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Magainins, a class of antimicrobial peptides from *Xenopus* skin: Isolation, characterization of two active forms, and partial cDNA sequence of a precursor

(vertebrate peptide antibiotics)

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ABSTRACT **A family of peptides with broad-spectrum antimicrobial activity has been isolated from the skin of the African clawed frog *Xenopus laevis*. It consists of two closely related peptides that are each 23 amino acids and differ by two substitutions. These peptides are water soluble, nonhemolytic at their effective antimicrobial concentrations, and potentially amphiphilic. At low concentrations they inhibit growth of numerous species of bacteria and fungi and induce osmotic lysis of protozoa. The sequence of a partial cDNA of the precursor reveals that both peptides derive from a common larger protein. These peptides appear to represent a previously unrecognized class of vertebrate antimicrobial activities.**

Over the past several years my laboratory has utilized the *Xenopus laevis* oocyte system to study RNA expression in eukaryotes (1–5). Ovaries used in these studies were removed surgically from anesthetized adult females. Incisions were made through both the skin and the nonadherent muscular layer of the abdomen into the peritoneum. After removal of the ovaries, the muscular wall and the skin were separately repaired with sutures. Despite the nonsterile surgical procedure and the microbially contaminated water-filled tanks to which the animals were returned immediately after surgery, it was extremely rare for these surgical wounds to develop infection. Indeed, sutures dissolved after several weeks, and normal healing of the scar almost always occurred. Infections were not seen on the cut margins of the wound, at the sites of suture placement, or within the communicating subdermal space or peritoneum. Healing occurred with little gross evidence of inflammation or cellular reaction at the wound sites. The absence of infection under these conditions was medically remarkable.

The manner in which wound healing occurs in this animal suggested that there might be a "sterilizing" activity in the skin. I report here the characterization of a family of potent antimicrobial peptides purified from female *X. laevis* skin. These peptides may be responsible for the extraordinary freedom from infection characteristic of wound healing in this animal and appear to constitute a previously unrecognized antimicrobial host-defense system.

MATERIALS AND METHODS

Purification. The skin of one adult female *X. laevis* was used for each preparation. All procedures were performed at 4°C. The animal was anesthetized by immersion in 0.1% tricaine for about 15 min, and the skin from the ventral surface including that overlying abdomen, thorax, and legs was surgically removed. The animal was subsequently sacrificed. The skin was homogenized in a chilled blender containing 4 vol (based on initial skin weight) of 0.2 M sodium acetate (pH 4.0), 0.2% Triton X-100/pepstatin A at 50 μg/ml, leupeptin at 50 μg/ml, and 3 mM phenylmethylsulfonyl fluoride added just prior to homogenization (phenylmethylsulfonyl fluoride was dissolved in 100% ethanol, so buffers contained the corresponding ethanol concentrations). The foamy gray homogenate was centrifuged at 20,000 × *g*, the supernatant was removed, and 2 mM phenylmethylsulfonyl fluoride was added. The supernatant then was frozen and stored at −70°C. I have noted that activity tends to increase with storage over the first several hours, suggesting that processing of the activity from a precursor occurs during storage of the crude preparation.

The supernatant was thawed, clarified by centrifugation at 20,000 × *g* for 15 min, and pumped onto a column bed of CM52 (Whatman) equilibrated with 0.2 M sodium acetate (pH 4.0). A bed volume equal to one-half of the supernatant volume was used. The column was washed with 0.2 M sodium acetate (pH 4.0) until the absorbance returned to baseline. The activity was eluted with 0.2 M ammonium acetate (pH 5.1–5.2). The eluted fraction was pooled and lyophilized to dryness. The fraction was resuspended in water and loaded onto a 1.6 × 10 cm column of Bio-Gel P-30 (Bio-Rad) in 0.2 M ammonium formate (pH 4.0). The activity has an apparent molecular weight of ≈7000. The peak of antibacterial activity was pooled and lyophilized. Portions (≈100 μg) were further purified by HPLC on a 0.46 × 25 cm Vydac C_4(214TP54) column (Separations Group, Hesperia, CA); 20 μl of 100% buffer B [70% (vol/vol) acetonitrile, 0.1% trifluoroacetic acid] was added to 50 μl of sample, and the sample was applied to the column and eluted with a gradient of 17.5% (vol/vol) buffer B to 70% (vol/vol) buffer B at 0.5 ml/min for 45 min at 40°C. Samples were dried under vacuum.

The standard antibacterial assay used *Escherichia coli* D31 (11). Bacteria were grown in LB (Luria) broth to an OD_{600} of 0.8, representing 10^9 colony-forming units/ml, and 10^6 bacteria were added to 8 ml of 0.7% agarose in LB broth and poured over a 150-mm Petri dish containing 50 ml of 1.5% agarose in LB broth. Standard LB broth was prepared as described (7). Antibacterial activity was assayed by suppression of bacterial growth dependent on application of fractions to the top agar surface. Other organisms (see text) were assayed in this manner or in liquid culture. For assays in liquid culture, fractions were added to 100 μl of a suspension of the organisms diluted from a midlogarithmic-phase liquid culture to a concentration of 10^5 cells per ml, in standard TSB broth. TSB was prepared from premixed components as described by the manufacturer (Baltimore Biological Laboratory) and adjusted to pH 7.5 with NaOH prior to autoclaving. After incubation at 37°C for 4 hr, OD_{600} was measured.

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Amino acid analysis, sequence determination, and carboxyl-terminal analysis will be described in detail elsewhere but followed standard methods (M.Z., B. Martin, and H. C. Chen, unpublished data).

cDNA Isolation. Based on the amino acid sequence of magainin 2, the following two contiguous nonoverlapping fully degenerate oligonucleotide probes were synthesized (by OCS Laboratories, Denton, TX): 5′ GCYTTNCCRAAYTTYTTNGC 3′ (probe 1) and 5′ RTTCATDATYTCNCCNCCNACRAA 3′ (probe 2); where Y is either thymidine or cytidine; N is adenosine, guanosine, thymidine, or cytidine; R is adenosine or guanosine; and D is adenosine, guanosine, or thymidine. They were labeled with ^{32}P at the 5′ end and used to screen a cDNA library constructed (8) from adult *X. laevis* skin in λgt11 (a generous gift of Klaus Richter, NICHHD). About 4×10^5 phage were screened utilizing duplicate sets of filters prepared from each Petri dish and either probe 1 or probe 2. Hybridization was done in 6× SSC, 5× Denhardt's solution, 0.5% $NaDodSO_4$, yeast tRNA at 200 μg/ml at 45°C for 15 hr, with the probe at 5×10^5 cpm/ml. (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin.) Filters were washed at 50°C in 6× SSC containing 0.5% $NaDodSO_4$, and autoradiographed against Kodak XAR-2 film at −70°C. All plaques that hybridized to both probe pools were selected and subcloned. Phage preparation and phage DNA isolation were as described (7). To subclone cDNA inserts, phage DNA was digested with *Eco*RI, and the unfractionated DNA digest was ligated into *Eco*RI-linearized pGEM1 (Promega Biotec, Madison, WI) and used to transform *E. coli* HB101 (7). Plasmids bearing cDNA inserts were identified by electrophoretic analysis of plasmid miniprep restriction enzyme digests (7). DNA sequencing was performed on minipreps of the appropriate plasmids by the dideoxy chain-termination method (6) utilizing primer oligonucleotides complementary to either the T7 or the SP6 promoter (Promega Biotec).

$NaDodSO_4$/gel electrophoresis was performed using the system of Laemmli (9), and gels were stained with Coomassie brilliant blue R-250.

Protein concentration was measured by the method of Bradford (10) using bovine serum albumin as standard.

RESULTS

Antimicrobial Activity in Skin Extracts. The assay designed to detect antibacterial activity involved inhibition of bacterial growth on an agarose support. The strain utilized was *E. coli* D31, a lipopolysaccharide-defective mutant that is considerably more sensitive to membrane-active antibiotic agents than wild-type *E. coli* (11). Fractions to be assayed were applied directly on the bacterial lawn, and suppression of growth was noted. My initial impression was that *X. laevis* might secrete an antimicrobial substance on its skin along with mucus-rich secretions. However, no such antimicrobial activity could be detected in these secretions (Fig. 1). In contrast, an extract of the ventral skin contained a clearly demonstrable antibacterial activity (Fig. 1). Furthermore, antibacterial activity could be shown directly in the subdermal and peritoneal fluids (Fig. 1).

Purification of the Skin Antibacterial Activity. The crude skin extract was further fractionated by ion-exchange chromatography on carboxymethyl-cellulose. The active fraction was recovered by a step elution (Fig. 1, "CMC pool" square), with an ≈10-fold increase in specific activity. This fraction was concentrated and fractionated further by gel filtration on Bio-Gel P-30 (Fig. 2). The active fractions were recovered (Fig. 2 *Upper*; assay is shown in Fig. 1, squares "10–29"). $NaDodSO_4$/gel electrophoresis of the active fractions revealed small peptides, between 2000 and 3000 in molecular weight (Fig. 2 *Lower*). In addition, the striking disparity between the Coomassie blue staining of these components and low UV absorbance at 280 nm (Fig. 2 *Upper*) suggested that these components lacked tyrosine and tryptophan.

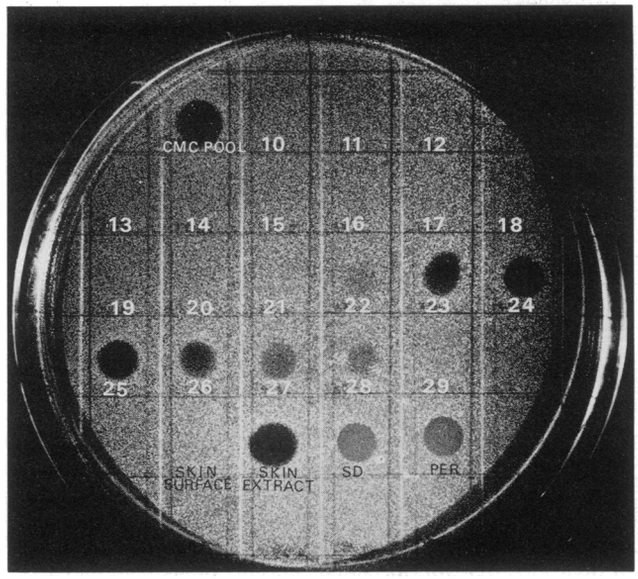

FIG. 1. Antibacterial activity of skin extract. A 10-μl aliquot of each fraction assayed was applied to a freshly poured lawn of *E. coli* D31. Skin extract, crude extract (80 μg). CMC pool, 0.2 M ammonium acetate eluate of the carboxymethyl-cellulose fractionation (10 μg). 10–29, fractions from a Bio-Gel P-30 fractionation of the CMC pool activity, corresponding to the fractions displayed in Fig. 2. Skin surface, mucus-rich secretion scraped from the ventral surface of an adult *X. laevis*. SD and PER, subdermal and peritoneal fluids, respectively (cellular components were removed from these fluids prior to assay by centrifugation at 5000 × *g* for 10 min).

The major peak (corresponding to fractions 23–26, Fig. 2) was pooled, concentrated, and further fractionated by reverse phase HPLC. At least five major components could be resolved in the pooled P-30 fraction. By assay, antibacterial activity coeluted specifically with each component (data not shown).

Primary Sequences of Antimicrobial Peptides. The HPLC fractions were separately analyzed with respect to sequence and composition. A full account of these analyses will be published elsewhere along with the demonstration of the antimicrobial activity of the synthetic peptides (M.Z., B. Martin, and H. C. Chen, unpublished data). The two components with highest specific activity were seen to be related but distinctly different peptides. The primary sequences of the two most active components are shown in Fig. 3. They have been designated "magainins" (derived from the Hebrew word "magain" meaning "shield"), reflecting their possible function as an antimicrobial shield.

A computer search comparing these peptide sequences to all published protein sequences in the GenBank* file revealed no significant homology to any prokaryotic or eukaryotic protein.

Properties of the Magainin Peptides. The antimicrobial spectrum was studied using HPLC-purified fractions. As shown in Table 1, magainin 2 displayed antibiotic activity against numerous Gram-negative and Gram-positive bacteria. A similar spectrum of activity was seen on assay of magainin 1 (data not shown). Only one fungal species,

*EMBL/GenBank Genetic Sequence Database (1985) GenBank (Bolt, Beranek, and Newman Laboratories, Cambridge, MA), Tape Release 38.

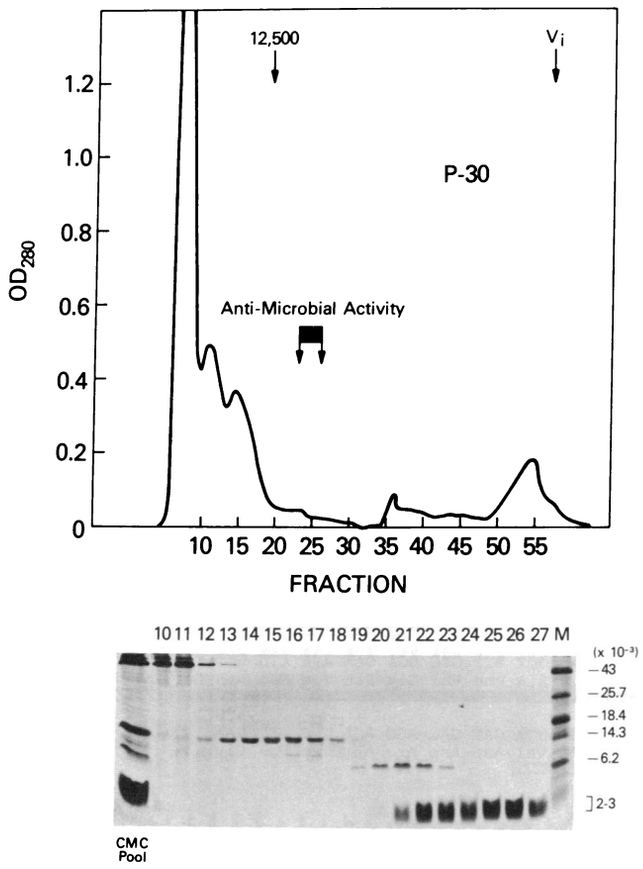

FIG. 2. Fractionation of the CMC pool by gel filtration. (*Upper*) The CMC pool was fractionated on a Bio-Gel P-30 column. Antibacterial activity was localized in the fractions noted based on the assay shown in Fig. 1. Elution volume of cytochrome *c* (12,500) along with the included volume of the bed (Vi) are noted. (*Lower*) A 10-μl aliquot of each fraction (fractions 10–27 are shown) were analyzed by $NaDodSO_4$/gel electrophoresis. Lane M, molecular weight markers (α-chymotrypsin, 25,700; β-lactoglobulin, 18,400; lysozyme, 14,300; bovine trypsin inhibitor, 6200; insulin A and B chains, 2000–3000).

Candida albicans, was tested quantitatively although *Cryptococcus neoformans* and *Saccharomyces cerevisiae* were also shown semiquantitatively to be similarly sensitive. Except for *E. coli* strain D31, all organisms tested (Table 1) were obtained from human clinical specimens (Clinical Microbiology, Clinical Center, National Institutes of Health).

It should be noted that several bacterial species appear to be resistant to magainin 2 (Table 1). Several independent clinical isolates of *Proteus mirabilis* were all found to be resistant, as were several strains of *Proteus morganii* and of *Proteus vulgaris*.

Magainin 2 also appears to be active against protozoa. When *Paramecium caudatum* were exposed to magainin 2 at 10 μg/ml in pond water (or in 1% TSB in distilled water), within several minutes, swelling of the contractile vacuoles was observed. The organism itself began progressively to swell and to subsequently burst. During this process, normal swimming behavior (and, hence, ciliary function) was apparently preserved. Similar effects were noted for other protozoans including *Amoeba proteus* and *Euglena gracilis* (M.Z., unpublished data). These observations suggested that the magainin peptides could perturb membrane functions responsible for osmotic balance in susceptible target organisms.

Table 1. Antimicrobial activity of magainin 2

Organism	Minimal inhibitory concentration, μg/ml
Escherichia coli (D31)	5
Klebsiella pneumoniae	10
Pseudomonas putida	10
Staphylococcus epidermidis	10
Citrobacter freundii	30
Enterobacter cloacae	50
Escherichia coli	50
Staphylococcus aureus	50
Candida albicans	80
Pseudomonas aeruginosa	100
Serratia marcescens	100
Proteus mirabilis	>100
Streptococcus fecalis	>100

The organisms, diluted from a midlogarithmic-phase liquid culture were inoculated into trypticase soy broth (Baltimore Biological Laboratory) to a concentration of 10^5 colony-forming units/ml. Magainin 2 was added to each culture at various concentrations up to 100 μg/ml in increments of 20 μg/ml. Microbial growth was assessed by increase in OD_{600} after 4 hr of incubation. The magainin 2 used was HPLC purified. The fraction assayed was at least 95% homogeneous based on amino acid composition and sequence analysis (M.Z., B. Martin, and H. C. Chen, unpublished data). Small amounts of contaminating acetonitrile and trifluoroacetic acid appear to have no significant effect in these assays since side fractions of the column effluent bracketing magainin 2 were fully inactive against the panel above.

The spectrum of antimicrobial activity exhibited by magainin 2 (Table 1) is very similar to the antimicrobial spectrum noted for the peritoneal and subdermal fluids of the adult *X. laevis* female (unpublished data). Furthermore, the activity responsible, although not yet purified, is similar or identical in molecular weight to the characterized magainins, based on gel filtration (data not shown). Thus, I believe that the species isolated from skin are similar or identical to the corresponding antimicrobial activities of the subdermal and peritoneal fluids, although proof awaits their purification and sequence analysis.

The magainin peptides are bactericidal. In the presence of magainin 2 at 10 μg/ml, *E. coli* D31 lost viability irreversibly (Fig. 4*A*).

The peptide sequence reveals that both magainin species can potentially exhibit large hydrophobic moments (12, 13). If this peptide adopts an α-helical conformation in solution, it should be strongly amphiphilic, exhibiting on one face a hydrophobic surface, and on the other a hydrophilic surface. Indeed, preliminary studies have shown these peptides to be extremely surface active (unpublished data).

Since these amphiphilic peptides may be membrane disruptive (12), magainin 2 was assayed for hemolytic activity against human erythrocytes (Fig. 4*B*). Unlike mellitin, a hemolytic, amphiphilic 26-amino acid peptide from bee

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                5                   10                  15                  20
Magainin 1: Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Gly-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Lys-Ser

                5                   10                  15                  20
Magainin 2: Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser
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FIG. 3. Primary sequences of magainin peptides. Residues that differ between the two peptides are underlined.

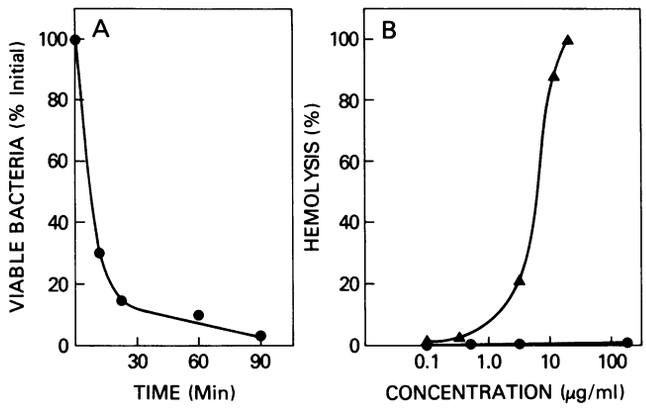

FIG. 4. Properties of magainin 2. (*A*) Bactericidal activity. Magainin 2 was added to *E. coli* D31 (10^5 cells per ml) in LB broth to a final concentration of 10 μg/ml. The suspension was incubated at 37°C, and at times noted aliquots were removed, diluted, and replated for determination of numbers of viable bacteria. (*B*) Hemolytic assay: Either magainin 2 (●) or mellitin (Sigma) (▲) was added to 100 μl of a 10% (vol/vol) suspension of human erythrocytes in phosphate-buffered saline. Samples were incubated with additions at the concentrations noted at 37°C for 10 min, centrifuged at 10,000 × *g* for 10 min to remove cells and debris; hemolysis was determined by measurement of OD_{350} of aliquots of the supernatant. Addition of 0.1% Triton X-100 to a suspension defined 100% hemolysis.

venom (12), magainin 2 was not hemolytic up to at least 150 μg/ml in phosphate-buffered normal saline (Fig. 4*B*). The absence of hemolytic activity of magainin 2 is striking in that it possesses a potential hydrophobic moment very similar to mellitin (12).

Partial cDNA Sequence of Magainin Precursor. To determine the sequence of the precursor of the magainin peptides, a corresponding cDNA was cloned from a λgt11 cDNA library constructed from the poly(A)-containing mRNA of adult *X. laevis* skin. The library was screened with two fully degenerate pools of synthetic oligonucleotides corresponding to amino acids 9–15 and 16–22 of the magainin 2 sequence. Plates were screened using duplicate filters; each filter was hybridized separately with either oligonucleotide pool. Phage that hybridized to both probe pools were considered to be putative positives. Screening 5×10^5 phage yielded ≈100 positive phage. Ten were subsequently purified and shown to have common restriction digest patterns. One phage, containing about 1 kilobase of cDNA insert, was further characterized and subsequently sequenced.

The DNA sequence of a portion of the cDNA cloned is presented in Fig. 5. Since a complete reading frame was not present on the cDNA (the 5′ end of the cDNA does not appear in the clone) I show only the critical portion of the coding region of the mRNA.

The cDNA encodes a 160-amino acid portion of a protein containing three segments bearing magainin sequences. Magainin 1 is encoded between nucleotides 94 and 162, whereas magainin 2 is found between nucleotides 231 and 300 and between nucleotides 370 and 437. From the deduced reading frame each magainin species is bracketed by a putative proteolytic cleavage site (14), an arginine at the amino terminus, and a Lys-Arg dipeptide at the carboxyl terminus. Each of the three magainins is bracketed, furthermore, by a common peptide leader sequence of 6 amino acids (denoted "leader" in Fig. 5) and a common trailer of 7 amino acids (denoted "trailer"). The sequences of the peptides that separate the magainin 2 species are perfectly duplicated. The peptide segment preceding magainin 1 is strikingly similar to the corresponding sequences that precede each of the magainin 2 peptides. Curiously, both the magainin peptides and the peptide sequences that bridge them in the precursor

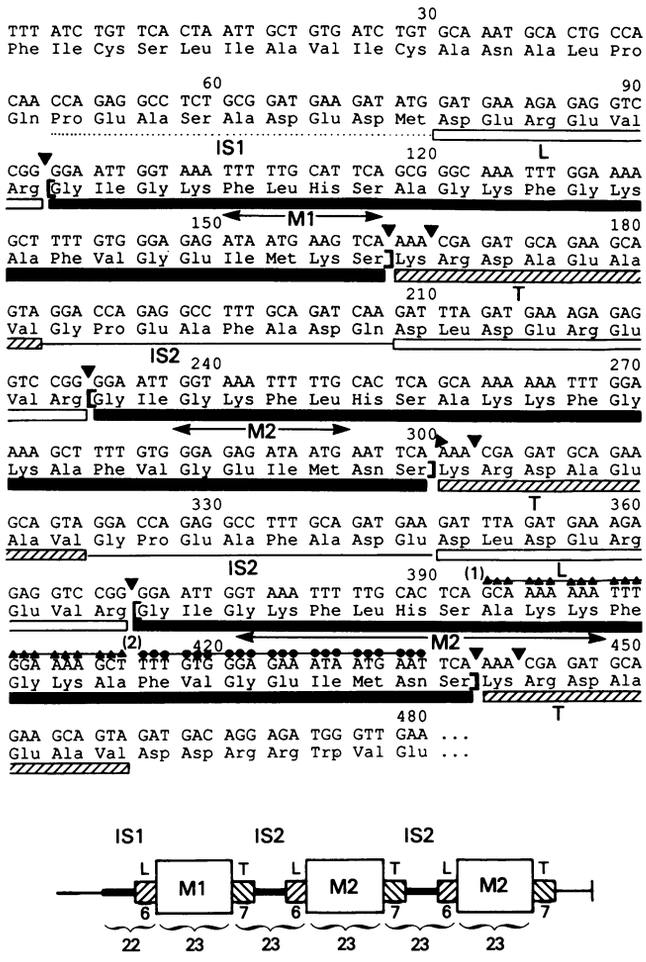

FIG. 5. (*Upper*) Nucleotide sequence of magainin precursor. Magainin sequences are noted between the heavy brackets. Putative proteolytic cleavage sites are noted by the wide arrows. (1) and (2) denote the sequences corresponding to the pools of synthetic oligonucleotides used in screening. L and T denote conserved leader and trailer peptide sequences bracketing magainin segments. IS2 denotes an internal spacer preceding each magainin 2 segment, while IS1 denotes a related segment preceding magainin 1. (*Lower*) A schematic of the peptide organization. Numbers refer to amino acid length of each segment.

are 23 amino acids long. These intervening segments show no relatedness to magainin and do not appear to be amphiphilic. No segment of the sequenced portion of the precursor bears significant homology to a GenBank* sequence.

DISCUSSION

I have described a family of antimicrobial peptides present in the skin of *X. laevis*. On the basis of protein yield, these peptides appear to be major components of *X. laevis* skin; at least 2 mg of magainin was purified from the ventral skin of a single frog [≈1 gm (wet weight)]. The data suggest that magainins are not secreted onto the external surface of the skin, but rather are released within the skin itself and possibly into body fluids that bathe the subdermal space and peritoneum.

The magainin family may be the vertebrate counterpart of the cecropins, a family of amphiphilic, nonhemolytic peptides, 37 amino acids long, which represent a major, inducible, antibacterial defense system of insects (15, 16). The cecropins, initially isolated from the hemolymph of *Cecropia* moths, provide primary antibacterial defense in these invertebrates, which lack both lymphocytes and immunoglobulins

(15). Vertebrate antimicrobial peptides have been identified over the past several years within granule-rich fractions of phagocytic cells (17–19). A peptide family called "defensins" has been isolated from human and rabbit neutrophils and rabbit alveolar macrophages (20, 21). These peptides are between 29 and 34 residues long, are highly conserved between mammalian species, have broad antimicrobial activity, are cystine-rich, and do not display an amphiphilic sequence. They are inactive at physiological ionic strength and are believed to function within the milieu of the phagocytic vacuole (20). They appear to be functionally and structurally distinct from the magainins.

The amphibian skin has been a source from which many biologically active peptides have been isolated (21). Most of these peptides appear to be analogs of hormones active within the gastrointestinal tract or the central nervous system of mammals (21–23). Because of the fundamental protective nature of the magainin peptides, I suspect they will also have closely related analogs in mammals. Candidate locations would be the mammalian gut, which normally serves as a "sheltered" residence to massive numbers of microorganisms and wet epithelial barriers including those exposed to the external environment, such as the mucous membranes of the oral cavity and the respiratory tract. Indeed, cystic fibrosis, a human disorder in which the airway becomes colonized soon after birth by organisms such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (24), may represent a lesion in such a system.

Lastly, the magainin family of peptides, because of their small size and antimicrobial potency, have therapeutic potential in the treatment of bacterial, fungal, and protozoan infections in man.

I thank Dr. Barbara Zasloff for the many thoughtful discussions, her perceptive suggestions, and her continued and persistent encouragement. I thank Dr. Jim Sidbury for his scientific criticism and support. I also thank Dr. Klaus Richter for the *X. laevis* skin cDNA library, Dr. Giuseppe Martini for assisting with the DNA sequence analysis, Dr. Lee Drinkard for participation during the very early phase of this work, and Ms. Colleen Genovese for typing the manuscript.

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