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Andersson, Emma; Rydengård, Victoria; Sonesson, Andreas; Mörgelin, Matthias; Björck, Lars; Schmidtchen, Artur

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PO Box 117  
221 00 Lund  
+46 46-222 00 00



# Antimicrobial activities of heparin-binding peptides

Emma Andersson<sup>1</sup>, Victoria Rydengård<sup>1</sup>, Andreas Sonesson<sup>1</sup>, Matthias Mörgelin<sup>2</sup>, Lars Björck<sup>2</sup> and Artur Schmidtchen<sup>1</sup>

<sup>1</sup>Department of Medical Microbiology, Dermatology and Infection, Section for Dermatology; <sup>2</sup>Department of Cell and Molecular Biology, Section for Molecular Pathogenesis, Lund University, Biomedical Center, Sweden

Antimicrobial peptides are effector molecules of the innate immune system. We recently showed that the human antimicrobial peptides  $\alpha$ -defensin and LL-37 bind to glycosaminoglycans (heparin and dermatan sulphate). Here we demonstrate the obverse, i.e. structural motifs associated with heparin affinity (cationicity, amphipaticity, and consensus regions) may confer antimicrobial properties to a given peptide. Thus, heparin-binding peptides derived from laminin isoforms, von Willebrand factor, vitronectin, protein C inhibitor, and fibronectin, exerted antimicrobial activities against Gram-positive and Gram-negative bacteria. Similar results were obtained using heparin-binding peptides derived from complement factor C3 as well as

consensus sequences for heparin-binding (Cardin and Weintraub motifs). These sequence motifs, and additional peptides, also killed the fungus *Candida albicans*. These data will have implications for the search for novel antimicrobial peptides and utilization of heparin–protein interactions should be helpful in the identification and purification of novel antimicrobial peptides from complex biological mixtures. Finally, consensus regions may serve as templates for *de novo* synthesis of novel antimicrobial molecules.

**Keywords:** antimicrobial; cathelicidin; defensin; heparin binding; glycosaminoglycan.

Multicellular organisms express a blend of antimicrobial peptides (AMP), which are ubiquitously distributed at biological boundaries prone to infection. These peptides, originally described in silk worms [1], occur in animals ranging from insects to mammals [2–6]. At present, more than 700 different AMP peptide sequences are known (<http://www.bbcm.univ.trieste.it/~tossi/search.htm>). AMPs kill bacteria by permeating their membranes, and thus the lack of a specific molecular microbial target minimizes resistance development. The fundamental principle of action of most peptides depends on the ability of these molecules to adopt a shape in which clusters of hydrophobic and cationic amino acids are organized in discrete sectors, creating an amphipatic  $\alpha$ -helical,  $\beta$ -sheet, extended coil, or cyclic structure [7,8]. Various pathogenic bacteria are responsible for release of glycosaminoglycans (GAG) from epithelia and connective tissues. We, and others, have shown that the  $\alpha$ -helical LL-37 [9],  $\beta$ -sheet-containing  $\alpha$ -defensin [10], and the linear PR-39 [11] are bound to and inactivated by GAGs, thus promoting bacterial

infection. Sulphation of GAGs and the presence of iduronic acid, typical features of dermatan sulphate (DS) and heparin/heparan sulphate, facilitated binding to LL-37 and  $\alpha$ -defensin [9,10]. These data indicate that the two peptides belong to the expanding group of heparin-binding molecules.

The structural prerequisite for heparin binding and the presence of heparin-binding motifs in various proteins is well documented. This group of molecules includes various laminin isoforms, fibronectin, coagulation factors, growth factors, chemokines, histidine-rich glycoprotein, kininogen and many others [12–14]. After examining a series of heparin-binding sequences Cardin and Weintraub proposed that these were arranged in the pattern XBBBXXBX or XBBXBX (where X represents hydrophobic or uncharged amino acids, and B represents basic amino acids). Molecular modelling of these consensus sites predicts the arrangement of amino acids into either  $\alpha$ -helices or  $\beta$ -strands [15]. Additional analyses of heparin-binding peptide sites have revealed that these consensus sequences may not constitute an absolute requirement. Sobel and coworkers proposed a third consensus sequence, XBBBXXBBBXXBBX [16], and recently an additional sequence, TXXBXXTBXXXTB (where T defines a turn), was found to occur in heparin-binding sites of growth factors [12,14]. Based on studies of heparin-binding sites, Margalit and coworkers [17] reported that a distance of approximately 20 Å between basic amino acids constituted a prerequisite for heparin binding irrespective of peptide conformation. Thus, spacing of basic amino acids in heparin-binding peptides facilitates formation of ion pairs with spatially defined sulfo- or carboxyl-groups in heparin and heparan sulphate. Furthermore, N-acetyl and hydroxyl groups in heparin and, to a greater extent, in heparan

Correspondence to A. Schmidtchen, Department of Medical Microbiology, Dermatology and Infection, Section for Dermatology, Biomedical Center B14, Tornavägen 10, SE-221 84 Lund, Sweden.  
Fax: + 46 46 157756, Tel.: + 46 46 2224522,  
E-mail: artur.schmidtchen@derm.lu.se

**Abbreviations:** AMP, antimicrobial peptide; DS, dermatan sulphate; c.f.u., colony forming units; GAG, glycosaminoglycan; low-EEO, low-electroendosmosistype; RDA, radial diffusion assay; TSB, trypticase soy broth.

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sulphate, require 'matching' residues, such as alanine, leucine, or tyrosine, and glutamine or asparagine, enabling hydrophobic interactions and hydrogen bonding, respectively [12]. Apparently, the requirements for heparin interaction of peptides (such as amphipaticity, cationicity, secondary structure) are strikingly similar to the structural features of many known AMPs (LL-37, defensins). Indeed, LL-37 forms an amphipatic helical structure and contains an XBBXB motif. These observations, in conjunction with the fact that LL-37 and defensin bind GAGs, made us raise the following questions: are heparin-binding peptide motifs, such as those described above, antibacterial? If so, may our knowledge of the vast amount of heparin-binding motifs be utilized for effective *de novo* synthesis of AMPs, or for the identification and purification of endogenous AMPs?

## Materials and methods

### Peptides

Cationic peptides (Table 1) were synthesized by Innovagen AB, Lund, Sweden. The purity and molecular mass of these peptides was confirmed by mass spectral analysis (MALDI-TOF Voyager).

### Microorganisms

*Enterococcus faecalis* 2374, *Escherichia coli* 37.4, *Pseudomonas aeruginosa* 15159, and *Proteus mirabilis* 4070 isolates, originally obtained from patients with chronic venous ulcers, and the fungus *Candida albicans* 4435 obtained from a patient with atopic eczema, were used in this study.

### Viable count analysis

*E. faecalis*, *P. aeruginosa*, *E. coli*, and *P. mirabilis* were grown to mid-logarithmic phase in Todd-Hewitt medium. Bacteria were washed and diluted in 10 mM Tris pH 7.4 containing 5 mM glucose. Bacteria (50 µL;  $2 \times 10^6$  bacteria per mL) were incubated at 37 °C for 2 h with the synthetic peptide at concentrations in the range 0.03–60 µM. To quantify bactericidal activity, serial dilutions of the incubation mixture were plated on Todd-Hewitt agar, followed by incubation at 37 °C overnight and the number of colony-forming units (cf.u.) was determined.

### Radial diffusion assay

Radial diffusion assays (RDA) were performed essentially as described earlier [18]. Briefly, bacteria (*E. coli*) or fungi

**Table 1.** Cationic peptides analysed.

Protein	Peptide	Sequence <sup>a</sup>	pI	Reference (heparin-binding)
Laminin				
α1	SRN16	SRNLSEIKLLISQARK(2079–2094)	11.0	[43]
α1	SRN29	SRNLSEIKLLISQARKQAASIKVAVSADR(2079–2107)	11.0	[43]
α1	KDF15	KDFLSIELFRGRVKV(2334–2348)	10.0	[44]
α1	SAV15	SAVRKKLSVELSIRT(2714–2728)	11.0	[45,46]
β1	RIQ17	RIQNLLKITNLRIFVKL(202–218)	12.0	[47]
α5	PPP25	PPPPLTSASKAIQVFLGGSRKRVL(2981–3005)	12.0	[48]
α5	LGT25	LGTRLRAQSRQRSRPGRWVKVSVRW(3373–3397)	12.8	[49], this report
α5	RLR22	RLRAQSRQRSRPGRWVKVSVRW(3376–3397)	12.8	[49], this report
α5	PGR11	PGRWHKVSVRW(3387–3397)	12.0	[49]
Fibronectin	QPP18	QPPRARITGYIIKYEKPG(1893–1910)	10.0	[50]
von Willebrand Factor	YIG23	YIGLKDRKRPSELRRIRASQVKYA(565–587)	10.5	[51]
Protein C Inhibitor	SEK20	SEKTLRKWLKMFKKRQLELY(264–283)	10.3	[47]
Vitronectin	AKK15	AKKQRFHRNRKGYR(347–361)	12.2	[47]
Complement Factor C3	LRK26	LRKCCEDGMRENPMRFSCQRRTRFIS(19–44)	9.8	This report
	LGE27	LGEACKKVFLDCCNYITELRRQHARAS(45–71)	8.7	This report
Cardin motifs	AKK24	AKKARAACKARAACKARAACKARA	12.5	[15,20]
	AKK18	AKKARAACKARAACKARA	12.3	[15,20]
	AKK12	AKKARAACKARA	12.0	[15,20]
	AKK6	AKKARA	11.2	[15,20]
	ARK24	ARKKAACAARKKAACAARKKAACA	12.3	[15,20]
	ARK16	ARKKAACAARKKAACA	12.0	[15,20]
	ARK8	ARKKAACA	11.3	[15,20]
Fibrinogen				
β-chain	GHR18	GHRPLDKKREEAPSLRPA(15–32)	10.0	Negative, this report <sup>b</sup>
α-chain	LVT19	LVTSGDKELRTGKEKVTS(414–432)	9.5	Negative, this report
Fibronectin control peptide	KNN15	KNNQKSEPLIGRKKT(1946–1960)	10.5	Negative, this report <sup>c</sup>

<sup>a</sup> Numbers indicate the position of the amino acids in the mature proteins. <sup>b</sup> Binding reported below 0.1 M NaCl for the larger 15–42 peptide, no binding for 18–31 [52]. <sup>c</sup> Weak heparin-binding in a competitive assay [50].

(*C. albicans*) were grown to mid-logarithmic phase in 10 mL full-strength (3% w/v) trypticase soy broth (TSB) (Becton Dickinson). The microorganisms were washed once with 10 mM Tris, pH 7.4 and then  $4 \times 10^6$  bacterial c.f.u. or  $1 \times 10^5$  fungal c.f.u. were added to 5 mL of the underlay agarose gel, consisting of 0.03% (w/v) TSB, 1% (w/v) low-electroendosmosistype (Low-EEO) agarose (Sigma) and a final concentration of 0.02% (v/v) Tween 20 (Sigma). The underlay was poured into an 85-mm Petri dish. After the agarose had solidified, 4 mm-diameter wells were punched and 6  $\mu$ L of test sample was added to each well. Plates were incubated at 37 °C for 3 h to allow diffusion of the peptides. The underlay gel was then covered with 5 mL of molten overlay (6% TSB, 1% Low-EEO agarose in dH<sub>2</sub>O). Antimicrobial activity of a peptide was visualized as a clear zone around each well after 18–24 h of incubation at 37 °C for bacteria and 28 °C for *C. albicans*. For *E. coli* the dose–response characteristics of the RDA was used and the linear relationship between zone diameter and log<sub>10</sub> concentration for LL-37 was determined by least mean squares regression analysis [18]. Synthetic peptides were tested in concentrations of 100  $\mu$ M to determine the antibacterial effect relative to the known peptide LL-37. To minimize variation between experiments, a LL-37 standard (100  $\mu$ M) was included on each plate. The antibacterial activity of the synthetic peptides is presented in LL-37 equivalencies, where the zone inhibition obtained using 100  $\mu$ M is indexed as 1. LL-37 yielded clear inhibition zones at concentrations of 10  $\mu$ M to  $\approx$  1000  $\mu$ M and at higher concentrations the peptide precipitated. Thus, the index expresses the level of antibacterial activity in relative terms [18]. The activities of the peptides are also presented in radial diffusion units (RDU) [(diameter of clear zone in millimetres – well diameter)  $\times$  (10)] [18].

### Heparin-binding assay

The synthetic peptides were tested for heparin-binding activities. Peptides (1, 2 and 5  $\mu$ g) were applied to nitrocellulose membranes (Hybond™-C, Amersham Biosciences). Membranes were blocked (NaCl/P<sub>i</sub> pH 7.4, 3% BSA) for 1 h and incubated with radiolabelled heparin ( $\approx$  10  $\mu$ g·mL<sup>-1</sup>) for 1 h in the same buffer. The radioiodination (<sup>125</sup>I) of heparin was performed as described earlier [19]. Unlabelled heparin (6 mg·mL<sup>-1</sup>) was added for competition of binding. The membranes were washed (3  $\times$  10 min in 10 mM Tris, pH 7.4). A Bas 2000 radioimaging system (Fuji) was used for visualization of radioactivity.

### Electron microscopy

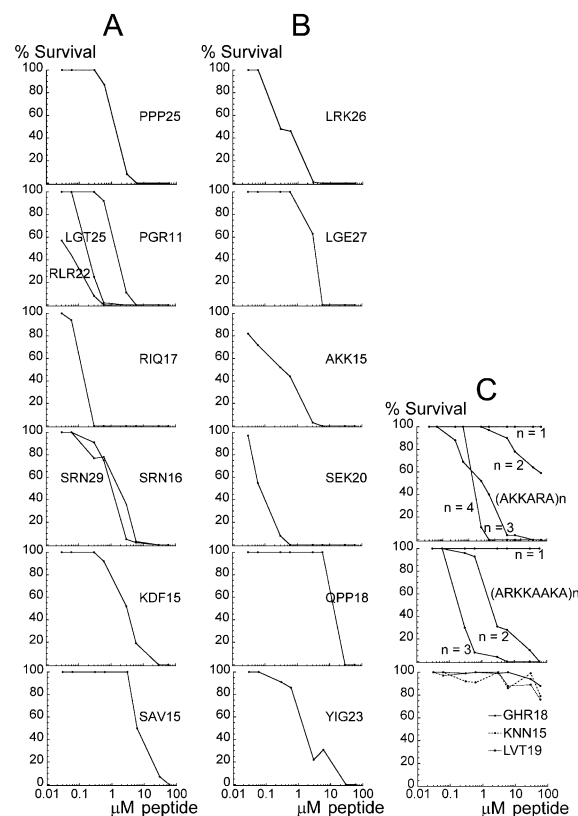
Suspensions of *P. aeruginosa* ( $1.6 \times 10^6$  per sample) were incubated for 2 h at 37 °C with different AMPs at  $\approx$  50% of their required bactericidal concentration (50% lethal dose, LD<sub>50</sub>). Peptides used were LL-37 (0.6  $\mu$ M), ARK24 (0.6  $\mu$ M), SEK20 (0.3  $\mu$ M), AKK24 (3  $\mu$ M), LGT25 (0.3  $\mu$ M). Each sample was gently transferred onto poly L-lysine-coated Nylaflo® (GelmanSciences) nylon membranes. The membranes were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate pH 7.2 for 2 h at 4 °C, and subsequently washed with 0.15 M cacodylate, pH 7.2. They were then postfixed with 1% osmium tetroxide (w/v) and 0.15 M sodium cacodylate, pH 7.2, for 1 h at 4 °C,

washed, and subsequently dehydrated in ethanol and further processed for Epon embedding. Sections were cut with a microtome and mounted on Formvar coated copper grids. The sections were postfixed with uranyl acetate and lead citrate and examined in a Jeol 1200 EX transmission electron microscope operated at 60 kV accelerating voltage.

## Results

### Antimicrobial activities of peptides

A series of cationic peptides (human sequences) of protein segments reported to have affinity for heparin (Table 1) were tested in bactericidal assays against an isolate of the Gram-positive species *E. faecalis*, originally obtained from a patient with a chronic skin ulcer [10]. Peptides of various lengths, derived from the large globular (LG) modules of laminin isoforms that occur in basement membranes of human skin ( $\alpha$ -1 and  $\alpha$ -5 in laminin 1 and 10/11, respectively) killed *E. faecalis* in antibacterial assays (Fig. 1A). The



**Fig. 1. Bactericidal effects of heparin-binding peptides (Table 1) on *E. faecalis*.** *E. faecalis* (isolate 2374;  $2 \times 10^6$  c.f.u.·mL<sup>-1</sup>) was incubated with peptides at concentrations in the range 0.03–60  $\mu$ M in a total volume of 50  $\mu$ L. (A) Synthetic peptides derived from the LG-domain of the laminin  $\alpha$ 5 chain (PPP25, LGT25, RLR22, PGR11), the laminin  $\alpha$ 1 chain (SRN16, SRN29, KDF15, SAV15) and the laminin  $\beta$ 1 chain (RIQ17). (B) Peptides derived from the complement factor C3 (LRK26, LGE27), vitronectin (AKK15), protein C inhibitor (SEK20), fibronectin (QPP18), and the von Willebrand factor (YIG23). (C) Antibacterial effects of heparin-binding consensus sequences (AKKARA)<sub>n</sub> ( $n = 1$ –4), and (ARKKAACA)<sub>n</sub> ( $n = 1$ –3) [15].

concentrations necessary to obtain 100% killing varied. The related peptides LGT25, RLR22, and PGR11 were highly active, and the required concentrations for killing were between 0.6  $\mu\text{M}$  and 3  $\mu\text{M}$ , which was comparable to the activity of LL-37. Similar results were obtained for other laminin-derived peptides (Table 1 and Fig. 1A) as well as heparin-binding peptides derived from human fibronectin (QPP18), von Willebrand factor (YIG23), protein C inhibitor (SEK20), and vitronectin (AKK15) (Fig. 1B). Additional peptides found to be antibacterial were derived from the cationic and heparin-binding amino terminus of complement component C3 (LRK26 and LGE27) (Fig. 1B). Next, we analysed whether heparin-binding consensus regions, the Cardin motifs, were antibacterial (Fig. 1C). Peptides of varying lengths, comprising multiples of AKKARA or ARKKAAKA [15,20] were synthesized and tested in bactericidal assays using *E. faecalis* as the test organism, and the results demonstrate that these peptides are also antibacterial. A correlation between peptide length and antibacterial activity was observed for the Cardin motifs (Fig. 1C), which corresponded well with data on the heparin affinities of these peptides [20]. Analogously, a correlation between peptide length and activity was recorded for the laminin-derived peptides RLR22 and PGR12, the former being more active; this group of peptides had  $\text{LD}_{50}$  values of 0.05–10  $\mu\text{M}$ . In the next series of experiments, peptides were screened for activity against the Gram-negative *E. coli* by using RDAs. A concentration of 100  $\mu\text{M}$  was selected for all peptides to determine the antibacterial level, and the results were presented as RDU [18], or relative to the known peptide LL-37 (indexing LL-37 as 1) [18]. As shown (Table 2), the results from these experiments correspond well with the data obtained from the experiments with *E. faecalis* (Fig. 1). It is of note that, except for LGE27, all of the peptides showing antibacterial activity are more potent against *E. coli* than the classical AMP LL-37 in the low-salt conditions used (Table 2). A typical result is obtained by RDA using a set of highly active peptides (Fig. 2). Interestingly, the fungus *C. albicans* exhibited marked sensitivity to these heparin-binding peptides (including the Cardin motifs), whereas LL-37 exerted little activity (Fig. 2B). Furthermore, in bactericidal assays these peptides also killed wound-derived *P. aeruginosa*, *E. coli*, and *P. mirabilis* isolates (data not shown).

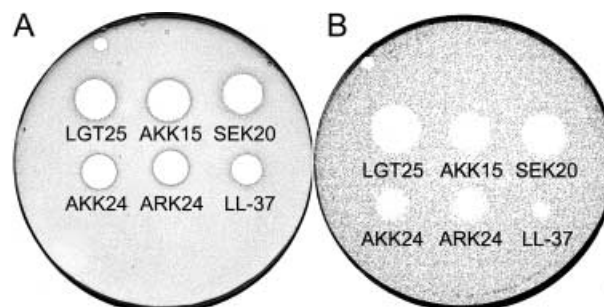
The heparin-binding peptides were investigated for a possible correlation between their pI and activity, but no correlation was detected for the group of AMPs studied (data not shown).

### Analysis of peptide effects by electron microscopy

Electron microscopy analysis of bacteria treated with peptides at  $\approx 50\%$  of the required bactericidal concentrations demonstrated clear differences in the morphology of treated bacteria in comparison with the control (Fig. 3A–F). LL-37 caused local perturbations and breaks along *P. aeruginosa* bacterial cell membranes, and occasionally, intracellular material was found extracellularly. Similar findings were noted after treatment of the bacteria with the peptides ARK24, SEK20 (adopting  $\alpha$ -helical conformations in anisotropic environments [21,22]), AKK24 ( $\beta$ -strand conformation suggested by the Chou-Fasman algorithm [23]), as well as the laminin-derived LGT25 (containing

**Table 2.** Antibacterial activity against *E. coli* obtained by RDA. Activity is expressed in LL-37 equivalences (index = 1 for LL-37); potent peptides have high numbers.

Protein	Peptide	Activity	RDA
hCAP-18	LL-37	1	50
Laminin			
$\alpha 1$	SRN16	10	77
$\alpha 1$	SRN29	6	71
$\alpha 1$	KDF15	4	65
$\alpha 1$	SAV15	9	75
b1	RIQ17	40	93
a5	PPP25	14	81
a5	LGT25	22	85
a5	RLR22	40	92
a5	PGR11	22	86
Fibronectin	QPP18	2	59
von Willebrand factor	YIG23	13	80
Protein C Inhibitor	SEK20	38	92
Vitronectin	AKK15	86	101
Complement Factor C3			
	LRK26	6	70
	LGE27	0,5	40
Cardin motifs			
	AKK24	4	67
	AKK18	3	62
	AKK12	2	56
	AKK6	0	0
	ARK24	8	74
	ARK16	2	55
	ARK8	0	0
Fibrinogen			
$\beta$ -chain	GHR18	0	0
$\alpha$ -chain	LVT19	0	0
Fibronectin	KNN15	0	0

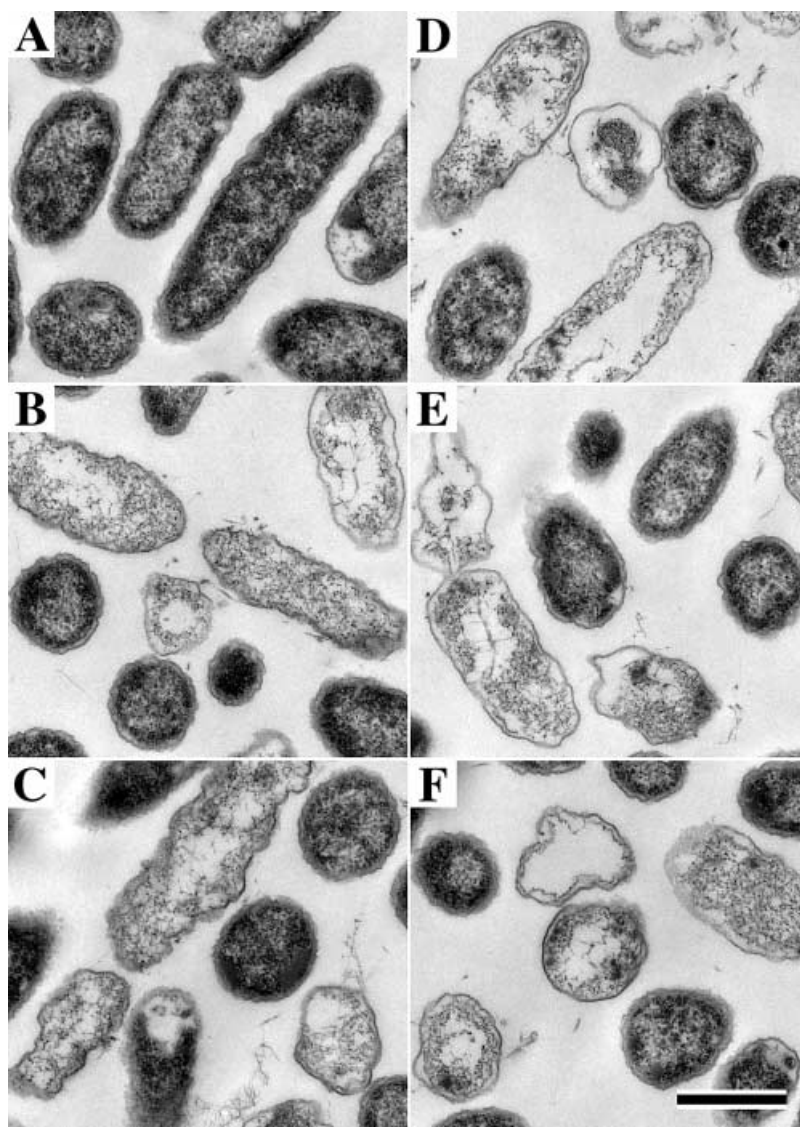


**Fig. 2.** Microbial growth inhibition tests using the RDA. (A) *E. coli* 37.4 and (B) *C. albicans* BM4435. Each 4 mm-diameter well was loaded with 6  $\mu\text{L}$  of 100  $\mu\text{M}$  peptide. The clearance zones correspond to the inhibitory effect of each peptide after incubation at 37  $^{\circ}\text{C}$  (*E. coli*) or 28  $^{\circ}\text{C}$  (*C. albicans*) for 18–24 h.

possible  $\alpha$ -helical and random coil regions as predicted by the GORIV algorithm at [us.expasy.org/tools](http://us.expasy.org/tools) [24]).

### Interaction of peptides with heparin and DS

In order to verify and extend previous studies on the GAG-binding of AMPs, we performed inhibition studies using the

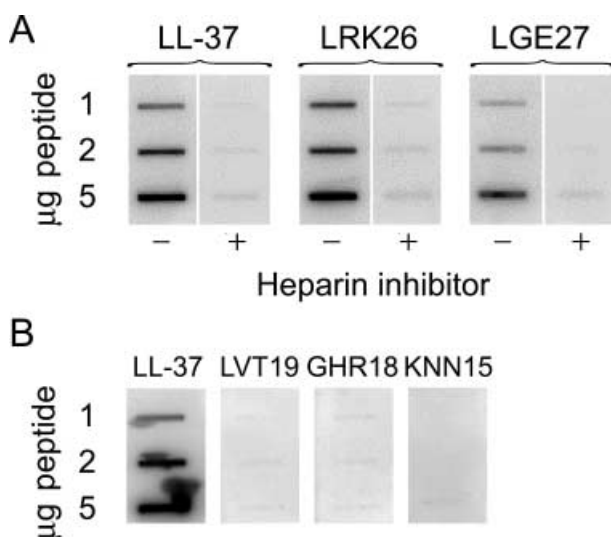


**Fig. 3.** Electron microscopy analysis of *Ps. aeruginosa* subjected to antimicrobial peptides. (A) Control. (B–F) Analysis of bacteria treated with peptides at  $\approx 50\%$  of the bactericidal concentration: (B) LL-37, (C) ARK24, (D) SEK20, (E) AKK24, (F) LGT25. Bar represents 1  $\mu\text{m}$ .

above peptides. Heparin is found exclusively in mast cells and the physiological ligand for various heparin-binding peptides *in vivo* is most likely DS or heparan sulphate. However, for simplicity, the commonly used term 'heparin-binding' is used herein for this peptide family. Thus, we tested the effect of equimolar amounts of DS added to the cationic peptides in RDAs. The antibacterial activity of all peptides was completely inhibited by DS (data not shown), and similar results were obtained with heparin. Thus, these experiments demonstrated, although indirectly, the heparin- (and DS-) binding activities of previously published peptides. The C3-derived cationic peptides LRK26 and LGE27 were selected based on the presence of  $\alpha$ -helical regions [25] and XBBXB motifs (Table 1), and in addition to being inhibited by DS and heparin, these peptides also bound heparin in a binding assay (Fig. 4A). Similar results were obtained using DS (data not shown). The cationic peptides LVT19, GHR18, and KNN15 did not bind heparin in the slot-binding assay (Fig. 4B), and exerted no antibacterial activity against *En. faecalis* and *E. coli* (Fig. 1C and Table 2).

## Discussion

Here we demonstrate that heparin-binding motifs of endogenous proteins exhibit antimicrobial activities. From a structural point of view, it is likely that the correspondence between heparin binding and AMP activity relates to the fact that many of the natural heparin-binding sequences studied so far show prominent amphipathic periodicities [14]. This assumption was substantiated by the fact that consensus heparin-binding peptide sequences [15] exhibited potent antibacterial effects, exerting similar effects on bacterial membranes as endogenous AMPs, such as LL-37. Many helical AMPs display complex and sequential interactions with various bacterial surface components, such as lipopolysaccharides (of Gram-negative bacteria), teichoic acid, peptidoglycans (of Gram-positive bacteria) and at the plasma membrane, phospholipid groups (both groups). The interaction with this anisotropic membrane environment promotes conformational changes, such as formation of an amphipathic helix, which in turn facilitates hydrophobic

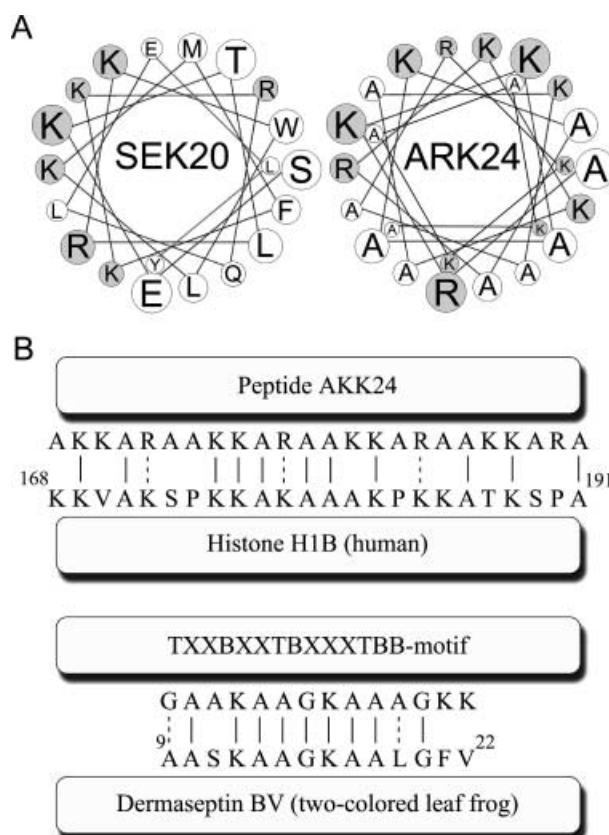


**Fig. 4. Heparin-binding activity of peptides derived from complement factor C3.** (A) Peptides (LRK26 and LGE27; 1–5 µg) were applied to nitrocellulose membranes and incubated in NaCl/P<sub>i</sub> (containing 3% BSA) with iodinated (<sup>125</sup>I) heparin. LL-37 was used as positive control. Unlabelled heparin (6 mg·mL<sup>-1</sup>) inhibited the binding of <sup>125</sup>I-labelled heparin to the C3-derived peptides and LL-37. (B) The peptides LVT19, GHR18, and KNN15 did not bind heparin.

membrane interactions, oligomerization, and finally, membrane destabilization and bacterial inactivation [26]. Interestingly, several heparin-binding peptides, being random in solution, assume  $\alpha$ -helical and amphipathic conformations in anisotropic environments. For example, a protein C inhibitor-derived peptide (SEK20) and the consensus sequence (ARKKAAGA)<sub>3</sub> adopt  $\alpha$ -helical amphipathic structures in presence of heparin [21,22]. A helical wheel representation of the peptides is depicted in Fig. 5A. Thus, secondary structure and ionic as well as nonionic interactions govern antimicrobial activity of a given AMP, in accordance with our finding that no clear correspondence was detected between pI and antimicrobial activity of heparin-binding peptides. Indeed, this observation parallels results obtained by systematic studies demonstrating that nonionic interactions (hydrophobic and hydrogen-bonding) confer both selectivity and specificity to heparin-binding peptides [12,14].

The finding that amphipathic motifs, such as the heparin-binding consensus sequences are antibacterial, prompted us to investigate whether similar motifs were present in endogenous human peptides or proteins (Fig. 5B). Indeed, a BLAST search indicated that regions similar to these peptides were found in numerous histone proteins. Histones normally interact with DNA, and as suggested by the database search, also with heparin. Histone-derived peptides have been identified in the human gastrointestinal tract and in extracts from human neutrophils, and they exert potent antibacterial effects [27,28]. Intriguingly, the heparin-binding motif TXXBXXTBXXXTBB, found in various growth factors [12,14], also occur in the AMP dermaseptin [29] (Fig. 5B).

Our data correspond with results demonstrating antimicrobial activities of angiogenins [30], chemokines [31],



**Fig. 5. Structural motifs of heparin-binding peptides.** (A) Given an  $\alpha$ -helical conformation, a helical wheel projection shows a perfect amphipathic structure of the protein C inhibitor-derived peptide SEK20 (left). A projection of the amphipathic ARK24 [(ARKKAAGA)<sub>3</sub>] is also shown (right). Grey circles represent basic amino acids. (B) A protein BLAST search shows homology between the heparin-binding consensus sequence AKK24 [(AKKARA)<sub>4</sub>] [15] and the reported antibacterial protein human histone H1B [28] (upper). Homology is also found between another heparin-binding motif (TXXBXXTBXXXTBB) [12,14] and the known antibacterial peptide dermaseptin [29] from the two-coloured leaf frog (lower).

and azurocidin [32], which contain heparin-binding regions involving clusters of basic residues [13,32,33]. We believe that improved understanding of the structures of GAG-binding AMPs, may aid in the search for novel endogenous AMPs from complex biological sources. It may also provide a logical rationale for evaluating possible antimicrobial properties of GAG-binding peptides or proteins not yet considered as AMPs. It must be emphasized though, that several important prerequisites, such as generation *in vivo* (proteolytically or *de novo*), activity at high ionic strength or in blood [34], or the intercellular micromilieu, and concentration gradients – all of which determine whether a given AMP will function *in vivo* – were not addressed in this study. Hence, ongoing analyses in our laboratories involve studies of antimicrobial and laminin-containing heparin-binding peptide fractions derived from human basement membranes, as well as characterization of heparin-binding peptides generated by proteolytic cleavage of human complement factor C3 and other plasma proteins.



During recent years it has become increasingly evident, that AMPs act as multifunctional effectors [35]. Their activities include chemotaxis (LL-37, defensins), apoptosis induction (lactoferricin), and angiogenesis (PR-39 and LL-37) [36–38]. In a broader perspective, a similar multifunctionality applies to the group of heparin-binding peptides as a whole. Biological effects of these peptides and proteins include growth stimulus and angiogenesis (various growth factors, angiogenins) [12,39], protease inhibition [40], antiangiogenesis (endostatin) [41], chemotaxis (chemokines, LL-37, defensins, C3a) [13,37,42], and antibacterial effects ('classical' AMPs and additional peptides). It is therefore tempting to speculate that generation of cationic and amphipathic peptides, with affinities for negatively charged eukaryotic cell surfaces and prokaryotic membranes, has promoted cellular communication as well as host defence during evolution.

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