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Temporins, antimicrobial peptides from the European red frog *Rana temporaria*

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A cDNA library from the skin of *Rana temporaria* has been screened using a cDNA fragment probe that encodes the signal peptide of the precursor of esculentin from the skin secretion of *Rana esculenta*. With this approach, the cDNAs encoding the precursors of three peptides were isolated. Subsequently, the peptides predicted from the sequence of the cloned cDNAs as well as several structurally related peptides could be isolated from the skin secretion of *R. temporaria*. These peptides, which were named temporins, have a length of 10–13 residues and show some sequence similarity to hemolytic peptides isolated from *Vespa* venom [Argiolas, A. & Pisano, J. J. (1984) *J. Biol. Chem.* 259, 10106–10111]. Natural and synthetic temporins have antibacterial activity against gram-positive bacteria, but they are not hemolytic. Temporins are the smallest antibacterial peptides hitherto found in nature.

Keywords: antimicrobial peptide; amphibian skin; *Rana temporaria*.

Skin secretions of frogs contain many different types of antibacterial peptides (see Barra and Simmaco, 1995, for a recent review). In particular, a variety of such peptides has been isolated from several *Rana* species. They all contain two cysteine residues close to the C-terminus, which form an intramolecular disulfide bridge. Four different groups of these peptides can be discerned. One is the brevinin 1 family, which includes brevinin 1 from *Rana brevipoda porsa* (Morikawa et al., 1992), brevinin 1E from *Rana esculenta* (Simmaco et al., 1994), ranalexin from *Rana catesbeiana* (Clark et al., 1994) and gaegurins 5 and 6 from *Rana rugosa* (Park et al., 1994). These peptides are composed of 20–24 amino acid residues. In addition to their antibacterial action, brevinin 1E and ranalexin also have high hemolytic activity. A second group consists of the brevinin 2 peptides, which contain 29–34 amino acids. Besides brevinin 2 from *R. brevipoda porsa* (Morikawa et al., 1992), several peptides from *R. esculenta* (Simmaco et al., 1994), the gaegurins 1–3 (Park et al., 1994) and rugosins A and B from *R. rugosa* (Suzuki et al., 1995) belong to this family. A third group consists of the 37-residue peptides esculentin 2 from *R. esculenta* (Simmaco et al., 1994) and gaegurin 4 (Park et al., 1994) and rugosin C from *R. rugosa* (Suzuki et al., 1995). Lastly, a group that consists of esculentin 1 from the skin secretion of *R. esculenta* (Simmaco et al., 1994), a 46-amino-acid peptide that has the highest antibacterial activity of all the *Rana* peptides characterized so far, has also been reported. In addition, this peptide is also active against *Candida albicans*, *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*.

It was also noted that in skin secretions of some of these frogs, such as *Rana erythraea* (Yasuhara et al., 1986) and *R. esculenta* (Simmaco et al., 1990a), short hydrophobic peptides containing only 13 amino acids were present. These were referred to as 'vespa-like' peptides, because of their structural similarity to some peptides present in venom of different wasp species (Miroshnikov et al., 1982; Argiolas and Pisano, 1984). It has been reported that some of these peptides act as chemotactic agents for human leucocytes and also possess hemolytic activity (Yasuhara et al., 1986; Simmaco et al., 1990a). However, it is not known whether they also have antimicrobial activity.

The sequence of the precursors of some antimicrobial peptides from *Rana* species has been determined via cDNA cloning (Simmaco et al., 1994; Clark et al., 1994). Surprisingly, the signal sequences of these precursors were very similar to those of the precursors of peptides with different biological activities isolated from *Phyllomedusa* species. These include the precursors of several opiate peptides (Richter et al., 1987, 1990a) and of the antifungal peptides dermaseptin and adenoregulin (Amiche et al., 1994). We decided to exploit this observation to search for the precursors of new peptides in the skin of *Rana temporaria*, a red frog found in many parts of Central Europe. A cDNA library prepared from the skin of this frog was screened with a DNA fragment encoding the signal peptide of the precursor of esculentin 1 from *R. esculenta*. With this approach, several clones could be isolated with inserts that potentially coded for the precursors of new peptides. Subsequently, peptides predicted from cloned cDNA sequences as well as additional members of this group could be isolated from skin secretion of *R. temporaria*. Some of these peptides, which were termed temporins, were found to have antibacterial activity.

MATERIALS AND METHODS

Enzymes and reagents. Analytical grade chemicals were from Merck, HPLC-grade solvents from Carlo Erba, sequenal-

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Abbreviation. LC, lethal concentration.
Enzyme. Carboxypeptidase Y (EC 3.4.16.5).
Note. The novel amino acid sequence data published here have been deposited with the EMBL nucleotide sequence data bank and are available under accession numbers Y09393, Y09394 and Y09395.
Note. This study is dedicated to Prof. Alessandro Ballio on the occasion of his 75th birthday.

grade chemicals from Perkin Elmer. Media for antimicrobial assays were from Difco; agarose (A6013) was from Sigma. The composition of medium E is 0.8 mM magnesium sulfate, 14 mM citric acid, 57 mM dipotassium hydrogen phosphate and 0.27 mM sodium ammonium hydrogen phosphate (Vogel and Bonnert, 1956). Restriction enzymes and DNA modifying enzymes were from Boehringer Mannheim; deoxyribonucleotides were from Pharmacia. DNA sequences were determined with a Sequenase kit (version 2.0, U.S. Biochemicals) using [α-^{35}S]-dATP[S]. Synthetic peptides were purchased from Tana laboratories (Huston, USA).

Isolation of RNA and cloning procedure. For these studies, the skin of two specimens of *R. temporaria* was used. The isolation of poly(A)-rich RNA by affinity chromatography over oligo(dT)−cellulose and the preparation of the cDNA library were performed according to Richter et al. (1990b).

A cDNA library comprising about 10000 clones was screened with a 240-bp fragment obtained by digestion of the esculentin 1 cDNA with *Hin*dIII (Simmaco et al., 1994). This fragment encodes the prepro region of the esculentin 1 precursor. The probe was labelled by random priming (Boehringer Mannheim). Hybridization was performed at 55°C for 16 h in 100 mM sodium phosphate, pH 7.2, containing 850 mM NaCl, 1 mM EDTA, 10×Denhardt's solution, 0.1% SDS, and 100 µg/ml yeast tRNA. Filter papers (Whatman 541, 11 cm×11 cm) were washed twice for 15 min at 50°C with NaCl/P_i/EDTA (0.3 M NaCl, 20 mM sodium phosphate, 2 mM EDTA, pH 7.4) that included 0.2% SDS. Positive clones were selected and analysed by cleavage with restriction enzymes and nucleotide sequencing.

Northern blot analysis. Poly(A)-rich RNA (5 µg) was fractionated by electrophoresis in 1.2% agarose gels containing 0.8 M formaldehyde (Arrand, 1985) and blotted directly onto Nytran sheets (Schleicher & Schüll). The insert of clone Rt-17 was labelled by random priming and used for probing the Northern blot. Filters were washed at 55°C in 0.1×NaCl/P_i/EDTA/0.1% SDS, and were used for autoradiography.

Collection and purification of skin secretions. Three specimens of *R. temporaria* (30−35 g each) were captured near Salzburg (Austria). They were maintained in a terrarium in our laboratory for 1 year and feed larvae of *Tenebrio molitor.* Skin secretions were collected at intervals of three weeks by a mild electrical shock (12 V, feet to head). The secretion was collected from the surface of a single frog by washing its dorsal region with 10 ml 0.05% (by vol.) acetic acid. The secretions of the three frogs were combined and lyophilized. Suitable aliquots were fractionated by HPLC on a Beckman model 332 system using a reverse-phase column (Aquapore RP-300, 7 mm×250 mm, Applied Biosystems) eluted with a gradient of 10−70% acetonitrile/isopropanol (4:1, by vol.) in 0.2% (by vol.) trifluoroacetic acid, at a flow rate of 1.8 ml/min. Elution of the peptides was monitored on a Beckman 165 spectrophotometer at 220 nm. Peak fractions were collected and lyophilized. A small aliquot of each peak was subjected to N-terminal analysis following derivatization with dansyl chloride and reverse-phase HPLC separation (Simmaco et al., 1990b). Further purification of peptides was achieved using a macroporous C_{18} column (4.6 mm ×150 mm, Supelco) developed with an appropriately modified gradient of the same solvent system as described above.

Structural analysis. Amino acid analyses were performed with a Beckman System Gold analyzer, equipped with an ion-exchange column and ninhydrin derivatization, after vapor phase hydrolysis of the peptides (1−2 nmol) in 6 M HCl for 24 h. Peptide sequences were determined by automated Edman degradation with a Perkin-Elmer model AB476A sequencer. In some cases, information on the amidation state of the C-terminus was confirmed by mass spectral analysis and/or carboxypeptidase Y digestion (Simmaco et al., 1990b).

Antimicrobial assay. The antibacterial activity was tested against *Bacillus megaterium* BM11, *Staphylococcus aureus* Cowan1, *Yersinia pseudotubercolosis*, *Streptococcus pyogenes β* hemolytic group A, *Pseudomonas aeruginosa* ATCC 15692, *Escherichia coli* D21, *E. coli* D21e7, *E. coli* D21f1, *E. coli* D21f2 and *E. coli* D22, using an inhibition zone assay on Luria-Bertani broth/1% agarose plates seeded with 2×10^5 viable bacteria, according to Hultmark et al. (1983).

The antifungal activity was determined on *Candida albicans* ATCC 10261; fresh cell cultures were inoculated in WB broth, pH 6.5, and grown at 37°C to an A_{600} of approximately 0.6. Before plating, cells were diluted 300-fold and incubated overnight at 30°C in the presence of the test peptide.

The concentration of the peptides was established by amino acid analysis. Inhibition zones were measured and the lethal concentration (LC, the lowest concentration that inhibits the growth) was calculated from the diameter of the zones obtained in serial dilutions of the test substance by using the formula given in Hultmark et al. (1983). Values are expressed as the mean of at least five experiments with a divergence of not more than one dilution step.

RESULTS

Analysis of cDNA clones encoding the precursors. A 240-bp *Hin*dIII fragment encoding the signal peptide and the propart of the esculentin 1 precursor was used as a probe to screen the cDNA library prepared from skin of *R. temporaria.* Six positive clones were detected. The sequences of the inserts present in clones Rt-5, Rt-6, and Rt-17 are shown in Fig. 1. Excluding the poly(A) tail, these cDNAs comprise 323, 356, and 329 nucleotides, respectively. After the first methionine codon, they contain open reading frames that can be translated into polypeptides containing 58 (Rt-6) or 61 amino acids (Rt-5 and Rt-17). The deduced sequences all have the typical features of peptide precursors. They start with a signal peptide containing a cluster of hydrophobic residues. The cleavage site for signal peptidase is most likely located after the cysteine residue at position 22 (Von Heijne, 1983). The sequences of the putative mature peptides are preceded by a Lys-Arg, a typical processing site for prohormone convertases. All these precursor polypeptides terminate with the sequence Gly-Lys. Hydrolysis by carboxypeptidases would expose a C-terminal glycine, which is required for the formation of C-terminal amides. The predicted end products would be amidated peptides containing 13 amino acids for clones Rt-5 and Rt-17, while Rt-6 has a 9 bp deletion in this region, and thus codes only for a decapeptide.

Northern blot analysis. In poly(A)-rich RNA from skin of *R. temporaria*, the probe derived from clone Rt-17 recognized an abundant message, detected as a single, rather broad band in the range 400−500 nucleotides. Under the same conditions, no signal could be obtained from the skin of other amphibian species such as *R. esculenta*, *Xenopus laevis* and *Bufo viridis*.

Isolation and analysis of peptides from skin secretion. After electrical stimulation of three specimens of *R. temporaria*, about 20 mg lyophilized material could be obtained. After a preliminary HPLC purification (Fig. 2), each fraction was subjected to N-terminal analysis, to identify those with N-terminal leucine or phenylalanine as predicted from the cDNA sequences. The relevant fractions were further purified by HPLC and subjected to amino acid and sequence analysis. Following this approach, the

Clone Rt-5

```
  1  ACAATTCTGAGCCAACTGAACCACCCGAGCCCAAAGATGTTCACCTTGAAGAAATCCCTG
                                                M  F  T  L  K  K  S  L
 61  TTACTCCTCTTTTTCCTTGGGACCATCAACTTATCTCTCTGTGAGGAAGAGAGAAATGCA
      L  L  L  F  F  L  G  T  I  N  L  S  L  C ↑E  E  E  R  N  A
121  GAAGAAGAAAGAAGAGATGAACCAGATGAAAGGGATGTTCAAGTGGAAAAACGACTTTTA
      E  E  E  R  R  D  E  P  D  E  R  D  V  Q  V  E  K  R  L  L
181  CCAATTGTTGGAAACCTGCTCAAGAGCTTGTTGGGAAAATAACCAAAAATGTTAAGAATG
      P  I  V  G  N  L  L  K  S  L  L  G  K  +
241  GAATTGGAAATCATCTGATGTGGAATATCATTTAGCTAAATGAGCAACAGATGTCTTATT

301  TAAAAAAATAAATATGTTCCATC
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Clone Rt-6

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  1  GCTTTGTAGGATAGACCTGCACTGAAGTCTTCCAGCCGTCTACATTCTGAGCACCAACTG

 61  AACTACCCGAGCCCAAAGATGTTCACCTTGAAGAAATCCCTGTTACTCCTCTTTTTCCTT
                              M  F  T  L  K  K  S  L  L  L  L  F  F  L
121  GGGACCATCAACTTATCTCTCTGTGAGGAAGAGAGAAATGCAGAAGAAGAAAGAAGAGAT
      G  T  I  N  L  S  L  C ↑E  E  E  R  N  A  E  E  E  R  R  D
181  GAACCAGATGAAAGGGATGTTCAAGTGGAAAAACGACTTTCACCAAACCTGCTCAAGAGC
      E  P  D  E  R  D  V  Q  V  E  K  R  L  S  P  N  L  L  K  S
241  TTGTTGGGAAAATAACCAAAAATGTTAAGAATGGAATTGGAAATCATCTGATGTGGAATA
      L  L  G  K  +
301  TCATTTAGCTAAATGCGCAACAGATGTCTTATTTAAAAAATAAATATGTTGCATAC
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Clone Rt-17

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  1  CCCCTCCAGCTGTCTACATTCTCATAACCAACTGAACCACCCGAGCCCAAAGATGTTCAC
                                                            M  F  T
 61  CTTGAAGAAATCCCTCTTACTCCTTTTCTTCCTTGGGACCATCAACTTATCTCTCTGTGA
      L  K  K  S  L  L  L  L  F  F  L  G  T  I  N  L  S  L  C ↑E
121  GGAAGAGAGAGATGCCGATGAAGAAAGAAGAGATGATCTCGAAGAAAGGGATGTTGAAGT
      E  E  R  D  A  D  E  E  R  R  D  D  L  E  E  R  D  V  E  V
181  GGAAAAGCGATTTTTTCCAGTGATTGGAAGGATACTCAATGGTATTTTGGGAAAATAACC
      E  K  R  F  F  P  V  I  G  R  I  L  N  G  I  L  G  K  +
241  AAAAAAAGTTAAAACTTTGGAAATGGAATTGGAAATCATCTAATGTGGAATGTCATTTAG

301  CTAAATGCACATCAAATGTCTTATAAAAA
```

Fig. 1. Nucleotide sequence of the insert present in the clones Rt-5, Rt-6, and Rt-17 and deduced amino acid sequence of the precursor polypeptides. The sequence of the mature peptides is underlined, as is the potential polyadenylation site at the 3′-end. Vertical arrows denote the probable site of cleavage by the signal peptidase.

three predicted peptides were found to be present in the secretion. Other molecules, structurally related to these peptides, were also isolated. The sequences of these peptides, which are termed temporins, are shown in Table 1. In Table 1, the amount of each peptide recovered from the secretion is also included. Along the HPLC profile reported in Fig. 2, the elution position of the various peptides is indicated. The structure of temporin E, with valine at its N-terminus, and which coeluted in part with temporin D, is also shown in the same table. Temporins are all amidated at their C-terminus, as predicted from the structure of the precursors (see above), and contain a prevalence of hydrophobic amino acids. Each of these peptides contains 13 residues, with the exception of temporins H and K, which are 10 residues long. Except for temporins C, D, and E, all of these peptides have at least one basic residue (either lysine or arginine). In the course of this analysis, a 22-residue peptide was also found in the skin secretion (see Table 1). Its sequence shows some similarity with

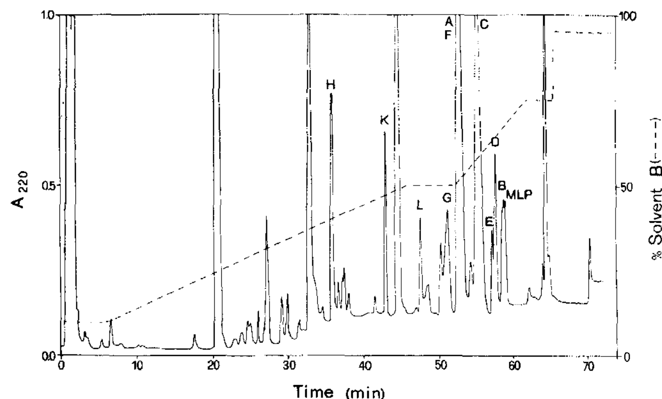

Fig. 2. Reverse-phase HPLC of skin secretion of *R. temporaria*. The elution position of the peptides reported in Table 1 is indicated by the corresponding letters. For details, see the Materials and Methods section.

Table 1. Sequences of *R. temporaria* skin peptides and relative amount in the secretion. Peptides for which the structure of the corresponding precursor has been predicted from cDNAs are marked (*). a indicates an amidated C-terminus. MLP, melittin-like peptide. Identical residues are in bold face. Gaps (−) were inserted to maximize identities.

Peptide	Sequence	Yield
		nmol/mg
Temporin A	FL**P**LI**G**RV**L**SGI**La**	14.5
Temporin B*	LL**P**IV**G**NL**L**KSL**La**	19.4
Temporin C	LL**P**IL**G**NL**L**NGL**La**	37.5
Temporin D	LL**P**IV**G**NL**L**NSL**La**	1.1
Temporin E	VL**P**II**G**NL**L**NSL**La**	1.2
Temporin F	FL**P**LI**G**KV**L**SGI**La**	13.5
Temporin G*	FF**P**VI**G**RI**L**NGI**La**	16.8
Temporin H*	LS**P**---NL**L**KSL**La**	8.7
Temporin K	LL**P**---NL**L**KSL**La**	9.8
Temporin L	FVQWFSKF**L**GRI**La**	3.6
MLP	F**IG**SA**LKVL**AGV**LP**SV**ISW**V**K**----**Qa**	5.1
Melittin	G**IG**AV**LKVL**TTG**LP**AL**ISW**I**K**RKRQ**Qa**	

that of melittin, a hemolytic peptide from bee venom (Habermann, 1972). It was thus named melittin-like peptide.

Assays for biological activity. The antimicrobial activity of the purified temporins was first tested against *B. megaterium* and *E. coli* D21. Temporins A, B, F, G, and L were active on both bacterial strains, whereas temporins C, D, E, H, and K only showed some activity against *B. megaterium*, the most sensitive bacterium.

The recovery of some of the temporins was too low to allow a detailed characterization of their biological properties. To confirm the structure and to obtain more material, temporins A, B, D, and H were chemically synthesised. The antimicrobial activity of synthetic temporins A and B, expressed as lethal concentration values, is reported in Table 2, together with the results obtained on red blood cell lysis. As reference, the activities of esculentin 1 from *R. esculenta* (Simmaco et al., 1994), cecropin from insect hemolymph (Steiner et al., 1981), and melittin from honeybee venom (Habermann, 1972) are also included in Table 2.

Synthetic temporins A and B showed the same activities as their natural counterparts, while both natural and synthetic temporins D and H were found to be without any biological activity of their own or as enhancers of other *Rana* peptides. Temporin

Table 2. Antimicrobial and lytic activity of *R. temporaria* peptides. Lethal concentrations were calculated from inhibition zones on agarose plates seeded with the respective organisms. The data for cecropin are from Hultmark et al. (1983). *S. pyogenes β* hem. group A and *P. aeruginosa* ATCC15692 are clinical isolates kindly provided by Dr Paolo Visca, Institute of Microbiology, University of Rome La Sapienza. n.t., not tested.

Organism and strain	Lethal concentration of					
	temporin A	temporin B	esculentin 1	cecropin A	MLP	melittin
	μM					
B. megaterium Bm11	1.2	2.8	0.1	0.5	n.t.	0.6
S. aureus Cowan1	2.3	6.0	0.4	>200	n.t.	0.2
Y. pseudotubercolosis	2.0	7.0	n.t.	n.t.	n.t.	n.t.
S. pyogenes β hem. group A	2.0	7.0	n.t.	n.t.	n.t.	n.t.
E. coli D21	11.9	21.0	0.2	0.3	n.t.	0.8
P. aeruginosa ATCC15692	>360	>360	0.7	n.t.	n.t.	n.t.
C. albicans	3.4	4.0	0.5	n.t.	n.t.	n.t.
Human red cells	>120	>120	>200	>400	0.5	0.9

Table 3. Antibacterial activity of temporins A and B against *E. coli* D21 and related lipopolysaccharide-modified strains. Assays were performed in Luria-Bertani broth/1% agarose, in the absence or in the presence of medium E (Vogel and Bonner, 1956). Bacterial strains were kindly provided by Prof. H. G. Boman, University of Stockholm.

Compound	Lethal concentration for				
	D21	D21e7	D21f1	D21f2	D22
	μM				
Temporin A	11.9	1.4	0.9	4.8	3.4
Temporin A + medium E	5.3	1.4	3.0	2.0	0.4
Temporin B	21.0	13.2	10.0	3.3	11.2
Temporin B + medium E	3.4	4.2	3.5	9.3	12.2

A is about three times more active than temporin B against *S. aureus* and *S. pyogenes*. In contrast, these two temporins were poorly active against *E. coli* D21 and completely inactive against *P. aeruginosa*. This indicates that temporins A and B act specifically against gram-positive bacteria.

Linear sulfur-free antibacterial peptides like cecropins are inactive against fungi, while the defensins (with three S-S bridges) show antifungal activity. Temporins A and B are active against *C. albicans* and their potency is of the same order as reported for dermaseptin from the South American frog *Phyllomedusa sauvagei* (Mor et al., 1994).

The antibacterial activity of temporins A and B was also tested against three cell-wall defective mutant strains of *E. coli* D21, i.e. D21e7, D21f1, and D21f2, which have lost increasing amounts of sugar residues of their lipopolysaccharide chain (Prehm et al., 1976). Strain D22 has a permeable outer membrane due to a mutation in the *envA* gene (Normark et al., 1969). The activities of the temporins were tested in the absence or in the presence of the basal medium E (Vogel and Bonner, 1956). From the results shown in Table 3, medium E was found to increase the activity of temporins in all strains tested. However, no similar effects were observed with gram-positive bacteria (data not shown). CD spectra showed that the increase in activity was correlated to an increased helix formation, as previously found for FALL-39 (Ageberth et al., 1995). Another likely explanation is that the citrate in the medium (unpublished results with citrate alone) can chelate divalent cations present in the outer membrane and thereby increase the sensitivity to temporins.

DISCUSSION

Cloning of cDNAs encoding the precursors of frog skin peptides yielded an unexpected result. Peptides with different sequences and biological function are sometimes derived from precursors with very similar signal sequences. Examples are opiate peptides/dermaseptins/adenoregulin/esculentin (Richter et al., 1987, 1990a; Amiche et al., 1994; Simmaco et al., 1994) and PGLa/xenopsin/caerulein (Kuchler et al., 1989). In the latter case, it was shown that a separate export exon encodes the signal peptide and part of the proregion.

We have used a cDNA fragment encoding the signal peptide and part of the proregion of esculentin from *R. esculenta* to screen a cDNA library prepared from skin of *R. temporaria*. Using this approach, we could isolate three cDNAs encoding new precursor polypeptides. The signal sequence and the proregion of temporin B and H precursors are identical, while those of temporin G differ in five positions. These precursors contain signal peptides very similar to the esculentin precursor (73% identity), yet the predicted end products are not related to esculentin. Rather, these are new peptides that contain 10 or 13 amino acids. These peptides and several related ones could subsequently be isolated from skin secretions of *R. temporaria*. These are hydrophobic peptides with a free N-terminal group and an amidated C-terminus. Skin secretion of these frogs also contain other peptides, such as bradykinin and some peptides related to the brevinins (Simmaco et al., 1994), but these have not been investigated in detail.

Temporins are related to peptides earlier isolated from wasp venoms (Miroshnikov et al., 1982; Argiolas and Pisano, 1984). Wasp peptides have hemolytic and chemotactic activities. Most of the temporins have antibacterial activity, in particular those that contain a positively charged amino acid and thus a net charge of +2. When the basic residue is replaced by an asparagine (like in temporins C, D, and E), the molecule becomes inactive. The activity is also lost when three residues inside the molecule are deleted, despite the fact that a basic residue is retained (temporins H and K). However, as opposed to the related peptides from wasp venom, the temporins are not hemolytic.

Temporins are the shortest natural antibacterial peptides that have been found to date. Five 13−15-residue cecropin-melittin hybrids were found to be almost equally active as the longer parental molecules (Merrifield et al., 1995). It seems that the minimal requirement for antibacterial activity is 13 residues and a net basic charge is also needed. To obtain specificity, that is to avoid self-damage, there must be other requirements, because a 13-residue helical fragment of seminalplasmin is both antibac-

terial and hemolytic (Sitaram et al., 1995), as also applies to several frog peptides.

Longer linear peptides have in several cases been found to form ionic channels in artificial membranes (reviewed by Boman, 1995). Membrane-spanning peptide channels would require a minimum length of about 23 residues. Thus, the temporins could only form channels if they were in a N-terminal tail-to-tail dimeric form. This is not known so far, but the activities recorded for the mutants of *E. coli* (Table 3) are consistent with a target inside the outer membrane. Additional support for this interpretation comes from the high activity against *C. albicans* and several gram-positive bacteria.

The *in vivo* role of the temporins remains to be elucidated: in particular, the possible synergistic effect of suitable combinations of the various peptide forms should be investigated. It will also be important to investigate the ability of the temporins to control microorganisms that normally interact with *R. temporaria*.

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REFERENCES

Agerberth, B., Gunne, H., Odeberg, J., Kogner, P., Boman, H. G. & Gudmundsson, G. H. (1995) FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis, *Proc. Natl Acad. Sci. USA 92*, 195–199.

Amiche, M., Ducancel, F., Mor, A., Boulain, J. C., Menez, A. & Nicolas, P. (1994) Precursors of vertebrate peptide antibiotics dermaseptin b and adenoregulin have extensive sequence identities with precursors of opioid peptides dermorphin, dermenkephalin and deltorphins, *J. Biol. Chem. 269*, 17847–17852.

Argiolas, A. & Pisano, J. J. (1984) Isolation and characterization of two new peptides, mastoparan C and crabrolin, from the venom of the European hornet, *Vespa crabro*, *J. Biol. Chem. 259*, 10106–10111.

Arrand, J. E. (1985) Preparation of nucleic acid probes, in *Nucleic acid hybridization: a practical approach* (Hames, B. D. & Higgins, S. J., eds) pp. 17–45, IRL Press, Oxford.

Barra, D. & Simmaco, M. (1995) Amphibian skin: a promising resource for antimicrobial peptides, *Trends Biotechnol. 13*, 205–209.

Boman, H. G. (1995) Peptide antibiotics and their role in innate immunity, *Annu. Rev. Immunol. 13*, 61–92.

Clark, D. P., Durell, S., Maloy, W. L. & Zasloff, M. (1994) Ranalexin, a novel antimicrobial peptide from bullfrog (*Rana catesbeiana*) skin, structurally related to the bacterial antibiotic, polymixin, *J. Biol. Chem. 269*, 10849–10855.

Habermann, E. (1972) Bee and wasp venoms, *Science 251*, 1481–1485.

Hultmark, D., Engström, Å., Andersson, K., Steiner, H., Bennich, H. & Boman, H. G. (1983) Insect immunity. Attacin, a family of antibacterial proteins from *Hyalophora cecropia*, *EMBO J. 2*, 571–576.

Kuchler, K., Kreil, G. & Sures, I. (1989) The genes for the frog skin peptides GLa, xenopsin, laevitide and caerulein contain a homologous 'export exon' encoding a signal sequence and part of an amphiphilic peptide, *Eur. J. Biochem. 179*, 281–285.

Merrifield, E. L., Mitchell, S. A., Ubach, J., Boman, H. G., Andreu, D. & Merrifield, R. B. (1995) D-Enantiomers of 15-residue cecropin A-melittin hybrids, *Int. J. Peptide Protein Res. 46*, 214–220.

Miroshnikov, A. I., Snezhkova, L. G., Nazimov, I. V., Rehsetova, I., Rozynov, B. V. & Gushchin, I. S. (1982) *Sov. J. Bioorg. Chem.* (Engl. Transl. *Bioorg. Chim.*) *7*, 787–796.

Mor, A., Hani, K. & Nicolas, P. (1994) The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms, *J. Biol. Chem. 269*, 31635–31641.

Morikawa, N., Hagiwara, K. & Nakajima, T. (1992) Brevinin-1 and brevinin-2, unique antimicrobial peptides from the skin of the frog, *Rana brevipoda porsa*, *Biochem. Biophys. Res. Commun. 189*, 184–190.

Normark, S., Boman, H. G. & Matsson, E. (1969) Mutant of *Escherichia coli* with anomalous cell division and ability to decrease episomally and chromosomally mediated resistance to ampicillin and several antibiotics, *J. Bacteriol. 97*, 1334–1342.

Park, J. M., Jung, J.-E. & Lee, B. J. (1994) Antimicrobial peptides from the skin of a korean frog, *Rana rugosa*, *Biochem. Biophys. Res. Commun. 205*, 948–954.

Prehm, P., Stirm, S., Jann, B., Jann, K. & Boman, H. G. (1976) Cell-wall lipopolysaccharides of ampicillin-resistant mutants of *Escherichia coli* K12, *Eur. J. Biochem. 66*, 369–377.

Richter, K., Egger, R. & Kreil, G. (1987) D-Alanine in the frog skin peptide dermorphin is derived from L-alanine in the precursor, *Science 238*, 198–202.

Richter, K., Egger, R., Negri, L., Severini, C. & Kreil, G. (1990a) cDNAs encoding [D-Ala^2]deltorphin precursors from skin of *Phyllomedusa bicolor* also contains genetic information for three dermorphin-related opioid peptides, *Proc. Natl Acad. Sci. USA 87*, 4836–4839.

Richter, K., Egger, R. & Kreil, G. (1990b) Molecular cloning of a cDNA encoding the bombesin precursor in skin of *Bombina variegata*, *FEBS Lett. 262*, 353–355.

Simmaco, M., De Biase, D., Severini, C., Aita, M., Falconieri Erspamer, G., Barra, D. & Bossa, F. (1990a) Purification and characterization of bioactive peptides from skin extracts of *Rana esculenta*, *Biochim. Biophys. Acta 1033*, 318–323.

Simmaco, M., De Biase, D., Barra, D. & Bossa, F. (1990b) Automated amino acid analysis and determination of amidated residues using pre-column derivatization with dansyl-chloride and reverse-phase high performance liquid chromatography, *J. Chromatogr. 504*, 129–138.

Simmaco, M., Mignogna, G., Barra, D. & Bossa, F. (1994) Antimicrobial peptides from skin secretion of *Rana esculenta*. Molecular cloning of cDNA encoding esculentin and isolation of new active peptides, *J. Biol. Chem. 269*, 11956–11961.

Sitaram, N., Subbalakshmi, C. & Nagaraj, R. (1995) Structural and charge requirements for antimicrobial and hemolytic activity in the peptide PKLLETFLSKWIG, corresponding to the hydrophobic region of the antimicrobial protein bovine seminalplasmine, *Int. J. Peptide Protein Res. 46*, 166–173.

Steiner, H., Hultmark, D., Engström, Å., Bennich, H. & Boman, H. G. (1981) Sequence and specificity of two antibacterial proteins involved in insect immunity, *Nature 292*, 246–248.

Suzuki, S., Ohe, Y., Okubo, T., Kakegawa, T. & Tatemoto, K. (1995) Isolation and characterization of novel antimicrobial peptides, rugosin A, B and C, from the skin of the frog, *Rana rugosa*, *Biochem. Biophys. Res. Commun. 212*, 249–254.

Vogel, H. J. & Bonner, D. M. (1956) Acetylornithinase of *Escherichia coli*: partial purification and some properties, *J. Biol. Chem. 218*, 97–106.

Von Heijne, G. (1983) Patterns of amino acids near signal-sequence cleavage sites, *Eur. J. Biochem. 133*, 17–21.

Yasuhara, T., Nakajima, T., Erspamer, V., Falconieri Erspamer, G., Tukamoto, Y. & Mori, M. (1986) Isolation and sequential analysis of peptides in *Rana erythraea* skin, in *Peptide chemistry 1985* (Kiso, Y., ed.) pp. 363–368, Protein Research Foundation, Osaka.