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Chemical synthesis and biological activity of a novel antibacterial peptide deduced from a pig myeloid cDNA

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Abstract

Several myeloid precursors of antibacterial peptides have recently been shown to share homologous pre- and pro-regions. Taking advantage of this homology, a novel cDNA was cloned from pig bone marrow RNA. This encodes a 166-residue polypeptide with highly conserved pre- (29 residues) and pro- (101 residues) sequences, followed by a unique, 36-residue C-terminal sequence. Structure analyses of this C-terminal region have identified a highly cationic sequence predicted to adopt an amphipathic α-helical conformation. A peptide corresponding to this sequence was chemically synthesized and shown to arrest the growth of both Gram-positive and Gram-negative bacteria. At least for *Escherichia coli*, the activity of this peptide appears to be mediated by its ability to permeabilize the bacterial membranes.

Key words: Antibacterial peptide; cDNA; Amphipathic helix; Cathelin; Myeloid cells

1. Introduction

cDNA sequencing of a variety of precursors of antibacterial peptides synthesized in bovine, porcine and rabbit myeloid cells, has predicted the existence of a family of precursors with highly homologous signal sequences and pro-regions [1–7]. In all these pre-proforms the C-terminal regions, which vary from 12 to 46 residues in length, are heterologous and correspond to known antibacterial/LPS binding peptides. The conserved pro-region also displays a striking level of homology (70–95%) with the cathepsin L inhibitor cathelin, isolated from pig leukocytes [8,9]. Studies carried out with pro-Bac5, one of the earlier proforms of this kind to be identified and purified [10], indicate that the antibacterial activity is expressed only after proteolytic release of the C-terminal region [11,12].

By using oligonucleotide primers derived from the conserved N-terminal pro-region, we have amplified several cDNAs from pig myeloid mRNAs, which encode polypeptides containing a cathelin-like domain. This domain precedes sequences corresponding to previously isolated antibacterial peptides in some cases [13,14], while in others it is followed by previously unknown sequences with structural characteristics consistent with antibacterial activity.

In this paper we report the cloning of a cDNA encoding a precursor with a pro-region that is 90% homologous to cathelin, and which contains a novel, highly cationic, 36 residue C-terminal region. The latter sequence was termed PMAP-36 from 'pig myeloid antibacterial peptide'. We synthesized a peptide corresponding to a stretch of 20 residues within PMAP-36 which was predicted to have a strong tendency to form an amphipathic α-helix, and which contained most of its cationic residues. Synthetic PMAP-36(1–20) was found to have a potent antibacterial activity against both Gram-negative and Gram-positive bacteria, and to permeabilize the inner membrane of *E. coli*.

2. Materials and methods

2.1. cDNA cloning, sequencing and Northern analysis

Total RNA was extracted from pig bone marrow cells with guanidinium thiocyanate [15]. The experimental conditions to obtain the 3′ and 5′ ends of PMAP-36 cDNA were as described in [5]. To obtain the 3′ end, the antisense primer adaptor 5′-TCGGATCCCTCGAGAAGC(T18)-3′ was used for reverse transcription. The sense oligonucleotide 5′-CGCGAATTCTGTGAGCTTCAGGGTG-3′, common to previously described precursors [1–5], and the antisense primer adaptor 5′-CGAGCTCGGATCCCTCGAGAAGCTT-3′ were used for PCR amplification. To obtain the 5′ end of PMAP-36 cDNA, the antisense oligonucleotide primer 5′-CACACCATTTACCCACAACCCA-3′, derived from the unique PMAP-36 cDNA sequence, was used for reverse transcription. The unique antisense oligonucleotide primer 5′-CCAGAATTCTTCAAAACCTTCCCGATCTTC-3′ and the sense oligonucleotide primer 5′-CAAGAATTCTCACCTGGGCACCATG-3′, derived from a 5′-conserved sequence of homologous precursors, includ-

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ing a short 5′-untranslated region [4], were used for PCR amplification. Amplified cDNAs were cloned in Bluescript SK$^+$ vector (Stratagene, San Diego, CA) and sequenced on both strands as reported [3].

Northern analysis was performed under experimental conditions previously described [5]. The 3′-end amplified PMAP-36 cDNA (nt 224–586), ^{32}P-labelled using standard protocols, was used to hybridize Northern blots of pig bone marrow total RNA.

2.2. Sequence analysis

cDNA sequence analysis was performed as described [3]. Homology searches were carried out on the Swiss-Prot database using the Seq, FastDB and Genalign programs in the Intelligenetics suite v. 5.4. Prediction of the secondary structure of the peptide was obtained using the PhD programme provided by the EMBL [16] and compared to that obtained with the algorithms of Chou and Fasman [17] and of Garnier et al. [18], as present in programs from the GCG and Intelligenetics suites.

2.3. Peptide synthesis and analytical assays

A Milligen 9050 synthesizer, loaded with Fmoc-Val substituted PEG-PS resin (0.1 mmol) (Milligen, Bedford, MA), was used for solid-phase synthesis. The 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 tetrafluoro-borate (Novabiochem) were added in a fivefold molar excess for each coupling step, except for residues 2, 5, 9, 13 where a sixfold excess was used. Side-chain protection was as follows: trityl (Gln), *t*-butyl (Glu, Asp, Thr), *t*-butyloxycarbonyl (Lys, Trp) and 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Arg). Cleavage from the resin and deprotection was carried out using a mixture of 95% TFA and 2.5% each of phenol and water. The peptide was repeatedly extracted with ether, and purified by RP-HPLC on a C18 column (Delta-Pak, Waters, Bedford, MA), using a 0–60% acetonitrile gradient in 0.05% TFA.

Peptide concentration was determined by absorbance at 214 nm as described by Buck et al. [19]. Amino acid analysis was performed using the Pico-Tag system (Waters) [20], and the molecular mass determined on 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, using 0.1 mg/ml of peptide in 5 mM sodium phosphate buffer, pH 7.0, containing 0–45% (v/v) trifluoroethanol. The α-helical content was evaluated by the CONTIN program [21] and according to Wu et al. [22], using mean molar residue ellipticities of -2000 and -32000 deg·cm^2·dmol^{-1} at 222 nm for 0% and 100% helix content, respectively.

2.4 Antibacterial and membrane permeabilization activities

The minimal inhibitory concentration (MIC) of the purified peptide was determined as described previously [10] against the following strains of bacteria: *Escherichia coli* ML35 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 by following the unmasking of β-galactosidase, as reported [23]. Hemolytic activity was determined by using human erythrocytes prepared from fresh, anticoagulated blood, as described previously [24]. Zero and 100% hemolysis were determined in the absence of additives and the presence of 0.2% Triton X-100, respectively. Melittin (Sigma Chemical Co., St. Louis, MO) was used as a positive control.

3. Results and discussion

PCR amplification of the 5′ and 3′ ends of pig bone marrow cDNA was performed using sense oligonucleotide primers corresponding respectively to nt -13 to $+2$ and 224 to 240 in Fig. 1. These derive from a 5′ mRNA sequence which is common to previously described antimicrobial peptide precursors [1–5]. Several cDNAs

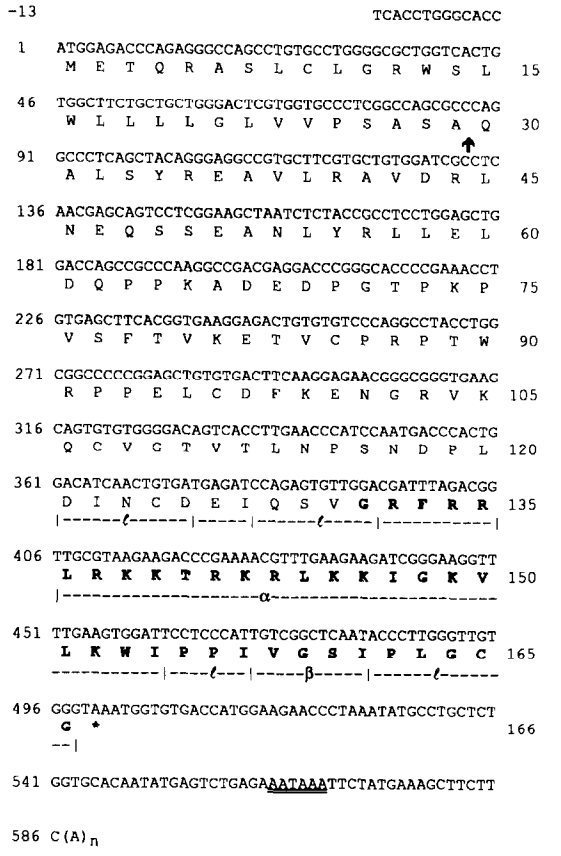

Fig. 1. Nucleotide and deduced amino acid sequence of pre-proPMAP-36. Nucleotides are numbered on the left and amino acids on the right. The end of the putative signal sequence, the stop codon, and the polyadenylation signal are indicated by an arrow, asterisk, and double underline, respectively. The C-terminal region following the cathelin domain is shown in bold and segments predicted to have α-helical (a), β-sheet (β) and loop (l) conformations are indicated.

with homologous 5′ regions were amplified. Three of these were found to encode the precursors of the antibacterial peptides PR-39 [4], protegrin PG-2 [5] and PMAP-23 (Zanetti, M. et al., unpublished), respectively. A fourth cDNA sequence showed an open reading frame of 166 codons, which corresponded to a transcript of about 0.8 kb, as detected by probing a Northern blot of pig bone marrow RNA with the 3′ end amplification product (not shown). The nucleotide and deduced amino acid sequence of this cDNA, obtained from two overlapping clones extending from nt -13 to $+457$ and from nt $+224$ to the polyadenylated tail, is shown in Fig. 1. The predicted polypeptide has a mass of 18648 Da and a calculated pI of 10.13. The N-terminal region of this polypeptide (residues 1 to 130) is 90–94% homologous to corresponding regions of previously described precursors of pig antibacterial peptides (Fig. 2), and includes a 29-residue signal peptide and a 101 residue pro-sequence which is about 88% homologous to pig cathelin (Fig. 2). The conserved region is followed by a unique,

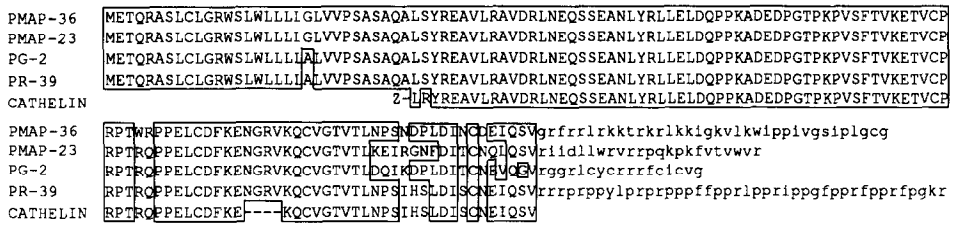

Fig. 2. Alignment of the precursor of PMAP-36 with other pig myeloid precursors and cathelin. The sequence is deduced from cDNA [4,5], except for cathelin, where it was determined by Edman degradation [9]. Residues homologous to those in PMAP-36 are boxed.

36-residue C-terminal sequence encompassing residues 131 to 166. This sequence is highly cationic, due to the presence of seven lysines and six arginines among the first 23 residues (Fig. 1). A valyl-residue is present at position 130, which is highly conserved in this precursor family [1,3–5], and is likely to be the proteolytic cleavage site responsible for release of antibacterial peptides from their precursors [1,3–5,12].

A structure prediction analysis was carried out on proPMAP-36 using three different algorithms (see section 2). The sequence between residues 132–154 obtained a high score for α-helix formation in all three methods. This region is comprised within the C-terminal PMAP-36 peptide and includes a segment which is by far the most cationic of the whole polypeptide. A helical wheel projection of the sequence between residues 131–150 (Fig. 3) shows the amphipathic nature of the predicted α-helix, a feature common to several antibacterial peptides [25,26]. This figure shows a dominant hydrophilic domain that subtends an angle of 260° on the projection, and a 100° hydrophobic domain. The only residues that do not fit this pattern, apart from Gly-1 and Val-20, which begin and end the amphipathic helix, are Gly-18 in the hydrophilic sector and Thr-10 in the apolar sector. Given the hydropathic characteristics of this two amino acids, they are unlikely to greatly affect the amphiphilicity of the helix.

This sequence between residues 131–150 shows a moderate homology (35%) to a corresponding region of rabbit leukocyte CAP18 [6], a LPS binding polypeptide with a cathelin-like domain. The equivalent peptide from CAP18 has been synthesized and shown to assume an amphipathic α-helical conformation and to exert a very potent antibacterial activity (Tossi A. et al., unpublished).

The structure prediction studies suggested thus that a peptide corresponding to residues 131–150 in Fig. 1 might possess antibacterial activity. This peptide, termed PMAP-36(1–20), was chemically synthesized and purified by HPLC with a reverse phase column. Mass spectrometry (measured mass of 2524.6 vs. the calculated mass of 2525.2 Da), amino acid analysis and analytical RP-HPLC (not shown) confirmed the identity and homogeneity of the purified peptide.

To test the predicted structure, circular dichroism spectra of PMAP-36(1–20) were recorded in aqueous environment and in the presence of trifluoroethanol. The spectra of the peptide in aqueous buffer were characteristic of an unordered structure, with no helical content (Fig. 4). Addition of trifluoroethanol induced a conformational change in PMAP-36(1–20), which exhibited an increasing level of helical folding (Fig. 4 and Fig. 4 inset). At 30% and 45% trifluoroethanol, respectively, α-helical content of 45% and 75% was estimated using both the

Table 1
Antibacterial activity of PMAP-36

Organism and strain	MIC (μM)
Escherichia coli ML 35	12
Escherichia coli ATCC 25922	12
Salmonella typhimurium ATCC 14028	48
Pseudomonas aeruginosa ATCC 25873	3
Bacillus megaterium (local isolate)	3
Staphylococcus aureus ATCC 25923	6

MIC was defined as the lowest concentration of peptide preventing visible bacterial growth after 18 h incubation with PMAP-36 at 37°C, as described previously [10]. Results, determined with approximately 1.5×10^5 colony forming units/ml, are the mean of at least five independent determinations with a divergence of not more than one MIC value with respect to those here reported.

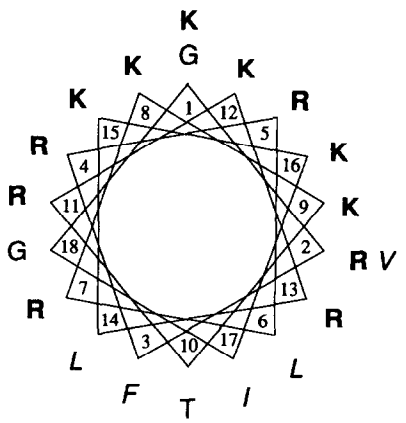

Fig. 3. Helical wheel representation of PMAP-36(1–20). Charged and strongly hydrophobic residues are indicated in bold and italics, respectively.

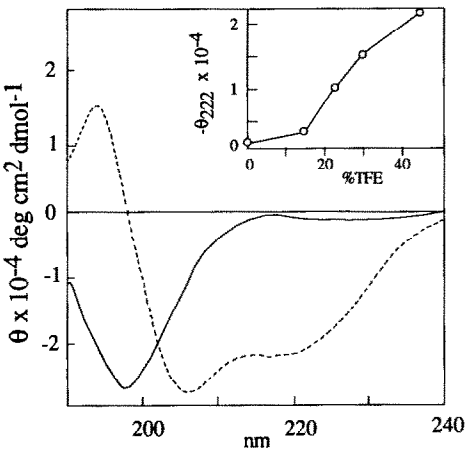

Fig. 4. CD spectra of PMAP-36(1–20). Spectra were taken in 5 mM phosphate buffer (pH 7) containing no additives (—) and 45% TFE (---). The change in mean residue ellipticity at 222 nm (Θ_{222}) with % TFE is shown in the inset.

CONTIN program [21] and the method of Wu et al. [22].

PMAP-36(1–20) displays in vitro antibacterial activity against several Gram-negative and Gram-positive microorganisms (Table 1). Maximal activity is exerted against *P. aeruginosa*, *B. megaterium* and *S. aureus* (MICs of 3–6 μM). What is peculiar is the high activity towards *P. aeruginosa*, which is usually more resistant than other species to antibacterial peptides [10,24,27], and the relatively poor activity against *S. typhimurium*, a species quite susceptible to other antibacterial peptides [10].

The amphipathic nature of PMAP-36(1–20) suggested it might act by permeabilizing the membranes of susceptible microbes. This was tested by measuring the unmasking of the cytosolic enzyme β-galactosidase in the *E. coli* ML35 strain. The peptide induces permeabilization of the bacterial inner membrane at 10–50 μM, while not inducing lysis of human erythrocytes even at a concentration of 100 μM. The kinetics of permeabilization is slower than for the Pro/Arg-rich antibacterial peptides Bac5 and Bac7 [23] or PMAP-23 (Zanetti, M. et al., unpublished), under the same conditions. The rate of reaction of the enzyme at steady state, relative to sonicated bacteria, is an order of magnitude lower for PMAP-36(1–20) than for Bac5. These observations may in part explain the somewhat lower activity of PMAP-36(1–20) towards *E. coli* ML35 (MIC of 12 μM) as compared to Bac5, Bac7 or PMAP-23 (MICs of 2 μM).

These results demonstrate that PMAP-36(1–20) is a potent, membrane-active antibacterial peptide. This peptide and the homologous peptide derived from rabbit CAP18 (Tossi, A. et al., unpublished) are new examples of α-helical antibacterial peptides. This structural motif is common in insect [25] and amphibian [26] antibacterial peptides, but to our knowledge was not found in mammalian antibacterial peptides, with the possible exception of porcine P1 cecropin [28]. Furthermore, PMAP-36 and the C-terminal domain of CAP18 are the only antibacterial peptides derived from cathelin-containing precursors with an amphipathic α-helical motif.

The identification of cDNAs encoding polypeptides with a cathelin-like pro-region is a rapid and powerful strategy for obtaining novel antimicrobial agents. This is achieved by combining structure prediction analysis of the deduced amino acid sequence with the chemical synthesis of the C-terminal region corresponding to the putative antibacterial domain.

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