn, phagocyte α-defensins evolved from Paneth cell defensins.

The production of many antimicrobial peptides in plants, amphibia, insects and mammals is inducible by microbial infection and other injurious stimuli. The similar regulatory pathways, involving members of the Rel/NF-κB (Rel homology domain/nuclear factor κ-binding) family, were described in plants by Antonio Molina (Polytechnic University of Madrid, Spain), in amphibia by Donatella Barra (University of Rome, Italy) and in insects by Jules Hoffmann (CNRS and University Louis Pasteur, Strasbourg, France).

Role of antibiotic peptides in health and disease

Two proposed functions for antibiotic peptides include the control of local flora and first-line defence against invasive microbes. Boman and Barra pointed out that our understanding of the regulation of resident microbial flora is very limited. They presented compelling evidence for the role of antibiotic peptides in the control of equilibrium among the different organisms found on the skin of frogs. In this system, the inhibition of peptide expression by topical treatment with glucocorticoids triggers fulminant bacterial overgrowth on the frog skin.

The second role of antimicrobial peptides, as a defence barrier, has been clearly demonstrated in plants, in which resistance to infection can be induced by genetic transfer of antimicrobial peptides from one species to another.

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For example, Cammue found that *Arabidopsis* mutants with defective pathways for the induction of plant defensins are more susceptible to fungal infection. Similarly, *Drosophila*, with mutations in the *Toll* signalling pathway that regulates the production of antifungal drosomycin, is also susceptible to fungal infection. Hoffmann described the complexity of the antibiotic peptide response triggered by infection in *Drosophila*, where only nine of the 40 peptides detected have been characterized. Depending on the invading pathogen, *Drosophila* switch on a particular set of peptides, governed by distinct signal-transduction pathways. Thus, the antifungal response is under the control of the *spätzle/Toll/cactus* cassette, whereas antibacterial defence is controlled by the immune deficiency (IMD) pathway, alone, or together with *Toll*. Fotis Kafatos [European Molecular Biology Laboratory (EMBL), Heidelberg, Germany] discussed the immune response of the *Anopheles* mosquito to *Plasmodium* infection. Both local and systemic induction of peptides has been observed, hence contradicting the previous notion that *Plasmodium* can enter the host without eliciting a reaction. These observations raise hopes that the peptide induction response can be used to curb the spread of malaria among mosquitos and their human targets.

Because of the multiplicity of antimicrobial peptide genes, overlapping host defence mechanisms, and species differences in antimicrobial peptide expression, the assignment of a specific function for a particular peptide is a difficult task in higher animals. Tomas Ganz, Michael Selsted (University of California, Irvine, CA, USA) and Charles Bevins presented data in support of the defensive function of antibiotic peptides. The local peptide concentration, either in specific mucosal locations or inside phagocytes, is sufficient to kill susceptible organisms.

As in *Drosophila* or plants, some mammalian antibiotic peptides are induced by pathogen components, such as lipopolysaccharide (LPS), or inflammatory cytokines, such as tumour necrosis factor-α or interleukin-1. In cystic fibrosis (CF), a human disorder of epithelial host defence, chronic bacterial colonization of the airways is caused by genetic defects in a chloride channel (the CF transmembrane regulator). According to a new model of the pathogenesis of this disease (Jeffrey Smith, University of Iowa, IA, USA), the defect leads to increased salinity of secretions, and inactivation of the local antibacterial peptides. *In vitro* and in human CF epithelial xenotransplants, the antimicrobial activity of the epithelia can be restored either by dilution of the salt or by gene therapy with a functional channel. Gennaro discussed some studies examining the toxicity of bactenecin analogues in an animal model. The therapeutic index for some of these analogues is higher than ten, suggesting potential for their clinical development.

The generation of transgenic mice for antibiotic peptides has allowed the characterization of elements that regulate their tissue-specific expression. Bevins described a mouse expressing human defensin-5 (HD-5), a Paneth cell defensin. Introduction of the HD-5 gene with 1.5 kb of 5′ flanking region produced a mouse that nearly exclusively matched the human pattern of HD-5 expression in Paneth cells of the small intestine.

Mechanism of action

Most of the known antimicrobial peptides are strongly cationic and adopt amphipatic structures in solution and/or upon contact with the phospholipids of the target membrane. The interactions of the peptides with their membrane targets do not generally involve a chiral receptor. Regardless of the specific mechanism, disruption of membrane integrity dissipates ionic gradients and is followed by the loss of internal homeostasis. Yechiel Shai (Weizmann Institute of Science, Rehovot, Israel) introduced two models for peptide–membrane interaction, the carpet model and the barrel-stave model. In the carpet mechanism, the peptide is initially inserted with its α-helical axis parallel to the membrane. However, as the number of inserted molecules increases, they may distort the membrane and destabilize its bilayer structure. The concept of self-promoted uptake and the formation of toroidal deformations in membranes were seen as extensions of the carpet model. In contrast, in the barrel-stave model, the peptide molecules form a hydrophilic pore that spans the membrane. The predominant mechanism for a specific peptide depends largely on the strength of monomer–monomer or monomer–membrane interactions, respectively. Selsted explained the role of dimerization of human defensins in the stabilization of the pore. Increasing the numbers of these pores leads to a gradual release of markers encapsulated in the liposomes. In contrast, plant-derived thionins (Félix Goñi, University of Basque Country, Bilbao, Spain) destabilize the liposomal membrane, inducing an all-or-nothing release of their contents. Katsumi Matsuzaki (University of Kyoto, Japan) studied the factors involved in pore stabilization. Magainin and PGLa, a 21 amino acid peptide starting with glycine and ending with an amidated leucine residue, isolated from *Xenopus laevis*, formed long-lasting pores that were inactivated by translocation across the lipid bilayers, whereas buforin, an amphibian peptide whose mechanism of action is based on its interaction with DNA, had a low membrane affinity and produced short-lived pores.

Peptide specificity and pathogen resistance

For membrane-active peptides, specificity is based on a differing lipid composition of prokaryotes and eukaryotes, with a higher percentage of acidic

phospholipids and an absence of sterols in bacteria. Cardiolipin is important for the activity of defensins and sapecin, whilst sterols also participate in the modulation of peptide activity.

The mechanism of action of the interaction between phospholipids and antibiotic peptides complicates rational drug design. It is not sufficient to develop peptides with an increased affinity for membranes, because higher affinity may lead to a loss of preference for microbial as opposed to host membranes. On the other hand, the emergence of resistance to peptides in target microbes is intrinsically a more complex process than resistance against classical antibiotics, which can often be because of a small number of mutations (Rennato Gennaro).

There is a clear correlation between virulence and peptide resistance in plants. Gram-negative bacteria may become more resistant by alteration of the LPS structure (*Ralstonia solanacearum*) or by mutation-activation of the *sapA-F* loci involved in peptide extrusion from the bacterium *Erwinia chrysanthemi* (Pablo Rodríguez-Palenzuela, Polytechnic University of Madrid, Spain). In eukaryotic cells such as *Leishmania* promastigotes [Luis Rivas, Consejo Superior de Investigaciones Cientifícas (CSIC), Madrid, Spain] or erythrocytes (Enrique Pérez-Payá, University of Valencia, Spain), membrane polyanionic components act as decoys for antimicrobial peptides. Promastigotes deficient in the biosynthesis of lipophosphoglycan, a membrane anionic oligosaccharide, have an increased susceptibility to cecropin A-melittin hybrids. Similarly, haemolysis caused by melittin analogues can be increased by previous erythrocyte desialylation.

Alternative and secondary mechanisms of action

Antimicrobial peptides that presumably act through alternative mechanisms include drosomycin, an insect antifungal peptide that does not permeabilize the membrane (Charles Hetru). Plant defensins (Bruno Cammue) are also unable to permeabilize model membranes, and their antifungal activity is based on Ca^{2+} influx into the target cell, triggered by direct or indirect interaction with a membrane Ca^{2+} channel. A higher *in vivo* activity, when compared with *in vitro* assays, suggests the involvement of mechanisms that are in addition to direct permeabilization of the pathogen. A sapecin analogue activates neutrophils through interaction with calreticulin (Shunji Natori, University of Tokyo, Japan), whilst in macrophages, intracellular *Leishmania* amastigotes are killed by nitric oxide production induced in macrophages by cecropin A-melittin hybrid peptides (Luis Rivas). Another insect peptide, 5-S-glutathionyl β-alanyl-L-dopa (5-S-GALD) is involved in H_2O_2 production and inhibition of tyrosine kinase (Shunji Natori). Monisha Scott (University of British Columbia, Vancouver, Canada) introduced the cecropin-melittin analogues as anti-endotoxin agents that prevent LPS-dependent macrophage activation. Such LPS-binding antibiotics may avoid the side effects of LPS release that takes place with conventional antibiotics.

Improvement of peptide activity and susceptibility

In most cases, structural determinants of optimal peptide activity towards a specific pathogen are defined by trial and error. The same structural features may not be optimal towards other pathogens or when introduced into other peptides. David Andreu (University of Barcelona, Spain) reviewed the optimization of cecropin A-melittin hybrid peptides against fungal pathogens and for antitumour activities and the presence of the VLKVL motif from melittin was found to be required for activity. However, Monisha Scott, working with other cecropin A-melittin analogues, did not find a good correlation between their antimicrobial or anti-LPS activity and peptide features such as charge, hydrophobicity or length. Selsted stressed the fine specificity observed in α-defensins, in which variation of a single amino acid can lead to dramatic changes in activity and specificity. The introduction of D-amino acids can alter peptide specificity. As discussed by Shai, introduction of D-amino acids into key positions of linear α-helical peptides decreased their α-helix content as well as their cytotoxicity, whilst antibacterial activity was preserved. Barra mentioned the natural presence of a residue of D-alloisoleucine in position 2 in H3–H5 members of bombinin-related peptides. The presence of this amino acid changes peptide specificity when compared with the analogue devoid of D-amino acid and interestingly, both analogues coexist in the frog. Hetru discussed the loss of activity of synthetic all-D-thanatin against Gram-negative bacteria, while activity against Gram-positive bacteria and fungi was maintained.

Conclusions

This workshop reviewed the significant recent advances in eukaryotic antibiotic peptide biology. Advances in peptide discovery, design and cheaper production, as well as key molecules in pathological disorders, generated both optimism about future pharmaceutical applications and new means for biological control of agriculturally important plant infections and insect-borne diseases.

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