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Molecular Cloning and Chemical Synthesis of a Novel Antibacterial Peptide Derived from Pig Myeloid Cells*

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Margherita Zanetti‡§¶, Paola Storici‡‖, Alessandro Tossi‖, Marco Scocchi‖, and Renato Gennaro§

From the ‡Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie, AREA Science Park, Padriciano, I-34012 Trieste, §Dipartimento di Scienze e Tecnologie Biomediche, Università di Udine, I-33100 Udine, and ‖Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, I-34127 Trieste, Italy

A group of myeloid precursors of defense peptides has recently been shown to have highly homologous N-terminal regions. Using a strategy based on this homology, a novel cDNA was cloned from pig bone marrow RNA and found to encode a 153-residue polypeptide. This comprises a highly conserved region encompassing a 29-residue signal peptide and a 101-residue prosequence, followed by a unique, 23-residue, cationic, C-terminal sequence. A peptide corresponding to this C-terminal sequence was chemically synthesized and shown to exert antimicrobial activity against both Gram positive and negative bacteria at concentrations of 2–16 μM. The activity of this potent and structurally novel antibacterial peptide appears to be mediated by its ability to damage bacterial membranes, as shown by the rapid permeabilization of the inner membrane of *Escherichia coli*.

Leukocytes are a key element in host defense against microbial infections. They act by killing microorganisms intracellularly, through both oxidative and nonoxidative pathways. The oxygen-independent system relies on an arsenal of cationic, granule-associated antimicrobial peptides and proteins, which are discharged into the phagocytic vacuoles concomitantly with the production of toxic oxygen derivatives (1–3). A variety of these peptides have been isolated and shown to exert potent *in vitro* cidal activity (4–8). Among these are the bactericidal/permeability-increasing protein (4), a cyclic dodecapeptide (9), indolicidin (10), a number of small, cysteine-rich peptides classified as defensins (5), β-defensins (11) and protegrins (12), the proline- and arginine-rich peptides Bac5 and Bac7 (13, 14), and the serprocidins (15). These peptides show significant diversity in structure, spectrum of activity, and species distribution.

cDNA and genomic sequences, when known, have shown that these peptides are synthesized in bone marrow cells as precursors (preproproteins) from which the mature peptides are derived by proteolytic processing (5, 15–23).

We have recently reported that the precursors of a number of structurally unrelated antimicrobial peptides share highly homologous N-terminal regions encompassing the signal peptide and the prosequence (17–21). The common prosequence is homologous to a polypeptide from porcine leukocytes, termed cathelin (24). A group of defense peptide precursors thus exists, in which a structurally highly varied C-terminal region, displaying antimicrobial (9, 10, 12, 13) and/or lipopolysaccharide binding activity (25) after processing, is attached to a highly conserved N-terminal region.

mRNA sequences encoding proteins with these features have been identified in bovine (17–19), porcine (20, 21), and rabbit (25, 26) bone marrow cells, suggesting that further unknown defense peptide precursors of this type might exist. In view of the high conservation of the mRNA 5′-sequence, a study was thus undertaken to identify mRNA sequences encoding the precursors of such novel antimicrobial peptides in pig myeloid cells. A similar approach was followed by Jones and Bevins (27) to obtain the cDNA of human defensin-5 from Paneth cells by exploiting the high conservation of mRNA sequences in the 5′-region of rabbit and human defensin precursors.

By using a molecular biological approach based on amplification of cDNA ends containing the conserved proregion homologous to cathelin, we were able to clone several porcine myeloid cDNAs coding for the precursors of potential antimicrobial peptides.

In this paper we report the cloning of one such cDNA encoding a putative antimicrobial peptide precursor with a unique, highly cationic, 23-residue-long C-terminal sequence. A peptide corresponding to this sequence was chemically synthesized and shown to possess a potent *in vitro* antimicrobial activity against Gram-positive and -negative bacteria. This peptide was termed PMAP-23, from "porcine myeloid antibacterial peptide" of 23 residues.

EXPERIMENTAL PROCEDURES

cDNA Cloning and Sequencing—Total RNA was extracted from pig bone marrow cells with guanidinium thiocyanate (28). The reagents and general methodology used to obtain the 3′-end cDNA were as previously described (18). In particular, reverse transcription was performed using the primer adaptor 5′-TCGGATCCCTCGAGAAGC(T18)-3′. Amplification was done with the antisense primer adaptor 5′-CGAGCTCGGATCCCTCGAGAAGCTT-3′ and a sense oligonucleotide 5′-CGCGAATTCTGTGAGCTTCAGGGTG-3′. The latter oligonucleotide was derived from a highly conserved sequence of the proregion of the Bac5 precursor cDNA (18), also present in other antimicrobial peptide precursors (17, 19–21). The experimental conditions to obtain the 5′-end cDNA were as described (21). Briefly, the 5′-region of PMAP-23 precursor cDNA was obtained by reverse transcription of porcine bone marrow mRNA with the antisense oligonucleotide primer 5′-TCTGACCCATACAGTCACAAAT-3′ complementary to nucleotides 438–459 of the 3′-end (Fig. 1), internal to the unique antibiotic domain of the PMAP-23 precursor. Amplification was performed using the sense oligonucleotide primer 5′-ACCGAATTCACCTGGGCACCATG-3′ from the highly conserved 5′-end (including nucleotides −13 to +3, Fig. 1) and an antisense oligonucleotide 5′-CCGAATTCCGACGTACTCTCCACAGCAG-3′ derived from nucleotides 403–423 in the antibiotic domain. The amplified cDNA was cloned in Bluescript SK+ vector (Stratagene, San Diego, CA) and sequenced as previously described (18).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L26053.

¶ To whom correspondence should be addressed: Laboratorio Nazionale Consorzio Interuniversitario Biotecnologie (CIB), AREA Science Park, Padriciano, 99, I-34012 Trieste, Italy. Fax: 39-40-398985.

Sequence Analyses—DNA sequence analysis was conducted with the aid of the IG suite version 5.4 (IntelliGenetics Inc., Mountain View, CA). Homology searches were carried out on the Swiss-Prot data base using the FastDB and Genalign programs. Prediction of the secondary structure of the peptide was obtained using the "PeptideStructure" and "Pep" programs in the GCG version 7 (Genetics Computer Group Inc., Madison, WI) and IG suites, respectively.

Northern and Primer Extension Analyses—Northern and primer extension analyses were performed as described (17). A synthetic antisense oligonucleotide 5′-TCTGACCCATACAGTCACAAAT-3′, ^{32}P-labeled using standard protocols, was used to hybridize a Northern blot of pig bone marrow total RNA and for primer extension analysis of PMAP-23 mRNA.

Peptide Synthesis—A Milligen 9050 synthesizer loaded with Fmoc-Arg[1] substituted PEG-PS resin (0.1 mmol) (Milligen, Bedford, MA) was used for solid-phase synthesis. For each coupling step, 0.8 mmol each of Fmoc-protected amino acid (Milligen or Novabiochem, Laufelfingen, Switzerland), *N*-hydroxybenzotriazole (Aldrich-Chemie, Steinheim, Germany), and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (Novabiochem) were used. Side chains were protected as follows: trityl (Gln), *t*-butyl (Glu, Asp, Thr), *t*-butyloxycarbonyl (Lys, Trp), and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Arg). Deprotection and cleavage from the resin were carried out using a mixture of 90% trifluoroacetic acid, 2% each of phenol, thioanisole, ethanedithiol, and triisopropylsilane, and 1% each of methanol and water. The peptide was then repeatedly extracted with ether and purified by reverse phase HPLC on a C18 column (Delta-Pak, Waters, Bedford, MA), using a 0–60% acetonitrile gradient in 0.05% trifluoroacetic acid.

Analytical Assays—The peptide concentration was determined from tryptophan absorbance (29), the amino acid analysis was performed using the Pico-Tag system (Waters) (30), and the molecular mass was determined with an API III ion spray mass spectrometer (PE SCIEX, Toronto, Canada). Circular dichroism was carried out on a Jasco J-600 spectropolarimeter with a cell path length of 2 mm. Peptide samples (0.1 mg/ml) in 5 mM sodium phosphate buffer, pH 7.0, containing 0–45% (v/v) trifluoroethanol were used.

Antibacterial and Membrane Permeabilization Activities—The minimal inhibitory concentration of the purified peptide was determined as previously described (13) against *Escherichia coli* ML-35 and ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus megaterium* (local isolate), and *Staphylococcus aureus* ATCC 25923 strains. Permeabilization of the inner membrane of the lactose permease-deficient, β-galactosidase-constitutive *E. coli* ML-35 strain was evaluated as reported (31) by following the unmasking of β-galactosidase. Total β-galactosidase activity was determined with bacteria lysed by ultrasonication. Hemolytic activity was determined as described (32) by using human erythrocytes prepared from fresh, anticoagulated blood. Zero (blank) and 100% hemolysis were evaluated in the absence of additives and the presence of 0.2% Triton X-100, respectively, while melittin (Sigma) was used as a positive control.

RESULTS AND DISCUSSION

A search was carried out for porcine bone marrow transcripts with sequence homology with those previously described for bovine (17–19) and rabbit (25, 26) defense peptide precursors. An approach based on polymerase chain reaction amplification of cDNA ends, using oligonucleotide primers derived from the 5′-region that is common to all these precursors, allowed the identification of several novel cDNA sequences. Two of these were found to encode the precursors of the antibacterial peptides PR-39 (20) and protegrin PG-2 (21), respectively, whereas a third encoded the putative precursor of a novel peptide, termed PMAP-23, with potential antibacterial activity. The sequence of this cDNA, shown in Fig. 1, was obtained from two polymerase chain reaction-generated, overlapping clones extending from nucleotide −13 to +423 (5′-end cDNA) and from nucleotide +224 to the polyadenylated tail (3′-end cDNA). A corresponding transcript of approximately 0.7 kilobase (not shown) was detected by probing a Northern blot of pig bone marrow total RNA with an antisense oligonucleotide derived

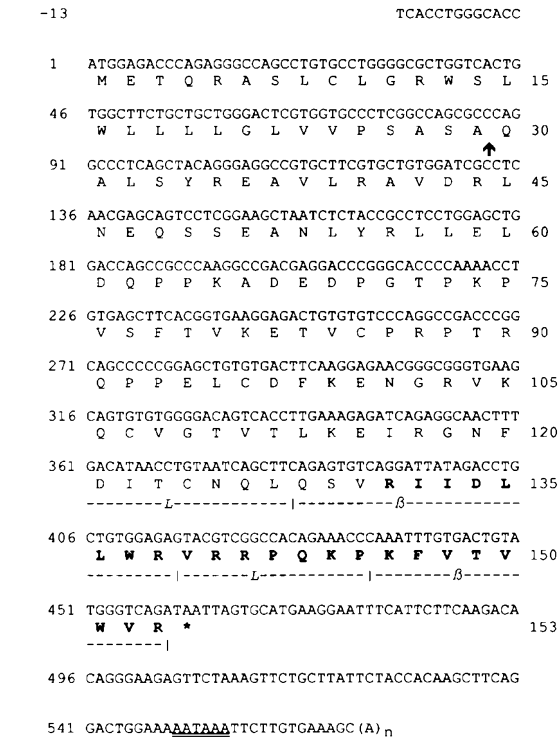

FIG. 1. **Nucleotide and deduced amino acid sequences and secondary structure prediction for PMAP-23.** Numbering is on the *left* for nucleotides and on the *right* for amino acids. The end of the putative signal sequence is indicated by an *arrow*, the stop codon by an *asterisk*, and the polyadenylation signal by a *double underline*. The sequence of the putative antibacterial peptide is shown in *boldface*. The position of the predicted β-sheet (β) and loop (*L*) conformations is also shown.

from a unique 3′-end sequence complementary to nucleotides 438–459 in Fig. 1. A short 5′-untranslated region of approximately 16 nucleotides was indicated by primer extension analysis of the mRNA.

Nucleotide sequence analysis predicted an open reading frame of 153 codons corresponding to a protein of 17,487 Da, with a calculated pI of 9.62. An extended N-terminal region of this putative protein (Fig. 1) was found to have all the structural features common to previously characterized porcine (20, 21) and bovine (17–19) defense peptide precursors with cathelin-like proregions. A comparison of this protein with the homologous porcine preproPG-2, preproPR-39, and cathelin is shown in Fig. 2. The conserved region includes a 29-residue-long putative signal peptide and a 101-residue-long prosequence that is 83% homologous to cathelin (24). A 23-residue C-terminal sequence follows this domain immediately after a valyl residue at position 130. This residue is conserved in most of the previously characterized members of this precursor group (Fig. 2 and Refs. 17, 18, 20, 21) and is likely to be the proteolytic cleavage site responsible for the release of active peptide (17, 18, 20, 21, 23). The 23-residue C-terminal sequence did not reveal any significant similarity to catalogued sequences in the Swiss Prot data base.

This sequence is highly cationic, containing 5 arginines and 2 lysines and only 1 negatively charged residue. The sequence also contains 11 highly hydrophobic residues, including 2 tryptophans. This suggests some form of amphipathic structure, which is typical of antibacterial peptides, but the presence of 2 prolines in the central region of the sequence precludes an extended α-helical conformation. Secondary structure prediction analysis indicated that PMAP-23 may consist of an antiparallel β-sheet connected by a loop in the region of residues 9–15, thus adopting a hairpin-like structure. This does not

[1] The abbreviations used are: Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HPLC, high pressure liquid chromatography.

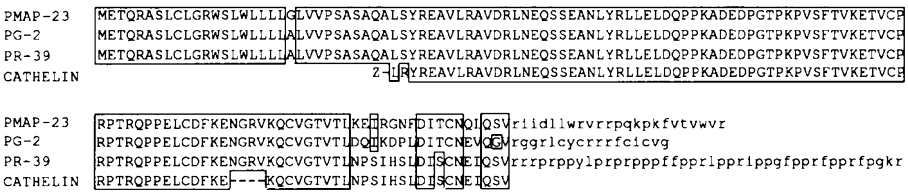

FIG. 2. **Alignment of PMAP-23 with PG-2, PR-39, and cathelin.** The amino acid sequence of preproPMAP-23, deduced from cDNA, is aligned with the sequences of preproPG-2 (21) and preproPR-39 (20), also deduced from pig myeloid cDNA, and that of cathelin determined by Edman degradation (24). *Boxed* residues reflect those common to preproPMAP-23 and at least one other polypeptide.

resemble the α-helical (33, 34) or disulfide bond-stabilized β-sheet (35) structures already found for antimicrobial peptides.

The peptide PMAP-23 (RIIDLLWRVRRPQKPKFVTVWVR) corresponding to the C-terminal residues 131–153 in Fig. 1 was synthesized by the automated solid-phase method and purified to homogeneity by HPLC. The peptide was shown to be correct by mass determination (2962.5 Da *versus* the calculated mass of 2962.7 Da) and amino acid analysis (not shown).

To test the predicted structure of synthetic PMAP-23, circular dichroism spectra were recorded in an aqueous environment and in the presence of trifluoroethanol, an organic solvent that mimics the membrane environment. The spectrum in aqueous buffer resembled that of a random coil peptide, but a considerable change was observed at 30% or more trifluoroethanol (not shown). The interpretation of these spectra and structural analysis were complicated by the presence of the two tryptophan residues, and no predominant conformation was evident. The spectra, however, did not preclude the presence of the predicted β-sheet and coil conformations. Attempts will be made to obtain the solution structure of PMAP-23 by NMR spectroscopy, as the synthetic approach allows the facile production of pure peptide in the amounts required for this technique.

PMAP-23 displays a remarkable *in vitro* antibacterial activity against several species of bacteria when tested by the minimal inhibitory concentration assay (Table I). Gram-negative (*E. coli* and *S. typhimurium*) and Gram-positive (*S. aureus* and *B. megaterium*) bacteria were equally susceptible to PMAP-23 at concentrations ranging from 2 to 8 μM. Even the growth of *P. aeruginosa*, a Gram-negative species often resistant to antimicrobial peptides, was suppressed at 16 μM.

The predicted amphipathic nature of PMAP-23 suggested that its activity might be related to bacterial membrane damage. Accordingly, permeabilization experiments were performed on the *E. coli* ML-35 strain, and a rapid, PMAP-23-induced permeabilization of the bacterial inner membrane was observed (Fig. 3). The kinetics of permeabilization was measured after addition of 1 and 10 μM peptide, and a steady state was reached at 9 and 7 min, respectively, after a lag time of about 2 min. This kinetics is comparable with that of the proline- and arginine-rich Bac5 and Bac7 (31) and considerably faster than that observed for human defensins (36), measured on similar systems. Conversely, the peptide failed to lyse human erythrocytes even at a concentration of 100 μM, suggesting a certain degree of target membrane specificity.

Our results thus clearly show that synthetic PMAP-23 is a potent, membrane-active antibacterial agent and suggest that a natural PMAP-23 derived from the precursor here described is a component of the leukocyte defense system.

The combined molecular biological/chemical synthesis approach we have devised has proved a powerful tool in rapidly identifying this new antibacterial peptide precursor with a cathelin-like prosequence (17–21, 25, 26). The usefulness of this approach is further supported by the identification of two additional cDNAs belonging to this group of precursors and encoding two novel antibacterial peptides.[2] The same strategy may thus be applied in determining how extensively this protein family is represented in different animal species.

TABLE I
Antibacterial activity of PMAP-23

The minimal inhibitory concentration (MIC) was defined as the lowest PMAP-23 concentration that prevented visible bacterial growth after 18 h of incubation with approximately 1.5×10^5 colony forming units/ml at 37 °C in Mueller-Hinton broth (13). These results are the mean of at least five independent determinations with a divergence of not more than one minimal inhibitory concentration value.

Organism and strain	MIC
	μM
E. coli ML35	2
E. coli ATCC 25922	4
S. typhimurium ATCC 14028	8
P. aeruginosa ATCC 27853	16
B. megaterium (local isolate)	2
S. aureus ATCC 25923	4

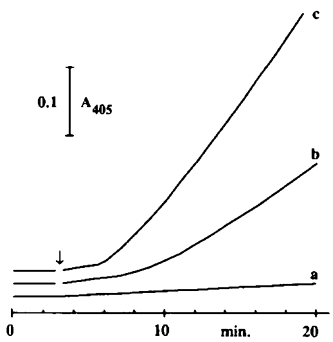

FIG. 3. **Permeabilization of *E. coli* ML-35 inner membrane by PMAP-23.** Permeabilization is determined by following the unmasking of cytoplasmic β-galactosidase activity spectrophotometrically at 405 nm. Bacteria (about 10^7/ml) were suspended in 10 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 1.5 mM substrate (30). *Trace a*, untreated bacteria; *traces b* and *c*, 1 and 10 μM PMAP-23, respectively. The *arrow* indicates the addition of PMAP-23. Results are representative of three independent experiments.

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