Anti-infection Peptidomics of Amphibian Skin*S

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Peptidomics and genomics analyses were used to study an anti-infection array of peptides of amphibian skin. 372 cDNA sequences of antimicrobial peptides were characterized from a single individual skin of the frog Odorrana grahami that encode 107 novel antimicrobial peptides. This contribution almost triples the number of currently reported amphibian antimicrobial peptides. The peptides could be organized into 30 divergent groups, including 24 novel groups. The diversity in peptide coding cDNA sequences is, to our knowledge, the most extreme yet described for any animal. The patterns of diversification suggest that point mutations as well as insertion, deletion, and “shuffling” of oligonucleotide sequences were responsible for the diversity. The diversity of antimicrobial peptides may have resulted from the diversity of microorganisms. These diverse peptides exhibited both diverse secondary structure and “host defense” properties. Such extreme antimicrobial peptide diversity in a single amphibian species is amazing. This has led us to reconsider the strong capability of innate immunity and molecular genetics of amphibian ecological diversification and doubt the general opinion that 20–30 different antimicrobial peptides can protect an animal because of the relatively wide specificity of the peptide antibiotics. The antimicrobial mechanisms of O. grahami peptides were investigated. They exerted their antimicrobial functions by various means, including forming lamellar mesosome-like structures, peeling off the cell walls, forming pores, and inducing DNA condensation. With respect to the development of antibiotics, these peptides provide potential new templates to explore further. Molecular & Cellular Proteomics 6:882–894, 2007.

Vertebrate immune system genes exhibit exceptionally high levels of polymorphism that is driven by selective pressure to detect a diversity of quickly evolving pathogens (1–8). Innate immunity uses gene-encoded antimicrobial peptides to form a first line of host defense against noxious microorganisms (4, 9). Granular glands in the skin of anuran amphibians, particularly those belonging to the families Pipidae, Hylidae, Hyperoliidae, Pseudidae, and Ranidae, synthesize and secrete a remarkably diverse array of antimicrobial peptides, 10–50 residues in length, that are released onto the outer layer of the skin to provide an effective and fast acting defense against harmful microorganisms (1, 2).

Extensive studies have been conducted on amphibian antimicrobial peptides of frogs belonging to the genus Rana. Members of the Rana genus comprise more than 250 species and are distributed worldwide except for the polar regions, southern South America, and most of Australia. About 160 antimicrobial peptides have been identified from more than 20 ranid amphibians (10). The ranid frogs synthesize and secrete multiple active components. The representative case is Rana palustris: 22 antimicrobial peptides belonging to eight different families were found in its skin secretions (11). Each of these antimicrobial peptides differs in size, charge, hydrophobicity, conformation, and spectrum of action. Based on these discoveries, it has been suggested that 20–30 different antimicrobial peptides are required to provide ranids with an adequate antimicrobial defense (7).

On the basis of broad structural characteristics, amphibian antimicrobial peptides have been grouped into various families including gaegurins (24–37 residues), brevinin-1 (17–24 residues) and -2 (30–34 residues), ranalexin (20 residues), ranatuerin-1 (25 residues) and -2 (33 residues), esculetin-1 (46 residues) and -2 (37 residues), palustrin (31 residues), japonicin-1 (14 residues) and -2 (21 residues), nigrocin-2 (21 residues), rugosins (33–37 residues), and temporin (10–14 residues) (1, 2, 10). Most amphibian antimicrobial peptides from ranid frogs share a conserved disulfide-bridged heptapeptide segment at the carboxyl-terminal end.

The ranid antimicrobial peptides have a common amino-terminal preproregion, which is highly conserved both intraspecifically and interspecifically, followed by a markedly different carboxyl-terminal domain that corresponds to the mature antimicrobial peptides. The conserved preproregion comprises a hydrophobic signal peptide of 22 residues followed by a 16–25-residue acidic propiece that terminates by a typical...
prohormone processing signal Lys-Arg. The remarkable similarity of preproregions of precursors that give rise to very different antimicrobial peptides in distantly related frog species suggests that the corresponding genes form a multigene family originating from a common ancestor. The diversification of antimicrobial peptide loci potentially might have evolved in response to selective pressure exerted by rapidly evolving microbial pathogens.

In this study, we report the purification and characterization of 107 novel antimicrobial peptides belonging to 30 different families, including 24 novel families from skin of a single individual of the frog Odorrana grahami. 372 different cDNAs encoding these antimicrobial peptides were identified from a skin cDNA library of O. grahami. 40 antimicrobial peptides belonging to the 30 families were synthesized to compare their properties. In addition, we suggest how this diversification occurred based on the nature of the characterized cDNAs.

**EXPERIMENTAL PROCEDURES**

**Collection of Frog Skin Secretions**—Adult specimens of O. grahami of both sexes (n = 30; weight range, 30–40 g) were collected in Yunnan province of China. Skin secretions were collected as follows. Frogs were put into a cylinder container containing a piece of absorbent cotton saturated with anhydrous ether. Following exposure to anhydrous ether for 1–2 min, the frog skin surface was seen to exude copious secretions. Skin secretions were collected by washing the dorsal region of each toad with 0.1 M NaCl solution (containing 0.01 M EDTA). The collected solutions (total volume, 500 ml) were quickly centrifuged, and the supernatants were lyophilized.

**Peptide Purification**—The lyophilized skin secretion sample of O. grahami (3.5 g, total A$_{280}$ nm of 1000) was dissolved in 10 ml of 0.1 M phosphate buffer, pH 6.0, containing 5 mM EDTA. The sample was applied to a Sephadex G-50 (superfine, Amersham Biosciences, 0.1 M phosphate buffer solution, pH 6.0, and purified further by a C$_{18}$ determined as indicated below. The protein peak containing antimi-

**Antimicrobial Bioweapon Array of Peptides**

SMART cDNA Synthesis—Total RNA was extracted using TRIzol (Invitrogen) from the skin of a single sample of O. grahami. cDNA was synthesized by SMART™ techniques by using a SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA). The first strand was synthesized by using cDNA 3’ SMART CDS Primer II A, 5’-AAGCGATGG-TATCAACGGAGTACT(30N)-1N-3’ (n = A, C, G, or T; N = A, G or C), and SMART II An oligonucleotide, 5’-AACAGTGGTATCA-AGCAGATAGGGGG-3’. The second strand was amplified using Advantage polymerase by 5’ PCR primer II A, 5’-AACAGTGGTATCAAGCAGATAGGGGG-3’. Screening of cDNA Encoding Antimicrobial Peptides—The cDNA synthesized by SMART techniques was used as template for PCR to screen the cDNAs encoding antimicrobial peptides. Two oligonucleotide primers, S$_1$ (5’-CCAAAG(C/G)ATGTTCACC(T/A)TGAAGAAA(T/C)-3’), in the sense direction, a specific primer designed according to the signal peptide sequences of antimicrobial peptides from ranid frogs, and primer II A as mentioned under “SMART cDNA Synthesis” in the antisense direction were used in PCRs. The DNA polymerase was Advantage polymerase from Clontech. The PCR conditions were as follows: 2 min at 94 °C followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C, and 40 s at 72 °C. Finally the PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI). DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

Evolutionary Analysis—Sequences were aligned using ClustalW (Version 1.82) (12), and gapped positions were omitted from subsequent analyses. The different domains including signal peptides, pro pieces, and mature peptides were aligned. In pairwise comparisons between nucleotide sequences within antimicrobial peptides from O. grahami, the number of synonymous substitutions per synonymous site ($d_S$) and the number of nonsynonymous substitutions per non-synonymous site ($d_N$) were estimated using the modified Nei-Gojobori model ($p$-distance) (13).

Bioassays—Antimicrobial, hemolytic, and anti-HIV activities were performed according to our previous methods (3). Nitric oxide release was tested as described previously (14) Mast cell degranulation was determined by measuring the release of β-g-glucosaminidase, which co-localizes with histamine, as proposed by Hide et al. (15). Histamine release assay was performed as described by Evans et al. (16).

**Circular Dichroism (CD) Spectroscopy**—CD data were acquired with a Jasco J-810 CD spectrophotometer using a 0.2-mm path length cylindrical cuvette. The response was measured using wavelength from 190 to 250 nm with 0.2-nm step resolution and a 1-nm bandwidth. The rate of 100 nm/min and a response time of 0.25 s were used, and the spectra were averaged over eight scans. Spectra were recorded at a peptide concentration of 70 μM in three different environments: water, 5 mM Tris-HCl (pH 8.0), and 5 mM Tris-HCl containing 160 μM SDS (pH 8.0). The experimental temperature was 25 °C. In each case, the circular dichroism spectrum of the solvent was subtracted from the spectrum of the peptide.

Transmission Electron Microscopy—Transmission electron microscopy was performed to study the possible mechanisms of action of these antimicrobial peptides on Gram-positive bacteria according to the methods described by Friedrich et al. (17) with minor modification. Exponential phase bacteria were treated with the peptides (100 μg/ml) for 30 min at 37 °C. This concentration was used to see an effect on a greater percentage of cells. After treatment, the bacteria were centrifuged at 300 × g for 10 min, and the pellets were fixed with 2.5% buffered glutaraldehyde for 1 h. The cells were then postfixed in 1% osmium tetroxide, washed three times with 1× phosphate buffered saline (pH 7.4), and dehydrated via an ethanol series. The specimens were then critical point dried, sputter-coated with gold-palladium, and examined on a Jeol JEM-2010 transmission electron microscope.

1 The abbreviations used are: RP, reverse phase; HIV, human immunodeficiency virus; CD, circular dichroism; NO, nitric oxide; Od, odorranain; SPD, signal peptide domain; MD, mature domain.
1% buffered osmium tetroxide for 1 h, stained en bloc with 1% uranyl acetate, dehydrated in a graded series of ethanol washes, and embedded in white resin. The buffer used was 0.1M sodium cacodylate, pH 7.4. Thin sections were prepared on copper grids using an LKB-V microtome and stained with 1% uranyl acetate and lead citrate. The resin and grids were purchased from Marivac (Halifax, Nova Scotia, Canada). Microscopy was performed with a JEM1011 microscope under standard operating conditions.

Synthetic Peptides—All of the peptides used for the assay bioactivities and CD analysis in this study were synthesized by a peptide synthesizer (433A, Applied Biosystems) at AC Scientific (Xi An) Inc. (Xi An, China) and analyzed by HPLC and MALDI-TOF mass spectrometry to confirm that the purity was higher than 95%. All peptides were dissolved in water.

RESULTS AND DISCUSSION

Many Antimicrobial Peptides Were Found in the Mixture of the Skin Secretion of O. grahami

The supernatant of O. grahami skin secretions was divided into six peaks by Sephadex G-50 as illustrated in Fig. 1A, and the antimicrobial activity occurred in peaks V and VI. The peaks with antimicrobial activity were applied to an RP-HPLC column, and 58 and 69 fractions were obtained from peak V and peak VI, respectively, as indicated by arrows in B (1–58) and C (1–69). The purified antimicrobial peptides are listed in Table I. Their mass fingerprint results are provided as Supplemental Figs. S1–S127. 56 fractions contained observed molecular weights that approximately matched the calculated molecular weights of 47 antimicrobial peptides predicted from cDNA sequences of O. grahami, respectively (Supplemental Table S).
amino sequences. odorranain-Q2, -L1, -O1, -N1, -M2, and nigrocin-OG10 are not presented because these antimicrobial peptides have no disulfide bonds in their longings to 17 different families (Table I). 13 of them are novel spectrometry. The 21 peptides are antimicrobial peptides be-
sequences by Edman degradation and analyzed by mass
O. grahami 47 antimicrobial peptides predicted from cDNA sequences of tations. 56 fractions contain observed molecular weights that Figs. S1–S127 reveal mass fingerprints of the total 127 frac-
tides. Furthermore the synthetic nigrocin-OG20, odorranain-A1, -B1, -H1, -J1, and -T1 peptides with a cysteine bridge had the same activities (Tables III and Table IV) and the same HPLC elution profiles2 as the native purified peptides. This demonstrated that the native peptides also contain a cysteine bridge like the synthetic peptides. It was noticed that the theoretical average mass values of two peptides (3348.03 Da for odor-
ranain-C6 and 4862.85 Da for esculentin-1-OG3) had a greater than 2.0-Da difference (Table I). The difference might result from isotopic resolution or less than 30-ppm mass accuracy using the instrument described under “Experimental Procedures.” In fact, oxidized odorranain-C6 and esculentin-1-OG3 had theoretical monoisotopic mass values of 3345.83 and 4859.65 Da, respectively, which were closer to their observed mass values (3344.61 and 4860.80 Da) than their average mass values. The mass differences between theoretical and experimental values of the antimicrobial peptides odorranain-A1 and odorranain-J1, −1.83 and −1.56 Da, respectively (Table I), might have been caused by isotopic res-
olution because the synthetic odorranain-A1 and odor-
ranain-J1 had the same mass fingerprints, HPLC profiles,2 and bioactivities (Table III) as the corresponding native pep-
tides. Another cause of all the molecular mass differences observed between theoretical and experimental values might be that the instrument was not well calibrated. Some other bioactive peptides such as bombesin-like peptides, bradyki-
nin-like peptides, and proteinase inhibitors were also identified from the skin secretion.²

### Sequence Diversity

We report 372 sequences (GenBank™ accession numbers DQ672724–DQ673095) of antimicrobial peptides deduced from cDNA sequences. These data increase the known nucleotide sequence diversity of amphibian antimicrobial peptides by 3-fold. cDNAs DQ672765, DQ672772, DQ673073, DQ673046, DQ672959, DQ673008, DQ672745, DQ672746, DQ673034–673039, DQ672792, DQ673055, DQ673023, DQ673024, DQ673042, DQ672916, DQ672793–672799, DQ672802–672805, DQ672875, DQ672940–672949, DQ673025–673033, DQ672723, DQ673076, DQ672729, DQ673052, DQ673053, and DQ672732 correspond to the peptides sequenced. The peptides could be assembled into 30 divergent groups containing 107 novel antimicrobial peptides (Fig. 2, A and B). Six groups of them belong to the antimicrobial peptide families that have been found in other amphibians; thus, they were designated as brevinin-1E-OG, brevinin-2E-OG, esculentin-1-OG, esculentin-2-OG, nigrocin-OG, and palustrin-OG, respectively, where OG is the abbreviation of *O. grahami* (Fig. 2A).

Particularly 23 members belonging to the nigrocin-OG family were found in this study. Only one member of nigrocin antimicrobial peptides has been found in other amphibians (18). The nigrocin-OG family reported here markedly increases the diversity of nigrocin antimicrobial peptides. Some of the peptides found in these families, such as brevinin-1E-OG6, brevinin-2E-OG6, esculentin-1-OG7, and nigrocin-OG10, have obvious size differences and different structural motifs compared with other members.

Extreme diversity of skin peptides could be observed in a single individual. Analysis of skin peptide cDNAs from a
single individual revealed the presence of at least 55 different peptides, representing each of the six families noted above (Fig. 2A).

**Sequence Diversification**

We purified and sequenced only 21 of the 107 peptides represented by the cDNAs we characterized. We suggest, but have not yet proven, that expressed mRNAs for which no peptide has yet been isolated are actually translated into stable peptides. However, to our knowledge, mRNA sequences isolated from frog skin universally are found to be translated into peptides. We assumed (but have not proven) this to be the case for our findings in the skin of *O. grahami*.

In the case of brevinin-1E-OG6, the stop codon (TGA) following the codon encoding cysteine, present in most members of the brevinin-1E-OG family, is mutated into the codon encoding arginine (AGA). The resulting reading frame is extended, creating a peptide of 40 amino acids. In contrast to brevinin-1E-OG6, brevinin-2E-OG6 has a length of 24 amino acid residues shortened from the original length of 33 amino acid residues due to the creation of a premature termination codon because the codon is terminated early. The same situation was seen in esculentin-1-OG7, foreshortened by a premature stop codon to a length of 14 amino acids. In addition, in this case, the truncated peptide has lost the carboxyl-terminal conserved disulfide-bridged heptapeptide segment. In the case of nigrocin-OG10, the mutation in the codon of the second cysteine disrupts the conserved disulfide bridge and extends the reading frame, creating a linear peptide of 28 amino acids rather than the 21-residue molecule containing a single carboxyl-terminal loop. A single point mutation in nigrocin-OG13 destroys the disulfide bridge by changing the second cysteine into an arginine while maintaining the original length of the peptide. An additional 24 peptide families reported here are novel groups (Fig. 2B). Among them, 12 groups contain two cysteine residues that form a disulfide bond. The others are linear peptides. The disulfide bond motif in the groups of odorranain-C, -D, -F, -G, -H, -P1, and -P is similar to that of other antimicrobial peptides, which contain a conserved disulfide-bridged heptapeptide segment at the carboxyl-terminal end. One exception is odorranain-H4 because its disulfide bond motif is changed. Interestingly odorranain-G, composed of 13 amino acid residues, is the shortest antimicrobial peptide containing a disulfide bond found in amphibians. It could be used as a potential template to study the structure-function relationship and design novel antimicrobial peptides.

Odorranain-P1 and odorranain-P2 contain four members. Odorranain-P1a is composed of 24 amino acid residues including a disulfide-bridged heptapeptide segment at its carboxyl-terminal end. Odorranain-P1b is related to odorranain-P1a by an extension of its amino terminus with a 17-residue segment, LKLNWKSSDVENHLAKC, whereas its disulfide-bridged segment is composed of 24 amino acid residues. A similar situation has also occurred in odorranain-P2a and -P2b, involving the splicing of the same 17-residue segment onto nigrocin-OG. In the two groups, domain shuffling and splicing of this 17-residue segment appear to have occurred in addition to further diversity resulting from point mutations within the “original” peptides.

The disulfide bond motifs in the groups of odorranain-A, -B, -J, -T, and -U possess disulfide-bridged segments, which differ in size from the conserved disulfide-bridged heptapeptide segment found in ranid frogs (1, 2). The disulfide-bridged segment in the groups odorranain-A, -J and odorranain-B, -T is composed of 12 and 11 amino acid residues, respectively, and the size of the disulfide-bridged segment in the group odorranain-U is 13 amino acid residues. To our knowledge, these sequences present the most diverse array of disulfide bridge motifs reported to date in a single species of frog; in addition, disulfide bridges composed of 11–13 residues have not yet been described in amphibians.

**Short, Cyclic 16-mers with Diversity**—As to the group odorranain-A, multiple isoforms were found, although these members are small peptides that are just composed of 16 amino acid residues. Only the first site (valine), the third (lysine), and two cysteines are conserved among the sequences. Evolution in this group seems very active. Some other groups such as odorranain-T and -U are also interesting templates or lead structures to design peptide antibiotics. The whole sequences of odorranain-T and -U are nearly in circles connected by disulfide bonds that are similar to some circular antimicrobial peptides.

**Linear Peptides**—Among the 24 novel groups of antimicrobial peptides reported in this study, 12 groups of antimicrobial peptides including odorranain-E, -K, -L, -M, -N, -O, -Q, -R, -S, -V, and -W are linear peptides without a disulfide bridge in their structures. Only a family of linear peptides, temporins, was found in ranid frogs in previous reports. Unexpectedly no temporin-like peptides were found in *O. grahami*. Some of these groups, such as odorranain-K and -I have similarity to esculentin-2-OG. Especially odorranain-K and esculentin-2-OG contain the same amino-terminal segment of GLFTLIK-GAAKLIGKTV. Other parts of odorranain-K and esculentin-2-OG show little similarity. This result suggests that the segment of GLFTLIK-GAAKLIGKTV might be an independent domain in its ancestor gene. By domain shuffling or splicing, multiple antimicrobial peptides are produced. Odorranain-I seems to be the product derived from esculentin-2-OG by deletion of the carboxyl-terminal disulfide-bridged heptapeptide segment. Odorranain-N, -R, -S, and -V are small peptides with 9–14 amino acid residues. Particularly odorranain-N is just composed of 9 amino acid residues with a high density of positive amino acids. Four of the 9 amino acid residues are lysine or arginine. It should be an excellent template to study the structure-function relationship of antimicrobial peptides and to design novel peptide antibiotics. Odorranain-S is char-
characterized by a high density of proline and aromatic amino acid residues in its primary structure. 11 of 12 amino acid residues in odorranain-V are hydrophobic with only one charged amino acid residue (arginine) in this peptide.

**Peptide Precursor Sequence Similarity**

The precursors encoding the 30 groups of antimicrobial peptide from *O. grahami* are aligned in Fig. 3. These antimicrobial peptides are from the precursors that have a common amino-terminal preproregion, which is highly conserved, followed by a markedly different carboxyl-terminal domain that corresponds to the mature antimicrobial peptides. The remarkable similarity of preproregions of precursors that give rise to very different antimicrobial peptides suggests that the corresponding genes form a multigene family originating from a common ancestor. Many gene diversifications to form a multigene family have been found. For example, 180 cDNA sequences of four-loop conotoxins have been reported from two species of the venomous gastropod *Conus* (19). It has been suggested that gene duplication and diversifying selection result in the formation of functionally variable conotoxins that are linked to ecological diversification and evolutionary success of this genus. The skeletons of the gene products are not changed, although they have experienced extensive diversification: all of the *Conus* toxins encoded by these diversification genes contain a “XCCXCCXCCX” cysteine “backbone” (19) where the X represents other residues. In contrast to the *Conus* toxins, the “backbones” in these products of the diversified genes reported in this study are extensively changed. Most of the groups of antimicrobial peptides have lost the cysteine disulfide loop. A similar type of precursor structural diversity is seen in the vertebrate cathelicidin family where the carboxyl-terminal antimicrobial peptides of the cathelicidins can include linear proline/arginine-rich peptides, linear α-helical peptides, and peptides with disulfide bonds, sometimes even from a single species (*i.e.* the pig).

The sequences encoding the antimicrobial peptides of *O. grahami* were divided into two domains in our experiments. The first domain (named SPD in this study) is composed of the signal peptide and propeptide, and the second domain (named MD in this study) is composed of mature peptide. Seven groups of antimicrobial peptides identified from *O. grahami* skin were aligned. Some antimicrobial peptide precursors share the same SPD, for example those belonging to the...
nigrocin-OG antimicrobial peptides in which there are only 13 different SPDs in its 21 members.

Examination of the specific nucleotide changes between two sequences can provide insights as to whether the mutations have been retained as a consequence of positive selection. We analyzed the rate of synonymous ($d_S$) and nonsynonymous ($d_N$) nucleotide substitutions in these different groups of antimicrobial peptides in O. grahami skin. These seven antimicrobial peptide groups displayed different nucleotide substitution patterns as visualized graphically in Fig. 4. In the groups brevinin-1E-OG, esculentin-2-OG, nigrocin-OG, and odorranain-C, most of the $d_N$ values were greater than the $d_S$ values in their MDs, whereas most of the $d_N$ values were equal to the $d_S$ values in their SPDs. The $d_N$ and $d_S$ values are illustrated in Fig. 4, A, D, E, and G, where most of the data points for MDs are above the equivalence 45° line. This implies positive selection in their MDs and neutral selection in their SPDs. Among these groups, the proportions of substitutions in the SPD and MD segments differed considerably from one another. The most substitutions (predominantly synonymous) occurred in SPDs of brevinin-1E-OGs. Nigrocin-OGs contained the greatest number of substitutions within the antimicrobial peptide sequence and were predominantly nonsynonymous. The diversity observed for the brevinin-1E-OG cDNA family appears to be the greatest of those cDNAs characterized. 23 members were found in this group compared with 10 or fewer in other antimicrobial peptide groups from O. grahami skin. Interestingly all the substitutions occurring in MD regions of odorranain-Cs were nonsynonymous, although the substitution rate was very low, implying that the sequence diversification in odorranain-C antimicrobial peptides is highly effective (Fig. 4G).

In contrast to the positive selection that occurred in the MD regions of groups brevinin-1E-OG, esculentin-2-OG, nigrocin-OG, and odorranain-C, the rate of most of $d_N/d_S$ in esculentin-1-OGs and odorranain-As was lower than 1 as illustrated in Fig. 4, C and F, so that most of the data points are lower than the equivalence 45° line. In the odorranain-A family, greater diversification occurred in the SPD regions than within the antimicrobial peptide segment, and all were nonsynonymous (Fig. 4F). The pattern observed in the brevinin-2E-OG group appears to be different from the two patterns described above (Fig. 4B). The rates of synonymous and nonsynonymous substitutions were equal, implying that neutral selection occurred in this group of antimicrobial peptides. However, these sequences are so short and the number of sequences is so small that the significance of this observation remains to be determined.

Secondary Structure Diversity

We studied 21 antimicrobial peptides belonging to 20 different groups. 18 of them are novel antimicrobial families reported in this study. Their secondary structures were examined by CD spectroscopy as listed in Table II. The CD results indicated the following. 1) Nigrocin-OG20, odorranain-F1, and
odorrannain-U1 predominantly adopted a combination of α-helix and β-turn, and their structures were affected by solution environments. 2) Nigrocin-OG13, odorrannain-A1, -B1, -G1, -H1, and -J1 predominantly adopted a combination of β-sheet and random coil in water, and their secondary structures were a combination of α-helix and β-turn in Tris-HCl (pH 8.0) and Tris-HCl-SDS (pH 8.0). 3) Odorrannain-A1 completely adopted a β-turn in water, whereas it adopted a combination of α-helix (25.3%) and random coil (74.7%) in Tris-HCl (pH 8.0). Interestingly its principal secondary structure component is an α-helix (90.8%) in Tris-HCl-SDS (pH 8.0). 4) Other antimicrobial peptides in this study adopted predominantly random structures. Odorrannain-L2, -N1, -Q2, and -S1 adopted fully random secondary structures in different solution environments. Odorrannain-R1 and -O1 exhibited primarily a random structure with some β-turn in three solution environments. The primary secondary structure of odorrannain-T1 in water and Tris-HCl (pH 8.0) was random, but it assumed 100% α-helix in Tris-HCl-SDS (pH 8.0).

Although nigrocin-OG13 and nigrocin-OG20 belong to the same group, the disulfide bridge is absent in nigrocin-OG13 because the second conserved cysteine in nigrocin is replaced by arginine, although its size is not changed. From the CD analysis, nigrocin-OG13 and nigrocin-OG20 adopted different secondary structures, most evident in water. In water, nigrocin-OG13 contained a combination of β-sheet and random coil, whereas nigrocin-OG20 adopted a combination of α-helix and β-turn. Therefore, the gene mutation that changes cysteine not only breaks the disulfide bridge but also changes the molecular conformation in solution.
AntimicrobialBioweaponArrayofPeptides

**TABLE III**

Bioactivities of antimicrobial peptides from *O. grahami*

The concentration of these peptides was 50 μg/ml. MIC, minimal peptide concentration required for total inhibition of cell growth; MCD, mast cell degranulation; RCH, red cell hemolysis; NOR, nitric oxide release; HR, histamine release; *E. coli*, *S. aureus*, *B. subtilis*, *C. albicans*. The percentages represent mean values ± S.D. of three independent experiments performed in duplicate. NA, no detectable activity.

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<td>NA</td>
<td>35.20</td>
</tr>
<tr>
<td>OdO1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>17.7</td>
</tr>
<tr>
<td>OdP1a</td>
<td>3.50</td>
<td>3.50</td>
<td>1.75</td>
<td>6.9</td>
</tr>
<tr>
<td>OdP2a</td>
<td>6.25</td>
<td>3.12</td>
<td>12.5</td>
<td>3.12</td>
</tr>
<tr>
<td>OdQ1</td>
<td>4.68</td>
<td>4.68</td>
<td>1.10</td>
<td>1.10</td>
</tr>
<tr>
<td>OdR1</td>
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<td>NA</td>
<td>NA</td>
<td>50.00</td>
</tr>
<tr>
<td>OdS1</td>
<td>42.50</td>
<td>21.25</td>
<td>85.00</td>
<td>21.25</td>
</tr>
<tr>
<td>OdT1</td>
<td>17.50</td>
<td>35.00</td>
<td>17.50</td>
<td>8.75</td>
</tr>
<tr>
<td>OdU1</td>
<td>&gt;100</td>
<td>75.00</td>
<td>9.37</td>
<td>9.37</td>
</tr>
<tr>
<td>OdV1</td>
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<td>18.00</td>
<td>18.00</td>
<td>9.00</td>
</tr>
<tr>
<td>OdW1</td>
<td>12.60</td>
<td>8.30</td>
<td>6.30</td>
<td>3.15</td>
</tr>
</tbody>
</table>

**TABLE IV**

Bioactivities of nigerocin-OG antimicrobial peptides from *O. grahami*

The concentration of antimicrobial peptide was 50 μg/ml. MIC, minimal peptide concentration required for total inhibition of cell growth in liquid medium; MCD, mast cell degranulation; RCH, red cell hemolysis; NOR, nitric oxide release; HR, histamine release; *E. coli*, *B. subtilis*, *C. albicans*. LPS, lipopolysaccharide. The percentages represent mean values ± S.D. of three independent experiments performed in duplicate. NA, no detectable activity.

<table>
<thead>
<tr>
<th>Antimicrobial activity (MIC)</th>
<th>E. coli</th>
<th>S. aureus</th>
<th>B. subtilis</th>
<th>C. albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/ml</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td><strong>Triton X-100</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nigrocin-OG2</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Nigrocin-OG4 &gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>1.10</td>
</tr>
<tr>
<td>Nigrocin-OG5</td>
<td>9.37</td>
<td>18.75</td>
<td>37.50</td>
<td>4.68</td>
</tr>
<tr>
<td>Nigrocin-OG13</td>
<td>4.68</td>
<td>9.37</td>
<td>18.75</td>
<td>1.10</td>
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<tr>
<td>Nigrocin-OG20</td>
<td>&gt;100</td>
<td>75.00</td>
<td>9.37</td>
<td>9.37</td>
</tr>
<tr>
<td>Nigrocin-OG21</td>
<td>4.68</td>
<td>9.37</td>
<td>18.75</td>
<td>4.68</td>
</tr>
</tbody>
</table>

Immunity, allergy, and inflammation (20–22). In specific IgE-mediated responses, they are activated by antigen to release chemical mediators such as histamine, proteases, prostaglandins, and cytokines, which in turn stimulate a complex local immune response (23–25). Most of the antimicrobial peptides we isolated from the skin of *O. grahami* induced mast cell degranulation. We speculate that the frog utilizes these peptides as an “innate immunity” alternative to the...
IgE-mediated pathway orchestrated by the adaptive immune system to mount a mast cell attack.

The functional significance of structure diversification among these different groups of antimicrobial peptide has been compared as mentioned above. Considering that the sequence diversification is not only present in these different

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**TABLE V**

Synergic effects of antimicrobial peptides from _O. grahami_

MIC, minimal peptide concentration required for total inhibition of cell growth in liquid medium; _E._, _Escherichia_; _B._, _Bacillus_; NA, no detectable activity; SI, selective index.

<table>
<thead>
<tr>
<th>Antimicrobial activity (MIC)</th>
<th><em>E. coli</em></th>
<th><em>S. aureus</em></th>
<th><em>B. subtilis</em></th>
<th><em>C. albicans</em></th>
<th>HIV (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nigrocin-OG13</td>
<td>4.68</td>
<td>9.37</td>
<td>18.75</td>
<td>1.10</td>
<td>NA</td>
</tr>
<tr>
<td>Nigrocin-OG21</td>
<td>4.68</td>
<td>9.37</td>
<td>18.75</td>
<td>4.68</td>
<td>NA</td>
</tr>
<tr>
<td>OdK1</td>
<td>4.68</td>
<td>4.68</td>
<td>1.10</td>
<td>1.10</td>
<td>NA</td>
</tr>
<tr>
<td>Nigrocin-OG13 + OdK1</td>
<td>1.10 + 1.10</td>
<td>4.68 + 4.68</td>
<td>1.10 + 1.10</td>
<td>0.26 + 0.26</td>
<td>7.2</td>
</tr>
<tr>
<td>Nigrocin-OG21 + OdK1</td>
<td>1.10 + 1.10</td>
<td>4.68 + 4.68</td>
<td>1.10 + 1.10</td>
<td>0.26 + 0.26</td>
<td>3.2</td>
</tr>
</tbody>
</table>

* Two kinds of antimicrobial peptides were cultured with microorganisms at the same time.

---

**Fig. 5. Ultrastructure of _S. aureus_ treated with the tested samples (100 µg/ml).** A, odorranain-E1; B, odorranain-C1; C, odorranain-U1; D, odorranain-H1; E, odorranain-K1; F, odorranain-J1; G, odorranain-M3; H, odorranain-P1a; I, brevinin-1E-OG6; J, odorranain-P2a; K, odorranain-G1; L, odorranain-H1.
groups of antimicrobial but is also present within the same group of antimicrobial peptide, we also checked the functional significance of structure diversification present in the same group of nigrocin-OG antimicrobial peptides. Six members belonging to the nigrocin-OG group were tested for their bioactivities as listed in Table IV. They displayed different specificity and potential.

Synergism

Synergy between antimicrobial peptides has been reported (26, 27). We conducted a study to explore the extent of synergy among a few of the peptides from O. grahami skin (Table V). Odorrnanain-K1 synergized with nigrocin-OG13 and nigrocin-OG21 to strengthen their antimicrobial potency. Unexpectedly although individually these antimicrobial peptides had no effect on HIV, in combination they did although with low selectivity. Although we have explored the potential of synergy between the newly discovered peptides to only a very limited extent, synergistic interactions within this diverse family could dramatically expand the potential antimicrobial spectrum of the already large and diverse population.

Variable Antimicrobial Mechanisms

To search for clues to possible mechanisms of action of the antimicrobial peptides on Gram-positive, Staphylococcus aureus ATCC 2592, transmission electron microscopy was performed on thin sections of bacteria that had been treated with the peptide for 30 min according to the methods described by Friedrich et al. (17). 12 families of antimicrobial peptides were tested in the experiments, and they had direct bacteria killing capability as illustrated in Fig. 5 by various mechanisms. Apparently odorrnanain-E1 (OdE1) severely destroyed the cell walls and membranes of bacteria with some mesosomes being found in the treated bacterium (Fig. 5A). The mesosomes likely led to the breaking of the cell wall. Mesosomes must be regarded as being indicative of cytoplasmic membrane alteration (17). Large laminar mesosomes were seen arising from the septa and cell wall of treated bacteria by OdC1, but the cell membrane and wall seemed intact as indicated in Fig. 5B. Multiple mesosomes were found in the bacteria treated by OdU1, and the interface between the cell walls and membranes was blurred, or it had even disappeared in some regions (Fig. 5C). Interestingly both holes and mesosomes were observed in the bacteria treated by Od1 (Fig. 5D). Fibers extending from the cell surface and growth ringlike structures were clearly observed in the OdK1-treated bacteria (Fig. 5E). Fibers and mesosomes were induced by OdJ1, and the cell membranes were severely destroyed as illustrated in Fig. 5F. Large holes were observed in the bacteria treated by OdM3 (Fig. 5G). OdP1a just led to mesosomes in the treated microorganisms as brevinin-1E-OG6 did (Fig. 5, H and I). Some peeling off of the cell walls was induced by OdP1a (Fig. 5J). Some large mesosomes were found near the cell membranes of bacteria treated by OdG1 and OdH1. Furthermore some DNA condensation was found (see Fig. 5, B–D and H–J). These results confirmed that the tested antimicrobial peptides interact with and disrupt the cytoplasmic membrane, cell walls, and DNA, resulting in cell death. It is not clear how antimicrobial peptides actually kill microbes, although many hypotheses have been presented. Our current results demonstrate that different antimicrobial peptides have different strategies to kill microbes.

Summary

372 cDNA sequences of antimicrobial peptides were characterized from a single individual skin of the frog O. grahami that encode 107 novel antimicrobial peptides. This is almost 10% of the number of currently reported antimicrobial peptides. The current discovery demonstrates the strong function of innate immunity and implies that the diversity of antimicrobial peptides may be a response to the diversity of microorganisms.

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