CMLS, Cell. Mol. Life Sci. 59 (2002) 1138–1150
1420-682X/02/071138-13 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

The short proline-rich antibacterial peptide family

L. Otvos, Jr

The Wistar Institute, 3601 Spruce Street, Philadelphia, Pennsylvania 19104 (USA), Fax +1 215 898 5821, e-mail: Otvos@wistar.upenn.edu

Received 12 December 2001; received after revision 11 February 2002; accepted 19 February 2002

Abstract. From the many peptide families that are induced upon bacterial infection and can be isolated from all classes of animals, the short, proline-rich antibacterial peptides enjoy particular interest. These molecules were shown to inactivate an intracellular biopolymer in bacteria without destroying or remaining attached to the bacterial cell membrane, and as such emerged as viable candidates for the treatment of mammalian infections. These peptides were originally isolated from insects, they kill mostly Gram-negative bacteria with high efficiency and they show structural similarities with longer insect- and mammal-derived antimicrobial peptides. However, while the distant relatives appear to carry multiple functional domains, apidaecin, drosocin, formaecin and pyrrhocoricin consist of only minimal determinants needed to penetrate across the cell membrane and bind to the target biopolymer. These peptides appear to inhibit metabolic processes, such as protein synthesis or chaperone-assisted protein folding. Pyrrhocoricin derivatives protect mice from experimental infections in vivo, suggesting the utility of modified analogs in the clinical setting. Sequence variations of the target protein at the peptide-binding site may allow the development of new peptide variants that kill currently unresponsive strains or species.

Key words. Conformation; antimicrobial peptide; delivery module; insect antibiotic; intracellular target; pharmacophore; protein folding; resistance.

Introduction

Antibacterial peptides come in all colors and sizes. These gene-encoded compounds are the first line of defense against microbial infections in higher organisms, such as mammals [1], as well as being significant effector molecules in lower organisms such as insects [2]. As non-peptidic antimicrobial drugs often exhibit discrete activity spectra, many antibacterial peptide families exist to cover almost all potential infection sources [3, 4]. For example, a single insect such as the fruit fly *Drosophila melanogaster* produces at least seven distinct groups of peptide antibiotics [5], each peptide assuming a completely different activity spectrum [6]. The best-known antibacterial peptide families are the multiple Cys-bridge-containing defensins exhibiting anti-Gram-positive activity [7, 8], linear α-helical peptides including the cecropins [9, 10] and the magainins [11, 12] with both Gram-positive and Gram-negative activity, the proline-rich peptides that almost exclusively kill Gram-negative bacterial strains [13] and the glycine-rich peptides that are similarly more active against Gram-negatives, but sometimes also kill Gram-positives [14, 15]. These cannot be regarded as strict categories: the diptericins feature a proline-rich amino terminus and glycine-rich C-terminal domains [16–18]. There are some additional antibacterial peptides without clear family connections, sometimes resembling others, sometimes not. For example, while thanatin from the spined stink bug, *Podisus maculiventris,* is important because it contains a single disulfide bridge [19] and displays sequence similarity to the brevinins, antimicrobial peptides isolated from frog

skin secretions [20], moricin, from *Bombyx mori,* apparently does not resemble any other antibacterial peptide [21].

In general, all antimicrobial peptides interact with the bacterial cell membrane. A direct correlation between antibiotic effect and membrane disruption has been found for defensins from mammals and insects, magainins and cecropins [22–24]. This is understandable based on the structure of the antibacterial peptides. They are composed of hydrophilic and strongly positively charged domains, which are proposed to initiate peptide interaction with the negatively charged bacterial surface and the negatively charged headgroups of bilayer phospholipids. The hydrophobic property would permit the peptides to enter the membrane interior. The interaction with the membrane involves either a 'carpet' mechanism by which the peptides cooperatively destroy the membrane barrier [25], or these biooligomers bind to the outer leaflet of model membranes and flip inward, carrying lipids along the way and creating brief disruptions in permeability [26]. However, the ability of various antimicrobial peptides to depolarize the cytoplasmic membrane potential of *Escherichia coli* varies widely, with certain peptides being unable to cause depolarization at the minimal inhibitory concentration (MIC), while others cause maximal depolarization below the MIC [27]. For all of the peptides, representing most of the structural classes, there is clearly no correlation between the concentrations leading to complete membrane permeabilization and the MIC.

When prolines are inserted into the sequences of α-helical antimicrobial peptides, the ability of the peptides to permeabilize the cytoplasmic membrane of *E. coli* decreases substantially as a function of the number of proline residues incorporated [28]. In this regard, that many very active native antibacterial peptides, at least against selected Gram-negative pathogens, belong to the proline-rich peptide family is intriguing [29]. Unlike strongly membrane disrupting peptides, such as the magainins or cecropins that kill bacteria in any stereochemical configuration [30, 31], the short, proline-rich peptides are inactive when made of D-amino acid residues, suggesting the presence of an intracellular target biopolymer with chiral properties, most likely protein or nucleic acid [32, 33]. This does not completely exclude some type of interaction with the bacterial membrane, and supports rather a model in which the penetration across the membrane layer is followed by the binding to and inhibition of a functional intracellular end molecule [34]. These unique properties explain the ever-growing interest in the short, proline-rich antibacterial peptides, and warrant continued research for many years to come.

Currently known members of the short, proline-rich peptide family

Most of the proline-rich peptides have been isolated from insects. They are remarkably similar in amino acid composition and sequence motif pattern. The names of the peptides reflect their origin rather than subdivision among the individual sequences. I argued earlier that the sequence diversity of the peptides arose from differences in the sequences of the target protein in the pathogens and the genuine combinatorial chemistry of the insects as they developed during evolution [35]. Insects have evolved an immune system that can distinguish different classes of pathogens [36]. The example of lebocins, longer proline-rich antibacterial peptides from various sources, indicate that different insect species have evolved specific antibacterial peptides adapted to the environment where they reside and the pathogens that threaten their existence [37]. The mature form of lebocin from the cabbage loofer *Trichoplusia ni* shares only 44% sequence homology with lebocin isolated from the silkworm *B. mori*. Even the same insect species produce different antimicrobial peptides depending upon the immunizing microorganism [38].

Drosocin [39] is the most frequently studied variant of these insect-derived peptides that have been isolated from Hymenoptera, Lepidoptera, Hemiptera and Diptera [35] (table 1). The main sequence characteristics are the repeated Pro-Arg-Pro tripeptide fragments that are symmetrically distributed along the 19-residue stretch of the mature gene product, and the Thr-linked carbohydrate located in mid-chain position. Although a series of different mono- and disaccharide glycoforms of drosocin can be detected in the hemolymph of *Drosophila*, the native peptide has never been observed to exist without the addition of a carbohydrate side chain [40]. The biological role of the sugar remains an enigma. Although addition of the most likely native sugar combination Gal-GalNAc in the typical $\alpha(1\rightarrow O)$ anomeric configuration increases the in vitro antibacterial activity of drosocin [33], other synthetic drosocin variants, in which the classical O-linked

Table 1. Amino acid sequences of selected short, proline-rich antibacterial peptides.

Drosocin	G	-	-	K	P	R	P	Y	S		P	R	P	T*	S	H	P	R		P	I	R	V
Formaecin 1	G	-	-	R	P	N	P	V	N		N	K	P	T*	P	H	P	R		-	L		
Pyrrhocoricin	V	-	D	K	G	S	-	Y	L		P	R	P	T*	-	P	P	R		P	I	Y	N	R	N
Apidaecin 1a	G	N	N	R	P	-	V	Y	I		P	Q	P	-		R	P	P	H	P	-	R	I		

* Site of glycosylation. Some other related peptide sequences discussed in the text in detail are:
PR-39: RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP,
Bac-5:
RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPFP
Penaeidin-2:
SLDKRYRGGYTGPIPRPPPIGRPPFRPVCNACYRLSVSDAR-NCCIKFGSCCHLVKG,
Buforin II: TRSSRAGLQFPVGRVHRLLRK.

sugars are replaced with oxime-linked carbohydrates, work equally well [41, 42]. Another typical member of the family, the 20-mer pyrrhocoricin, was isolated from the European sap-sucking bug *Pyrrhocoris apterus* [43]. In pyrrhocoricin, the PRP tripeptide is repeated twice and Thr11 is also O-glycosylated. However, synthetic O-glycosylated pyrrhocoricin is less active than the synthetic unglycosylated counterpart [44]. Although a couple of arginines are still present in the peptides, in formaecin from the bulldog ant *Myrmecia gulosa*, the repeated Pro-Arg-Pro fragments are replaced with Pro-Asn-Pro and Pro-His-Pro sequences. Synthetic formaecin (16 residues) without carbohydrate is considerably less active than the parent, O-glycosylated analog carrying sugar on Thr11 [45]. Remarkably, all three native peptides show high selectivity toward Gram-negative organisms, mostly from the Enterobacteriaceae family (table 2). The formaecins are reported to kill only *E. coli* strains at low micromolar concentrations [45]. This limited activity likely reflects the small number of bacterial strains studied rather than a very narrow activity spectrum. Based on published data, both native drosocin and pyrrhocoricin kill *E. coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Agrobacterium tumefaciens* in the mid nanomolar to low micromolar concentration range [33, 44]. Additional Gram-negative drosocin-susceptible strains include *Enterobacter cloacae* and *Erwinia carotovora carotovora*. Pyrrhocoricin is highly potent against *Haemophilus influenzae* in the liquid growth inhibition assay [M. Cudic et al., unpublished data]. From the Gram-positive strains, both peptides kill *Micrococcus luteus*, and pyrrhocoricin shows weak activity against *Bacillus megaterium*. The short, proline-rich peptides in their native form do not appear to kill the major Gram-positive threats to humans, such as *Staphylococcus aureus*, *Streptococcus pneumoniae* or *Streptococcus pyogenes* or fungi. Nevertheless, an exciting new study found that drosocin is expressed by *Drosophila* upon experimental infection with parasites [46].

A series of small proline-rich peptides were originally isolated from bees, and they are collectively called apidaecins [47]. The insects include the honey bee *Apis mellifera*, the bumblebee *Bombus terrestris*, the cicada killer *Sphecius speciosus*, the bald-faced hornet *Dolichovespula maculata*, the yellowjacket *Vespula maculitrons* and the wasp *Coccygomimus disparis* [48]. These are 18- to 20-residue peptides with a highly conserved motif PRP-PHPRI/L in the carboxy terminus. While many of the N-terminal substitutions are conservative in nature (Arg → Lys), intriguingly, Gln is either replaced for hydrophobic residues such as Ile or Val or for a hydrophilic one, like Lys. Actually, the authors suggested that the constant domains are responsible for the general antibacterial activity, and the variable domains are responsible for the bacteria-specific activity spectrum. Indeed, huge activity differences can be found among the apidaecin peptides, with those from the honeybee strongly killing *Yersinia enterocolitica* but not affecting *Campylobacter jejuni*, and the analogs isolated from the wasps acting in exactly the opposite way [48]. If these modifications are made in nature to combat insect-specific invaders, the apidaecins are prime examples of the combinatorial chemical capability of insects [35] and strongly suggest the existence of an intracellular target protein containing sequence variations among the assaulting bacterial strains. Worth mentioning here is that recombinant apidaecin has been successfully expressed in *E. coli* [49]. This indicates that a phage library can be used to produce large numbers of analogs of the antibacterial peptides, and the sequences can be screened and optimized against each individual bacterial strain, including those which are currently non-responsive to natural versions of the proline-rich peptide family, but possess similar functional target macromolecules. This library screening would also identify the analogs

Table 2. In vitro antibacterial activity of the insect-derived proline rich peptides.

Bacterial strain	Drosocin	Pyrrhocoricin	Apidaecin	Formaecin	Lebocin	Abaecin	Diptericin
Gram negative							
Escherichia coli	+++	+++	+++	++	++	++	+++
Pseudomonas aeruginosa	–	+/–	++				–
Salmonella typhimurium	+++	++	+++				+++
Klebsiella pneumoniae	++	++	++				+
Enterobacter cloacae	++	+					+
Agrobacterium tumefaciens	+++	+++	+++			+	–
Gram positive							
Micrococcus luteus	+++	+					–
Bacillus megaterium	–	++	+			++	
Staphylococcus aureus	–	–			+		
Streptococcus pyogenes	–	–					

The various publications used different assay types and conditions, therefore numerical values cannot be compared. For qualitative comparison, +++ indicates strong (nanomolar) activities, ++ medium (low micromolar) and + weak (hardly detectable) activities. The – sign indicates that the peptide was inactive under the used assay conditions.

most active against the currently responsive bacteria, including *E. coli*. However, when an attempt was made to produce thanatin in a similar fashion, the *E. coli* hosts were killed [50]. Even slight leakiness of the expression of the thanatin gene product, exhibiting a much stronger bactericidal activity than apidaecin, was suggested to lead to killing of the host cells. In turn, this means that the most active peptide variants, the ones that are the most desirable to identify, will never be expressed, rendering such a library inferior to de novo design of novel molecules, once the target protein and the contact residues are discovered.

It was argued that the 32- to 39-residue-long abaecins and lebocins do not belong to the group defined by drosocin, pyrrhocoricin, the formaecins and apidaecins, partly because of the increased size and partly because the longer peptides can apparently affect membrane permeability [51]. While these contentions are certainly true, the remarkable sequence similarities between the two groups justify briefly mentioning these longer peptides here. Abaecins from the honeybee *A. mellifera* [52] or the bublebee *Bombus pascorum* [53] are made up from Pro residues, interspersed with Arg, Val, Leu, Phe, Asn or Gln, amino acids characteristic for the smaller peptide family. However, abaecin is less active than apidaecin in vitro, and the composition of the assay medium greatly affects antibacterial potency [52]. This may not reflect negligible in vivo efficacy. In our hands, the in vivo activity of pyrrhocoricin against *E. coli* ATCC 25922 was not mirrored by extraordinary in vitro efficacy, at least not in the validated assay conditions preferred by pharmaceutical companies [35]. Nevertheless, the lack of Gram-negative selectivity of abaecin indeed signals a mode of action different from that of the apidaecins, the closest sequence relatives [52]. The sequences of the lebocins contain relatively less proline residues than any previously discussed peptides, but still feature the O-glycosylated PTPP tetrapeptide fragment, present in pyrrhocoricin [51]. Interestingly, a monosaccharide-containing derivative exhibits increased antibacterial potency, but addition of a second carbohydrate moiety (resulting in a pyrrhocoricin-like Gal-GalNAc structure) reduces the in vitro antibacterial activity below that of the unglycosylated variant [51]. Curiously, when the pyrrhocoricin variants were assayed for in vitro biological activity, only zero and two sugar derivatives were tested, never the monosaccharide-containing analog. Pushing the size of the peptides to the extreme, diptericin, an 82-mer peptide (small protein) contains an N-terminal quarter almost identical to the apidaecin-drosocin-pyrrhocoricin trio, and a C-terminal region resembling the glycine-rich protein attacin [17]. Diptericin from *Phormia terranovae* is also glycosylated on Thr10 and Thr54, but the sugars are not needed at all for the antimicrobial activity [54, 55]. Although diptericin was shown to disrupt bacterial membrane integrity, the authors concluded that the molecule is unlikely to function primarily as a pore-former [55].

Non-insect relatives

If we follow the thread of the arguments above, there are no known non-insect relatives of the short, proline-rich antibacterial peptides. All mammalian equivalents are significantly longer, and fall outside the arbitrary <21 residue size limit. However, this review cannot be complete without discussing some of these longer mammalian analogs, especially as peptide PR-39 (for proline-arginine-rich with a size of 39 residues) is frequently identified as the mammalian relative of apidaecin [56]. This categorization is based on the very similar amino acid sequence patterns and the proposed similar mechanism of action. Peptide PR-39 was first isolated from pig intestine and showed antibacterial activity against *E. coli* and *B. megaterium* [57]. Later, similar porcine analogs were isolated from neurophils [58] and spleens [59]. Peptide PR-39 belongs to the proline-rich branch of the cathelicidin family of antibacterial peptides, one of the two major antibacterial families in mammals (the other is the defensin family) [60]. This branch also includes Bac-5 and Bac-7, even longer peptides from bovine neutrophils, but with very similar amino acid composition [61]. The bactenecins are 75% homologous with peptide PR-39 [62] and their sequences feature an Arg-rich N-terminal region and successive repeats of the Arg-Pro-Pro-Ile tetrapeptide fragment [63]. Similar genes encoding cathelicidin-type antimicrobial peptides have been identified in sheep [64]. Clearly, to kill bacteria, the length of these peptides does not need to be as long as they appear in nature: for the antimicrobial activity of the bactenecins, an arginine at or near the N terminus and a chain length of at least 15 residues is satisfactory [63]. The minimally active domain of Bac-5 was mapped to the 7–23 fragment [63]. Likewise, the N-terminal 1–26 fragment of peptide PR-39 is a better antibiotic than the full-sized parent analog [65] and even a 15-residue PR-39 fragment exhibits antibacterial activity [66]. A precursor of the prophenins, proline-arginine rich antibacterial peptides (proteins), was isolated from porcine bone marrow [67]. Although the full prophenin-1 peptide is as long as 79 amino acid residues, its N-terminal 60 residues consist of three perfect and three nearly perfect repeats of a decamer, FPPPNFPGPR [68].

The proline-arginine-rich cathelicidins (and prophenins as well) in their native forms exhibit an activity spectrum remarkably similar to the short, insect-derived proline-rich peptides, killing *E. coli*, *Salmonella enteritidis*, and *K. pneumoniae* strains at high nanomolar concentrations [60]. From the Gram-positive bacteria, the sensitive strains include *B. megaterium*, *Bacillus subtilis* and *Lis-*

teria monocytogenes [57, 61, 65]. This would indicate an intimate relationship with drosocin, pyrrhocoricin and the apidaecins. However, the D-form of peptide PR-39 retains activity against *E. coli,* even improves the activity against *Pseudomonas aeruginosa* and actually becomes active against *S. aureus*, a pathogen that does not respond to the native form [31]. The penaeidins are antibacterial peptides longer than 50 residues. They were isolated from the penaeid shrimp *Penaeus vannamei* [69] and their amino acid composition features two clear distinct domains. Their N-terminal sequence starts with a proline-rich domain, but as in diptericin, this region alone lacks antimicrobial activity [70]. Clearly, the biological activity is carried by the C-terminal defensin-like domain. Indeed, chitin binding is observed only for the C-terminal region [71]. Interestingly, a 'big defensin' molecule consisting of 79 amino acid residues also exists, and is made up from independently functioning domains [72]. Data on antimicrobial activities of recombinant panaeidins demonstrate that these peptides (small proteins) have broad-spectrum antifungal properties associated with a fungicidal activity [73]. Just like the widespread smaller defensins, the shrimp penaeids kill mostly Gram-positive strains. This raises the question: what is the biological role of the proline-rich amino-terminal region?

If we want to maintain the connection between peptide PR-39 and the short, proline-rich insect peptide family, we have to explain the aberrant activity spectrum of PR-39 as the result of the rest of the peptide that is added in excess to the apidaecin-analog sequence. In support of this, peptide PR-39 binds to negatively charged membranes but appears not to form discrete pores in artificial membranes or to lyse *E. coli* cells [74, 75]. An initial defensin-like membrane uptake is postulated [76], an effect which, due to the increased length of the peptide, may be stronger than that elicited by the shorter insect-derived variants. In addition to antimicrobial properties, peptide PR-39 was shown to participate in many in vitro and in vivo regulatory processes [77, 78]. In support of the idea that the apidaecin analog N terminus is the protein-binding domain and the defensin-like C terminus is the membrane-active part, while modification of the N-terminal region interferes with the intrinsic SH3-binding ability, C-terminal amino acid changes have a greater influence on the antibacterial activity [79]. Apparently, the non-bactericidal activities of PR-39 were investigated in significantly more detail than similar properties of the short, proline-rich antibacterial peptides. Whether any or all of these diverse biological activities are due to the sequence motifs shared with drosocin, pyrrhocoricin or apidaecin remains to be seen, and will be studied if derivatives of these peptides reach the clinical trial stage, requiring the performance of considerably more and more diverse assays on them.

Toxicity and in vivo activities

Despite the realization that apidaecin-based peptides could be used clinically to treat infections [48], there is very little information available on the in vivo efficacy of the proline-rich antibacterial peptides. Nevertheless, since these molecules are not expected to interfere with eukaryotic cell assembly, they are attractive drug leads. Generally speaking, the interaction of most peptides with eukaryotic membranes is inhibited by the lack of negatively charged lipids on the surface of such cells, by the rather low membrane potential across the plasma membrane and by the presence of cholesterol, in contrast to bacterial membranes which are abundant in anionic surface phospholipids, have high transmembrane potential and lack cholesterol [80]. However, peptides with strong membrane-disintegrating activity may overcome these features. For example, cecropin is deadly to mice at a dose of 100 mg/kg [81]. Even less clearly membrane active peptides, like PR-39, pose a danger; this peptide rapidly enters human microvascular endothelial cells [77]. These peptides become toxic if they stay attached to the membrane and destroy the eukaryotes. However, if the peptides are detached from the membrane after penetrating into eukaryotic cells, and inhibit a bacteria-specific intracellular target, their value is very high because in this case they can selectively kill obligate and facultative intracellular pathogens that live inside mammalian cells. The potential of short, proline-rich peptides as therapeutics is indicated by the fact that drosocin is completely non-toxic to healthy animals when added intravenously in two 100 mg/kg doses [44].

However, drosocin fails to protect mice from experimental systemic *E. coli* infection in the 25–100 mg/kg dose range, and we explained this as due to the low stability of the native glycopeptide in animal blood [44]. The peptide is significantly more stable in insect hemolymph, at least under laboratory conditions [44]. Therefore, for pharmaceutical development, we searched for other family members that are more stable in mammalian body fluids. Pyrrhocoricin appears to be degraded more slowly than drosocin in human and mouse sera, and although it still shows a fast decomposition rate, characteristic for peptides, some peptide apparently remains intact after a few hours, a time period needed to kill bacteria by this family of antimicrobial agents [29, 54]. Pyrrhocoricin does not lyse sheep erythrocytes up to the studied 256 µM concentration, and is non-toxic to COS cells of mammalian origin up to the studied 50 µM concentration. The in vivo toxicity and activity of pyrrhocoricin and Chex-pyrrhocoricin-Dap(Ac) (an analog in which Val1 is replaced with 1-amino-cyclohexane-carboxylic acid and Asn20 is replaced with β-acetyl-diamino-propionic acid) were studied in mice. The N- and C-terminal residues in the latter analog were replaced with non-natural amino acids to prevent exopeptidase cleavage. The peptides showed no toxicity up to the maximum ap-

plied dose of 50 mg/kg. The native peptide protected mice against *E. coli* infection at intravenous doses of 10 or 25 mg/kg applied 1 h after infection (with a boost 5 h after infection), but at a dose of 50 mg/kg was toxic to compromised animals. Similarly, drosocin also showed some toxicity to infected animals. This toxicity could be rationalized as due to the inhibition of a mammalian chaperonine which is increasingly produced upon infection [82]. The Chex-pyrrhocoricin-Dap(Ac) derivative protected all mice in a 10–50 mg/kg dose range [29] without any toxic effects, in line with the blockage of the mammalian chaperonine-binding site [G. Kragol et al., unpublished data]. Most recently, we studied the efficacy of a backbone-protected pyrrhocoricin dimer during a local (lung) *H. influenzae* infection [M. Cudic et al., unpublished data). The designed dimer added as a single 20 mg/kg dose intranasally significantly reduced the bacterial count in the bronchoalveolar lavage of all four mice compared to all five untreated animals.

More detailed literature data can be found on the in vivo and ex vivo activities of the distant mammalian analog PR-39. Based on the suppression of the NF-κB-dependent expression of adhesion molecules [83], the activity of PR-39 was studied in a rat heart ischemia-reperfusion model. In this assay, the peptide effectively inhibits myocardial ischemia-reperfusion injury, an effect mediated by inhibition of NF-κB-dependent adhesion molecules [84]. Because the in vivo active domain is located at the N-terminal 1–11 fragment, we can speculate that this activity may be repeated by the short, proline-rich insect antibacterial peptides. In a related study, PR-39 added as a bolus intravenous injection abolished ischemia-reperfusion-induced leukocyte adhesion and emigration [85].

Mechanism of action

The activity of apidaecin is reduced when tested in nutrient-free buffer, providing early clues for the mode of action [47]. Formaecin was then clearly documented to kill only growing cells, suggesting that the short, proline-rich antibacterial peptides interrupt metabolic processes [45]. The same applies for diptericin, and for the target metabolic processes, the syntheses of proteins, cell wall or nucleic acids were proposed [55]. As in most research subjects in the field, apidaecin led the way to the identification of the precise mode of action. As early as 1994, evidence was presented indicating the lack of membrane permeabilization, at concentrations exceeding lethal doses by four orders of magnitude, and undiminished sensitivity of apidaecin-resistant mutants to pore-forming peptides [32]. Since the D-enantiomer is completely devoid of antibacterial activity, the authors proposed that apidaecin stereoselectively recognizes a chiral cellular target. Recently, they went further and in an impressive set of experiments postulated a five-step mechanism by which apidaecin exerts its antimicrobial activity [56]. According to this study, the peptide first binds to an outer membrane component of *E. coli*, followed by invasion of the periplasmic space, and by a specific and essentially irreversible engagement with a receptor/docking molecule that may be inner membrane bound or otherwise associated, most likely a component of a permease-type transporter system. In the final step, the peptide is translocated into the interior of the cell where it meets its ultimate target, perhaps one or more components of the protein synthesis machinery [56]. A somewhat similar mode of action was suggested for peptide PR-39. PR-39 requires a lag period of about 8 min to penetrate the outer membrane of wild-type *E. coli*; subsequent killing is quite fast [86]. Isotope incorporation experiments indicate that PR-39 kills bacteria by a mechanism that stops protein and DNA synthesis and results in degradation of these components [86]. This idea was later challenged, citing the increased size of this peptide and noting that PR-39 remains attached to the membrane almost quantitatively [65]. However, this latter finding may just reflect the existence of two functional domains of PR-39, leaving open the possibility that the N-terminal active domain does have an intracellular target biopolymer. Because PR-39 induces the expression of a transmembrane proteoglycan at the concentration at which it lyses bacteria, this peptide and other members of the proline-rich peptide family were suggested to function as signaling molecules in complex cellular behaviors [87].

When identifying the biopolymers involved in the process of killing bacteria, we observed that pyrrhocoricin, drosocin and apidaecin bind the bacterial lipopolysaccharide (LPS) and the 70-kDa heat shock protein DnaK in a specific manner, and the 60-kDa bacterial chaperonin GroEL non-specifically [82]. The inactive D-stereoisomer does not bind to DnaK. Table 3 compares

Table 3. Hypothetical pathways of bacterial cell entry and intracellular interactions of the short, proline-rich antibacterial peptides.

Event	Player/incident suggested by ref. 56	Alternatively by refs 82, 88
1) Entry to cells	binding to an outer membrane component	interaction with negatively charged surfaces/LPS
2) Invasion of periplasmic space	receptor/docking/transporter molecule	GroEL
3) Binding to target	inhibition of protein synthesis – ribosomes	inhibition of protein folding – DnaK

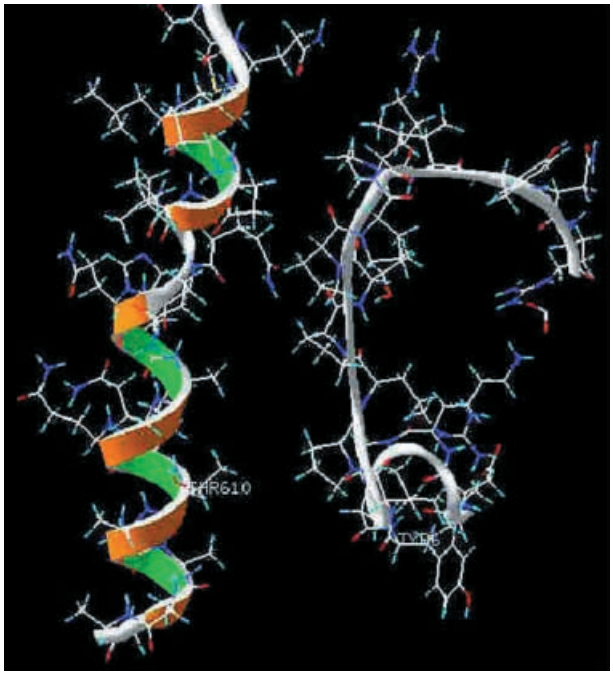

Figure 1. Characteristic structures of pyrrhocoricin and the D-E helix region of *E. coli* DnaK as they are generated by the flexible docking process [88]. The helical domain on the left corresponds to the synthetic peptide fragment representing DnaK with helix D located at the top and helix E at the bottom. The right structure corresponds to pyrrhocoricin which is positioned with its amino terminus at the bottom, and its carboxy terminus at the top. Tyr6, in the antibacterial peptide and the opposing residue of the protein, Thr610, are marked to show the relative distances on DnaK. Note that in this model, the E helix has been extended all over the length of the synthetic peptide fragment for computational purposes; in reality, the C-terminal residues may assume a less ordered, and consequently more elongated structure.

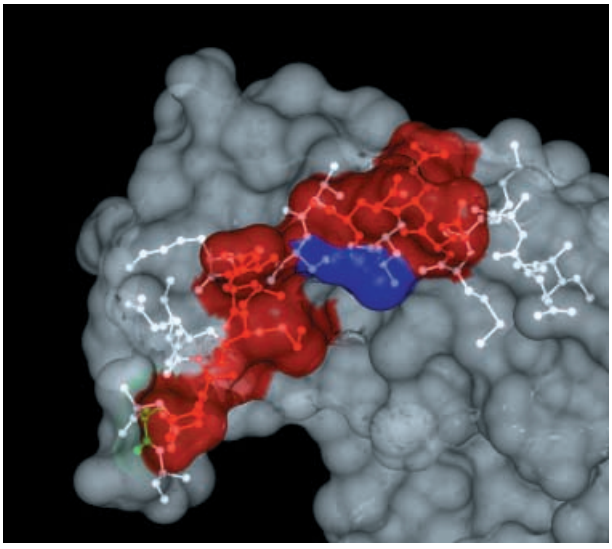

Figure 2. Schematic representation of the published structure [122] of the *E. coli* DnaK D-E helix fragment in a ball-and-stick model and with the protein surface shown as semi-transparent. Red residues correspond to those which were identified by a mutational analysis to be important for pyrrhocoricin binding. The mutational analysis failed to link the residue printed in blue (Ala591) as interacting with the antimicrobial peptide, but associated the residue printed in green (Gln604) with pyrrhocoricin binding. However, Gln604 appears to be located outside the surface identified by the rest of the important amino acids.

the hypothetical pathways of cell entry and intracellular activity of the proline-rich antibacterial peptides. Recently, we identified the DnaK-binding sites of pyrrhocoricin. According to these studies, the extended N-terminal half of the peptide binds to the hinge between helices D and E, located just above the conventional peptide-binding pocket [88] (fig. 1). A mutational analysis of the synthetic *E. coli* DnaK D-E helix fragment identified a probable pyrrhocoricin-binding surface on the protein (fig. 2). Enzyme assays support the notion that pyrrhocoricin inhibits chaperone-assisted protein folding [88]. In addition, pyrrhocoricin and its N-terminal half block the inherent ATPase activity of DnaK [88]. The rest of the peptide, especially the positively charged residues, is needed for the peptide to enter into the cytoplasm. This hypothesis is supported by the fact that the replacement of the positively charged residues, Lys3, Arg14 and Arg19, results in partial or complete loss of antibacterial activity. Taken together, pyrrhocoricin, drosocin and apidaecin are likely to carry independent modules for entry into bacterial cells (in Gram-negative strains this takes place by interacting with LPS) and binding to bacterial DnaK [G. Kragol et al., unpublished data]. If GroEL serves as a non-specific vehicle for intracellular trafficking, small fragments of the peptides may be present to enhance interaction with GroEL.

Conformation and role of the proline residues

Finally, we reached the question as to the roles played by the increased number of proline residues in defining the structure or function of these peptides. In simple structure-activity relationship studies, several Pro residues, including Pro9 in apidaecin, were found to be responsible for the antibacterial activity of the peptide [49, 89].
Consecutive prolines in a sequence usually assume a left-handed polyproline II helix structure, as was shown for the classical examples, the mucins [90]. Indeed, circular dichroism spectra of Bac-5 [63, 91] and PR-39 [74] resemble that of polyproline II helices [92]. Drosocin [33] and dipericin [54] also exhibit similar spectra, but without the small positive band around 220 nm. The problem is that these spectra also resemble that of polylysine at pH 5.7 in unordered conformation [93]. Higher-resolution nuclear magnetic resonance spectroscopy (NMR) cannot resolve the question, because the lack of intramolecular hydrogen bonding renders the polyproline II conforma-

tion of free peptides in solution indistinguishable in ^{1}H-NMR from an irregular backbone structure by [94]. Accordingly, no significant secondary structure was detected for the polyproline II helical mucin peptides by NMR [90] and, likewise, the predominantly unordered structure of drosocin as detected by NMR [95] may, but not necessarily, reflect the presence of an underlying polyproline II helix conformation. In support of this, energy calculations of Bac-5 suggest a structure more extended than that of a polyproline II helix [91]. Circular dichroism (CD) and Fourier-transform infrared spectroscopy have been used to investigate the secondary structure of PR-39 in the absence or presence of lipids [74]. According to the CD data, this structure is not altered upon incubation of PR-39 with negatively charged vesicles, although the infrared spectra suggest that the hydrogen bond pattern is modified upon peptide-lipid interaction.

When studying the effect of the native disaccharide on the conformation of drosocin, we observed subtle differences in the small populations of folded conformers between the glycosylated and non-glycosylated peptides [95]. In particular, the turn at residues 10–13 tends toward a more extended structure upon glycosylation, while there is some tightening of the downstream turn at residues 17 and 18. There are a significant number of nuclear Overhauser enhancement contacts between the sugar moiety and the peptide near the glycosylation site, consistent with a close association between them. On the basis of the biological activity of drosocin fragments and the structural studies, we built a model in which we hypothesized that the interaction of drosocin with its target protein may involve the locally structured regions 4–7 and 17–19 and that glycosylation may aid in correctly orienting these separate binding regions. Like drosocin, the structure of pyrrhocoricin appears to be largely random coil and there is little change in the backbone conformation upon glycosylation [35]. For pyrrhocoricin, however, there is a subpopulation with organized structures at both the N and C termini, indicating the presence of reverse turns at the pharmacologically important terminal regions.

The general role of prolines in the cell-penetrating ability of antibacterial peptides was studied in buforin II [96], a histone H2A-derived peptide that kills bacteria by binding to nucleic acids [97]. Buforin and its truncated analogs penetrate the cell membrane, but like the proline-rich peptides do not permeabilize it. The sequence of buforin II consists of 21 amino acid residues with a single proline in mid-chain position [98]. The peptide penetrates the bacterial cell membrane and accumulates in the cytoplasm, in contrast with proline-free magainin that remains associated with the inner leaflet of the lipid bilayer after translocation of the artificial membrane [99]. However, when the buforin single proline residue is replaced with an alanine, the mode of action is changed from one featuring an intracellular target to magainin-type membrane destruction [96]. Taken together, a proline hinge was postulated, a key structural element for the cell-penetrating property without permanent membrane association. If a single proline can do the trick for buforin, the multiple proline residues in the short, proline-rich peptides should do it much more efficiently. An interesting proposal is that the prolines might function as targeting residues for a proline porter en route to the intracellular uptake of apidaecin [56].

Although the size of pyrrhocoricin cannot be shortened without a major loss of antibacterial activity [44], at least against *E. coli* [100], the amino acids in the C-terminal half can be more or less substituted, indicating that this region is not involved in binding to the target protein DnaK [G. Kragol et al., unpublished data]. No substitutions are allowed in the N-terminal half. The architecture of cathelicidins, the group to which peptides PR-39, Bac-5 and Bac-7 belong, is remarkably similar. Bovine myeloid cathelicidins carry precursors of broad-spectrum antimicrobial peptides characterized by N-terminal amphipathic α helices and C-terminal hydrophobic tails [101]. The C-terminal tails of the cathelicidins are not needed for the antibacterial activity, with the truncated peptides containing only the amphipathic helices being equally active against a selection of both Gram-negative and Gram-positive bacteria [101]. However, a 5- to 10-fold higher concentration of the truncated analogs is required to achieve a kinetics of permeabilization comparable with that of the respective parent peptides, likely indicating a somewhat less effective initial interaction with the target bacterial membranes [101]. Significantly, the hydrophobic tail sequences (PVIPLLHR and PIIVPIIRI) reveal remarkable similarities to C-terminal tails of pyrrhocoricin (PPRPIYNRN), drosocin (SHPRPIRV) and apidaecin (PHPRI). Apparently, no matter how the peptides actually kill bacteria, the highly similar delivery modules initiate a binding to the membrane surface and help in the internalization of the peptides. Here we have to return to the peneaidins. Assuming that the peneadin proline-rich region is not directly responsible for the antimicrobial properties, a possible role of the prolines was proposed to be membrane targeting [73]. Yet again, pushing this theme to the extreme, the prophenoloxidase-activating enzyme form the freshwater crayfish, *Pacifastacus leniusculus*, is composed of a C-terminal serine proteinase domain and an N-terminal glycine- and another N-terminal proline-rich domain, the latter being highly homologous to the short, proline-rich insect antibacterial peptides [102]. If this sequence pattern can be a non-functional part of a protein, it must carry a very general secondary function, such as being the delivery, targeting or conformational module.

The native proline-rich antibacterial peptides are not the only polyamides that can penetrate into cells. The delta-

sleep-inducing peptide, WAGGDASGE, was reported to be able to permeate biological barriers, although it does not cross the gastrointestinal epithelium-mimicking Caco-2 cell layer system [103]. Other cell-penetrating protein-derived peptides include penetratin, RQIKIWFQNRRMKWKK, signal-sequence-based peptides exhibiting highly hydrophobic α-helical structures and GRKKRRQRRRPPQ, corresponding to the 48–60 fragment of the Tat protein from human immunodeficiency virus-1 [104–107]. Synthetic analogs are transportan, the amphiphilic model peptide made of oligomers of KLAL and, most recently, a Tat analog made of β-amino acid residues [108]. Newly described cell-penetrating peptides come from virus proteins, leucine zipper proteins and a yeast transcription factor [109]. Except for the helical signal-sequence-based peptides, the common structural characteristic of these protein fragments and synthetic constructs is the abundance of positively charged residues, arginine and lysine, often allowing these peptides to bind to not only negatively charged membrane surfaces but nucleic acids as well (compare with buforin). In fact, hepta- or octaarginine appears to be the best carrier for translocation of cargo into cells [109, 110]. However, increasingly efficient cell penetration is often accompanied by destruction of the cell membrane and, hence, toxicity [111]. Remarkably, most of these cell-penetrating peptides lack proline residues known to break helices. Antimicrobial peptides with helical structures in a lipid environment and organic solvents are associated with membrane disintegration, as documented for a series of such peptides including insect cecropin [112, 113]. Likewise, the above-listed cell-permeating peptides are also helical in membrane environments [107]. In contrast, drosocin fails to form α-helix structure, even in the strongly helix promoting solvent trifluoroethanol [33]. Apparently, the positive charges in the short, proline-rich antimicrobial peptides enhance bacterial cell entry [100], and the interspersed prolines may prevent helix formation and toxicity to the host.

Concluding remarks

Ideally, we hope to identify a protein present in bacteria which is needed for the survival of the microorganism, but is absent or non-homologous in human cells [114]. This target may form the basis of rational drug design efforts, as was first suggested concerning apidaecin [32]. Some antibacterial peptides are known to act as inhibitors of enzymes produced by the bacteria either by serving as a pseudo-substrate or by tight binding to the active site eliminating the accessibility of the native substrate [1]. Inhibition of chaperone-assisted protein folding by the proline-rich cationic antibacterial peptides [88] appears to represent a bold new way of fighting bacteria that are resistant to conventional antibiotics. It is necessary to note here that specific amino acid combinations and conformational motifs that preferentially bind DnaK were selected from peptide libraries [115, 116], and these amino acid combinations strikingly resemble the common sequence motifs of pyrrhocoricin, drosocin and apidaecin. Based on screening of these DnaK-bound libraries, DnaK recognizes extended peptide strands within and positively charged residues outside the substrate-binding cavity [117], structural features clearly present in pyrrhocoricin. Incidentally, two groups have reported peptide-binding at the C-terminal area of DnaK, outside the conventional peptide-binding pocket. One of these identified the 518–545 residue stretch, helix B, [118] that serves as part of the multihelical lid over the peptide-binding pocket. According to the other, the highly negatively charged extreme C-terminal tetrapeptide of human Hsp70 binds a peptide substrate and affects ATPase activity [119]. DnaK shares only 50% sequence homology with eukaryotic Hsp70 [120], with some well-conserved N-terminal regions and some less conserved C-terminal ones [121]. The variable sequence domain of DnaK appears to offer a sensible target for antibacterial peptide development, and the pyrrhocoricin-drosocin-apidaecin peptide family is an excellent starting point for these drug development efforts. The sequence differences around the specific pyrrhocoricin-binding site allow the design of strain-specific antimicrobial peptides and peptidomimetics. Longer-term applications of the species-specific inhibition of chaperone-assisted protein folding include the control not only of bacteria, but also fungi, parasites, insects and, perhaps, rodents.

Acknowledgements. I would like to thank Dr. Ralf Hoffmann for the mutational analysis and Dr. Johan Rosengren for the preliminary figure of the possible pyrrhocoricin-interacting domain of *E. coli* DnaK. Details of these studies will be published later.

1 Andreu D. and Rivas L. (1998) Animal antimicrobial peptides: an overview. Biopolymers **47:** 415–433
2 Engstrom Y. (1999) Induction and regulation of antimicrobial peptides in *Drosophila*. Dev. Comp. Immunol. **23:** 345–358
3 Meister M., Lemaitre B. and Hoffmann J. A. (1997) Antimicrobial peptide defense in *Drosophila*. BioEssays **19:** 1019–1026
4 Barra D., Simmaco M. and Boman H. G. (1998) Gene encoded peptide antibiotics and innate immunity: do 'animacules' have defence budgets? FEBS Lett. **430:** 130–134
5 Hoffmann J. A., Hetru C. and Reichhart J.-M. (1993) The humoral antibacterial response of *Drosophila*. FEBS Lett. **325:** 63–66
6 Bulet P. (1999) *Drosophila* antimicrobial peptides. Med. Sci. **15:** 23–29
7 Hoffmann J. and Hetru C. (1992) Insect defensins: inducible antibacterial peptides. Immunol. Today **13:** 411–415
8 Hughes A. L. (1999) Evolutionary diversification of the mammalian defensins. Cell. Mol. Life Sci. **56:** 94–103
9 Steiner H., Hultmark D., Engstrom A., Bennich H. and Boman H. G. (1981) Sequence and specificity of two antibacter-

ial proteins involved in insect immunity. Nature **292:** 246–248
10 Boman H. G., Faye I., Gudmundsson G. H., Lee J. Y. and Lidholm D. A. (1991) Cell-free immunity in *Cecropia*: a model system for antibacterial proteins. Eur. J. Biochem. **201:** 23–31
11 Zasloff M. (1987) Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. USA **84:** 5449–5453
12 Giovannini M. G., Poulter L., Gibson B. W. and Williams D. H. (1987) Biosynthesis and degradation of peptides derived from *Xenopus laevis* prohormones. Biochem. J. **243:** 113–120
13 Bulet P., Hetru C., Dimarcq J. L. and Hoffmann D. (1999) Antimicrobial peptides in insects: structure and function. Dev. Comp. Immunol. **23:** 329–344
14 Ando K. and Natori S. (1988) Inhibitory effect of sarcotoxin IIA, an antibacterial protein of *Sarcophaga peregrina*, on growth of *Escherichia coli*. J. Biochem. **103:** 735–739
15 Asling B., Dushay M. S. and Hultmark D. (1995) Identification of early genes in the *Drosophila* immune response by PCR-based differential display: the Attacin A gene and the evolution of attacin-like proteins. Insect Biochem. Mol. Biol. **25:** 511–518
16 Ishikawa M., Kubo T. and Natori S. (1992) Purification and characterization of a dipteran homologue from *Sarcophaga peregrina* (flesh fly). Biochem. J. **287:** 573–578
17 Bulet P., Hegy G., Lambert J., Dorsselaer A. van, Hoffmann J. A. and Hetru C. (1995) Insect immunity: the inducible antibacterial peptide diptericin carries two O-glycans necessary for biological activity. Biochemistry **34:** 7394–7400
18 Dimarcq J. L., Zachary D., Hoffmann J. A., Hoffmann D. and Reichhart J. M. (1990) Insect immunity: expression of the two major inducible antibacterial peptides, defensin and diptercin, in *Phormia terranovae*. EMBO J. **9:** 2507–2515
19 Fehlbaum P., Bulet P., Chernysh S., Briand J. P., Roussel J. P., Letellier L. et al. (1996) Structure-activity analysis of thanatin, a 21-residue inducible insect defense peptide with sequence homology to frog skin antimicrobial peptides. Proc. Natl. Acad. Sci. USA **93:** 1221–1225
20 Conlon J. M., Halverson T., Dulka J., Platz J. E. and Knoop F. C. (1999) Peptides with antimicrobial activity of the brevinin-1 family isolated from skin secretions of the southern leopard frog, *Rana sphenocephala*. J. Pept. Res. **54:** 522–527
21 Hara S. and Yamakawa M. (1995) Moricin, a novel type of antibacterial peptide isolated from the silkworm, *Bombyx mori*. J. Biol. Chem. **270:** 29923–29927
22 Steiner H., Andreu D. and Merrifield R. B. (1988) Binding and action of cecropin and cecropin analogues: antibacterial peptides from insects. Biochim. Biophys. Acta **939:** 260–266
23 Wimley W. C., Selsted M. E. and White S. H. (1994) Interactions between human defensins and lipid bilayers: evidence for formation of multimeric pores. Protein Sci. **3:** 1361–1373
24 Ludtke S., He K. and Huang H. (1995) Membrane thinning caused by magainin 2. Biochemistry **34:** 16764–16769
25 Shai Y. (1995) Molecular recognition between membrane-spanning polypeptides. Trends Biochem. Sci. **20:** 460–464
26 Matsuzaki K., Mitani Y., Akada K. Y., Murase O., Yoneyama S., Zasloff M. et al. (1998) Mechanism of synergism between antimicrobial peptides magainin 2 and PGLa. Biochemistry **37:** 15144–15153
27 Wu M., Maier E., Benz R. and Hancock R. E. W. (1999) Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. Biochemistry **38:** 7235–7242
28 Zhang L., Benz R. and Hancock R. E. W. (1999) Influence of proline residues on the antibacterial and synergistic activities of alpha-helical peptides. Biochemistry **38:** 8102–8111
29 Otvos L. Jr, Bokonyi K., Varga I., Otvos B. I., Hoffmann R., Ertl H. C. J. et al. (2000) Insect peptides with improved protease-resistance protect mice against bacterial infection. Protein Sci. **9:** 742–749
30 Wade D., Boman A., Wahlin B., Drain C. M., Andreu D., Boman H. G. et al. (1990) All-D amino acid-containing channel-forming antibiotic peptides. Proc. Natl. Acad. Sci. USA **87:** 4761–4765
31 Vunnam S., Juvvadi P. and Merrifield R. B. (1997) Synthesis and antibacterial action of cecropin and proline-arginine-rich peptides from pig intestine. J. Pept. Res. **49:** 59–66
32 Casteels P. and Tempst P. (1994) Apidaecin-type peptide antibiotics function through a non-poreforming mechanism involving stereospecificity. Biochem. Biophys. Res. Commun. **199:** 339–345
33 Bulet P., Urge L., Ohresser S., Hetru C. and Otvos L. Jr (1996) Enlarged scale chemical synthesis and range of activity of drosocin, an O-glycosylated antibacterial peptide from *Drosophila*. Eur. J. Biochem. **238:** 64–69
34 Gallo R. L. and Huttner K. M. (1998) Antimicrobial peptides: an emerging concept in cutaneous biology. J. Invest. Dermatol. **111:** 739–743
35 Otvos L. Jr (2000) Antibacterial peptides isolated from insects. J. Pept. Sci. **6:** 497–511
36 Lemaitre B., Nicolas E., Michaut L., Reichhart J. M. and Hoffmann J. A. (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell **86:** 973–983
37 Liu G., Kang D. and Steiner H. (1995) *Trichoplusia ni* lebocin, an inducible immune gene with a downstream insertion element. Biochem. Biophys. Res. Commun. **269:** 803–807
38 Lemaitre B., Reichhart J.-M. and Hoffmann J. A. (1997) *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proc. Natl. Acad. Sci. USA **94:** 14614–14619
39 Bulet P., Dimarcq J.-L., Hetru C., Lagueux M., Charlet M., Hegy G. et al. (1993) A novel inducible antibacterial peptide from *Drosophila* carries an O-glycosylated substitution. J. Biol. Chem. **268:** 14893–14897
40 Uttenweiler-Joseph S., Moniatte M., Lagueux M., Van Dorsselaer A., Hoffmann J. A. and Bulet P. (1998) Differential display of peptides induced during the immune response of *Drosophila*: a matrix-assisted laser desorption ionization time-of-flight mass spectrometry study. Proc. Natl. Acad. Sci. USA **95:** 11342–11347
41 Rodriguez E. C., Winans K. A., King D. S. and Bertozzi C. R. (1997) A strategy for the chemoselective synthesis of O-linked glycopeptides with native sugar-peptide linkages. J. Am. Chem. Soc. **119:** 9905–9906
42 Marcaurelle L. A., Rodriguez E. C. and Bertozzi C. R. (1998) Synthesis of an oxime-linked neoglycopeptide with glycosylation-dependent activity similar to its native counterpart. Tetrahedron Lett. **39:** 8417–8420
43 Cociancich S., Dupont A., Hegyi G., Lanot R., Holder F., Hetru C. et al. (1994) Novel inducible antibacterial peptides from a hemipteran insect, the sap sucking-bug *Pyrrhocoris apterus*. Biochem. J. **300:** 567–575
44 Hoffmann R., Bulet P., Urge L. and Otvos L. Jr (1999) Range of activity and metabolic stability of synthetic antibacterial glycopeptides from insects. Biochim. Biophys. Acta **1426:** 459–467
45 Mackintosh J. A., Veal D. A., Beattie A. J. and Gooley A. A. (1998) Isolation from an ant *Myrmecia gulosa* of two inducible O-glycosylated proline-rich antibacterial peptides. J. Biol. Chem. **273:** 6139–6143
46 Boulanger N., Ehret-Sabatier L., Brun R., Zachary D., Bulet P. and Imler J.-L. (2001) Immune response of *Drosophila melanogaster* to infection with the flagellate parasite *Crithidia* spp. Insect Biochem. Mol. Biol**. 31:** 129–137

47 Casteels P., Ampe C., Jacobs F., Vaeck M. and Tempst P. (1989) Apidaecins: antibacterial peptides from honeybees. EMBO J. **8:** 2387–2391

48 Casteels P., Romagnolo J., Castle M., Casteels-Josson K., Erdjument-Bromage H. and Tempst P. (1994) Biodiversity of apidaecin-type peptide antibiotics: prospects of manipulating the antibacterial spectrum and combating acquired resistance. J. Biol. Chem. **269:** 26107–26115

49 Taguchi S., Ozaki A., Nakagawa K. and Momose H. (1996) Functional mapping of amino acid residues responsible for the antibacterial action of apidaecin. Appl. Environ. Microbiol. **62:** 4622–4655

50 Taguchi S., Kuwasako K., Suenaga A., Okada M. and Momose H. (2000) Functional mapping against *Escherichia coli* for the broad-spectrum antimicrobial peptide, thanatin, based on an in vivo monitoring assay system. J. Biochem. **128:** 745–754

51 Hara S. and Yamakawa M. (1995) A novel antibacterial family isolated from the silkworm, *Bombyx mori*. Biochem. J. **310:** 651–656

52 Casteels P., Ampe C., Riviere L., Damme J. van, Elicone C., Flemning M. et al. (1990) Isolation and characterization of abaecin, a major antibacterial response peptide in the honeybee (*Apis mellifera*). Eur. J. Biochem. **187:** 381–386

53 Rees J. A., Moniatte M. and Bulet P. (1997) Novel antibacterial peptides isolated from the European bumblebee, *Bombus pascorum* (Hymenophtera, Apoidea). Insect Biochem. Mol. Biol. **27:** 413–422

54 Cudic M., Bulet P., Hoffmann R., Craik, D. J. and Otvos L. Jr (1999) Chemical synthesis, antibacterial activity and conformation of diptericin, an 82-mer peptide originally isolated from insects. Eur. J. Biochem. **266:** 549–558

55 Winans K. A., King D. S., Rao V. K and Bertozzi C. R. (1999) A chemically synthesized version of the insect antibacterial glycopeptide, diptericin, disrupts bacterial membrane integrity. Biochemistry **38:** 11700–11710

56 Castle M., Nazarian A., Yi S.-S. and Tempst P. (1999) Lethal effects of apidaecin on *Escherichia coli* involve sequential molecular interactions with diverse targets. J. Biol. Chem. **274:** 32555–32564

57 Agerberth B., Lee J. Y., Bergman T., Carlquist M., Boman H. G., Mutt V. et al. (1991) Amino acid sequence of PR-39: isolation from pig intestine of a new member of the family of proline-arginine-rich antibacterial peptides. Eur. J. Biochem. **202:** 849–854

58 Shi J., Ross C. R., Chengappa M. M. and Blecha F. (1994) Identification of a proline-arginine-rich antibacterial peptide from neurophils that is analogous to PR-39, and antibacterial peptide from the small intestine. J. Leukoc. Biol. **56:** 807–811

59 Bonetto V., Andersson M., Bergman T., Sillard R., Morberg A., Mutt V. et al. (1999) Spleen antibacterial peptides: high levels of PR-39 and presence of two forms of NK-lysin. Cell. Mol. Life Sci. **56:** 174–178

60 Gennaro R. and Zanetti M. (2000) Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. Biopolymers **55:** 31–49

61 Gennaro R., Skleravaj B. and Romeo D. (1989) Purification, composition, and activity of two bactenecins, antibacterial peptides of bovine neutrophils. Infect. Immun. **57:** 3142–3146

62 Scocchi M., Romeo D. and Zanetti M. (1994) Molecular cloning of Bac-7, a proline- and arginine-rich antimicrobial peptide from bovine neutrophils. FEBS Lett. **26:** 197–200

63 Tokunaga Y., Niidome T., Hatakeyama T. and Aoyagi H. (2001) Antibacterial activity of bactenecin 5 fragments and their interaction with phospholipid membranes. J. Pept. Sci. **7:** 297–304

64 Huttner K. M., Lambeth M. R., Burkin H. R., Burkin D. J. and Broad T. E. (1998) Localization and genomic organization of sheep antimicrobial peptide genes. Gene **206:** 85–91

65 Shi J., Ross C. R., Chengappa M. M., Sylte M. J., McVey D. S. and Blecha F. (1996) Antibacterial activity of a synthetic peptide (PR-26) derived from PR-39, a proline-arginine-rich neutrophil antimicrobial peptide. Antimicrob. Agents Chemother. **40:** 115–121

66 Chan Y. R., Zanetti M., Gennaro R. and Gallo R. L. (2001) Anti-microbial activity and cell binding are controlled by sequence determinants in the anti-microbial peptide PR-39. J. Invest. Dermatol. **116:** 230–235

67 Strukelj B., Pungercar J., Kopitar G., Renko M., Lenarcic B., Berbic S. et al. (1995) Molecular cloning and identification of a novel porcine cathelin-like antibacterial peptide precursor. Biol. Chem. Hoppe Seyler **376:** 507–510

68 Harwig S. S. L., Kokryakov, V. N., Swiderek K. M., Aleshima G. M., Zhao C. and Lehrer R. I. (1995) Prophenin-1, an exceptionally proline-rich antimicrobial peptide from porcine leukocytes. FEBS Lett. **392:** 65–59

69 Destoumieux D., Bulet P., Loew D., Van Dorsselaer A., Rodriguez J. and Bachere E. (1997) Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). J. Biol. Chem. **272:** 28398–28406

70 Destoumieux D., Munoz M., Bulet P. and Bachere E. (2000) Penaeidins, a family of antimicrobial peptides from penaeid shrimp (Crustacea, Decapoda). Cell. Mol. Life Sci. **57:** 1260–1271

71 Destomieux D., Munoz M., Cosseau C., Rodriguez J., Bulet P., Comps M. et al. (2000) Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge. J. Cell Sci. **113:** 461–469

72 Saito T., Kawabata S., Shigenaga T., Takayenoki Y., Cho J., Nakajima H. et al. (1995) A novel big defensin identified in horseshoe crab hemocytes: isolation, amino acid sequence, and antimicrobial activity. J. Biochem. **117:** 1131–1137

73 Destomieux D., Bulet P., Strub J-M., Dorsselear A. van and Bachere E. (1999) Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp. Eur. J. Biochem. **266:** 335–346

74 Cabiaux V., Agerberth B., Johansson J., Homble F., Goormaghtigh E. and Ruysschaert J. M. (1994) Secondary structure and membrane interaction of PR-39, a Pro+Arg-rich antibacterial peptide. Eur. J. Biochem. **224:** 1019–1027

75 Linde C. M., Hoffner S. E., Refai E. and Andersson M. (2001) In vitro activity of PR-39, a proline-arginine-rich peptide, against susceptible and multi-drug resistant *Mycobacterium tuberculosis*. J. Antimicrob. Chemother. **47:** 575–580

76 Lehrer R. I., Barton A., Daher K. A., Harwig S. S., Ganz T. and Selsted M. E. (1989) Interaction of human defensins with *Escherichia coli*: mechanism of bactericidal activity. J. Clin. Invest. **84:** 553–561

77 Chan Y. and Gallo R. L. (1998) PR-39, a syndecan-inducing antimicrobial peptide, binds and affects p130Cas. J. Biol. Chem. **273:** 28978–28985

78 Shi J., Ross C. R., Leto T. L. and Blecha F. (1996) PR-39, a proline-rich antibacterial peptide that inhibits phagocyte NAPDH oxidase activity by binding to Src homology 3 domains of p47phox. Proc. Natl. Acad. Sci. USA **93:** 6014–6018

79 Chan Y. R., Zanetti M., Gennaro R. and Gallo R. L. (2000) Anti-microbial activity and cell binding are controlled by sequence determinants in the anti-microbial peptide PR-39. J. Invest. Dermatol. **116:** 230–235

80 Hancock R. E. W. and Scott M. G. (2000) The role of antimicrobial peptides in animal defenses. Proc. Natl. Acad. Sci. USA **97:** 8856–8861

81 Moore A. J., Devine D. A. and Bibby M. C. (1994) Preliminary experimental anticancer activity of cecropins. Pept. Res. **7:** 265–269

82 Otvos L. Jr, O I., Rogers M. E., Consolvo P. J., Condie B. A., Lovas S. et al. (2000) Interaction between heat shock proteins and antimicrobial peptides. Biochemistry **39:** 14150–14159

83 Gao Y., Lecker S., Post M. J., Hietaranta A. J., Li J., Volk R. et al. (2000) Inhibition of ubiquitin-proteasome pathway-mediated I kappa B alpha degradation by a naturally occurring antibacterial peptide. J. Clin. Invest. **106:** 439–448

84 Bao J., Sato K., Li M., Gao Y., Abid R., Aird W. et al. (2001) PR-39 and PR-11 peptides inhibit ischemia-reperfusion injury by blocking proteasome-mediated IκBα degradation. Am. J. Physiol. Heart Circ. Physiol. **281:** H2612–H2618

85 Korthuis R. J., Gute D. C., Blecha F. and Ross C. R. (1999) PR-39, a proline/arginine rich antimicrobial peptide, prevents postischemic microvascular dysfunction. Am. J. Physiol. **277:** H1007–H1013

86 Boman H. G., Agerberth B. and Boman A. (1993) Mechanisms of action on *Escherichia coli* of cecropin P1 and PR-39, two antibacterial peptides from pig intestine. Infect. Immun. **61:** 2978–2984

87 Gallo R. L., Ono M., Povsic T., Page C., Eriksson E., Klagsbrun M. et al. (1994) Syndecans, cell surface heparan sulfate proteoglycans, are induced by a proline-rich antimicrobial peptide from wounds. Proc. Natl. Acad. Sci. USA **91:** 11035–11039

88 Kragol G., Lovas S., Varadi G., Condie B. A., Hoffmann R. and Otvos L. Jr (2001) The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. Biochemistry **40:** 3016–3026

89 Taguchi S., Nakagawa K., Maeno M. and Momose H. (1994) In vivo monitoring system for structure-function relationship analysis of the antibacterial peptide apidaecin. Appl. Environ. Microbiol. **60:** 3566–3572

90 Spencer D. I., Missailidis S., Denton G., Murray A., Brady K. and Matteis C. I. (1999) Structure/activity studies of the anti-MUC1 monoclonal antibody C595 and synthetic MUC1 mucin-core-related peptides and glycopeptides. Biospectroscopy **5:** 79–91

91 Niidome T., Mihara H., Oka M., Hayashi T., Saiki T., Yoshida K. et al. (1998) Structure and property of model peptides of proline/arginine-rich region in bactenecin 5. J. Pept. Res. **51:** 337–345

92 Ladokhin A. S., Selsted M. E. and White S. H. (1999) CD spectra of indolicin antimicrobial peptides suggest turns, not polyproline helix. Biochemistry **38:** 12313–12319

93 Greenfield N. and Fasman G. D. (1969) Computed circular dichroism spectra for the evaluation of protein conformation. Biochemistry **8:** 4108–4116

94 Siligardi G. and Drake A. F. (1995) The importance of extended conformations, and in particular, the PII conformation for the molecular recognition of peptides. Biopolymers **37:** 281–292

95 McManus A., Otvos L. Jr, Hoffmann R. and Craik D. J. (1999) Conformational studies by NMR of the anti-microbial peptide, drosocin and its non-glycosylated derivative: effects of glycosylation on solution conformation. Biochemistry **38:** 705–714

96 Park C. B., Yi K., Matsuzaki K., Kim M. S. and Kim S. C. (2000) Structure-activity analysis of buforin II, a histone H2A-derived antimicrobial peptide: the proline hinge is responsible for the cell-penetrating ability of buforin II. Proc. Natl. Acad. Sci. USA **97:** 8245–8250

97 Park C. B., Kim H. S. and Kim S. C. (1998) Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. Biochem. Biophys. Res. Commun. **244:** 253–257

98 Park C. B., Kim M. S. and Kim S. C. (1996) A novel antimicrobial peptide from *Bufo bufo gargarizans*. Biochem. Biophys. Res. Commun. **218:** 408–413

99 Matsuzaki K. (1998) Magainins as paradigm for the mode of action of pore forming polypeptides. Biochim. Biophys. Acta **1376:** 391–400

100 Bencivengo A. M., Cudic M., Hoffmann R. and Otvos L. Jr (in press) The efficacy of the antibacterial peptide, pyrrhocoricin, is finely regulated by its amino acid residues and active domains. Lett. Pept. Sci.

101 Skerlavaj B., Gennaro R., Bagella L., Merluzzi L., Risso A. and Zanetti M. (1996) Biological characterization of two novel cathelicidin-derived peptides and identification of structural requirements for their antimicrobial and cell lytic activities. J. Biol. Chem. **271:** 28375–28381

102 Wang R., Lee S. Y., Cerenius L. and Soderhall K. (2001) Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*. Eur. J. Biochem. **268:** 895–902

103 Augustijns P. F. and Borchardt R. T. (1995) Transport and metabolism of delta sleep-inducing peptide in cultured human intestinal epithelial cell monolayers. Drug Metab. Dispos. **23:** 1372–1378

104 Lindgren M., Hallbrink M., Prochiantz, A. and Langel U. (2000) Cell-penetrating peptides. Trends Pharmacol. Sci. **21:** 99–103

105 Derossi D., Chassaing G. and Prochiantz A. (1998) Trojan peptides: the penetratin system for intracellular delivery. Trends Cell. Biol. **8:** 84–87

106 Suzuki T., Futaki S., Niwa M., Tanaka S., Ueda K. and Sugiura Y. (2002) Possible existence of common internalization among arginine-rich peptides. J. Biol. Chem. **277:** 2437–2443

107 Magzoub M., Kilk K., Eriksson G., Langel U. and Graslund A. (2001) Interaction and structure of cell penetrating peptides in the presence of phospholipid vesicles. Biochim. Biophys. Acta **1512:** 77–89

108 Umezawa N., Gelman M. A., Haigis M. C., Raines R. T. and Gellmann S. H. (2002) Translocation of a β-peptide across cell membranes. J. Am. Chem. Soc. **124:** 368–369

109 Futaki S., Suzuki T., Ohashi W., Yagami T., Tanaka S., Ueda K. et al. (2001) Arginine-rich peptides: an abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. J. Biol. Chem. **276:** 5836–5840

110 Rothbard J. B., Garlington S., Lin Q., Kirschberg T., Kreider E., Grane P. L. et al. (2000) Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation. Nat. Med. **6:** 1253–1257

111 Hallbrink M., Floren A., Elmquist A., Pooga M., Bartfai T. and Langel U. (2001) Cargo delivery kinetics of cell-penetrating peptides. Biochim. Biophys. Acta **1515:** 101–109

112 Holak T. A., Engstrom A., Kraulis P. J., Lindeber G., Bennich H., Jones T. A. et al. (1988) The solution conformation of the antibacterial peptide cecropin A: a nuclear magnetic resonance and dynamical simulated annealing study. Biochemistry **27:** 7620–7629

113 Marassi F. M., Opella S. J., Juvvadi P. and Merrifield R. B. (1999) Orientation of cecropin A helices in phospholipid bilayers determined by solid-state NMR spectroscopy. Biophys. J. **77:** 3152–3155

114 Giglione C., Pierre M. and Meinnel T. (2000) Peptide deformylase as a target for new generation, broad spectrum antimicrobial agents. Mol. Microbiol. **36:** 1197–1205

115 Gragerov A., Zeng L., Zhao X., Burkholder W. and Gottesman M. E. (1994) Specificity of DnaK-peptide binding. J. Mol. Biol. **235:** 848–854

116 Rudiger S., Germeroth L., Schneider-Mergener J. and Bukau B. (1997) Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. EMBO J. **16:** 1501–1507

117 Rudiger S., Buchberger A. and Bukau B. (1997) Interaction of Hsp70 chaperones with substrates. Nat. Struct. Biol. **4:** 342–349

118 Zhang J. and Walker G. C. (1998) Interactions of peptides with DnaK and C-terminal DnaK fragments studied using fluorescent and radioactive peptides. Arch. Biochem. Biophys. **356:** 177–186

119 Freeman B. C., Myers M. P., Schumacher R. and Morimoto R. I. (1995) Identification of a regulatory motif in Hsp70 that affects ATPase activity, substrate binding and interaction with HDJ-1. EMBO J. **14:** 2281–2292
120 Bardwell J. C. and Craig E. A. (1984) Major heat shock gene of *Drosophila* and the *Escherichia coli* heat-inducible dnaK gene are homologous. Proc. Natl. Acad. Sci. USA **81:** 848–852
121 Karlin S. and Brocchieri L. (1998) Heat shock protein 70 family: multiple sequence comparisons, function and evolution. J. Mol. Evol. **47:** 565–577
122 Zhu X., Zhao X., Burkholder W. F., Gragerov A., Ogata C. M., Gottesman M. E. et al. (1996) Structural analysis of substrate binding by the molecular chaperone DnaK. Science **272:** 1606–1014