Protein Chemistry and Structure:

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Biological Characterization of Two Novel Cathelicidin-derived Peptides and Identification of Structural Requirements for Their Antimicrobial and Cell Lytic Activities*

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Cathelicidins are a family of myeloid antimicrobial peptide precursors that have been identified in several mammalian species (Zanetti, M., Gennaro, R., and Romeo, D. (1995) *FEBS Lett*. 374, 1–5). Two novel bovine congeners have been deduced from cDNA. Their C-terminal sequences of 27 and 28 residues correspond to putative antimicrobial peptides with a cationic N-terminal region predicted to assume an amphipathic α-helical conformation followed by a hydrophobic C-terminal tail. Peptides corresponding to these sequences have been chemically synthesized and shown to exert a potent antimicrobial activity against Gram-negative and Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, and fungi. Both peptides are also cytotoxic to human erythrocytes and neutrophils, although at higher than microbicidal concentrations. The target selectivity has been improved by synthesizing truncated analogues, comprising only the 18 N-terminal residues, which show a great reduction in cytotoxic, but not in antimicrobial activity. The involvement of the C-terminal hydrophobic tail in the cytotoxic activity has been further demonstrated by inducing a major loss of activity in an analogue after replacing highly hydrophobic residues with more hydrophilic ones.

Research on naturally occurring antimicrobial peptides has resulted in the recognition of a surprisingly large number of peptides that are produced by animals and plants so as to fight infections (1, 2). In recent years, an additional impulse to the identification of novel antimicrobial peptides has come from the demand for new drugs ensuing from the emergence of multidrug resistant pathogens (3). Accordingly, the conversion of naturally occurring antimicrobial peptides into drugs is a goal that is currently being pursued by several research groups and biotechnology companies.

We and others have recently described a number of novel mammalian antimicrobial peptides that are derived from a group of precursors named cathelicidins (4). Precursors of this family are stored in the neutrophil granules of various mammalian species. They show a highly conserved N-terminal prosequence and a markedly varied C-terminal domain that exhibits antimicrobial activity after the propiece has been removed. Cathelicidin-derived antimicrobial peptides range in length from 12 to about 100 residues, and include α-helical peptides, *e.g.* human LL-37/hCAP18 (5–7) and pig PMAP-37 (8); linear peptides with one or two predominant amino acids, *e.g.* the bovine Pro- and Arg-rich Bac5 and Bac7 (9) and the Trp-rich indolicidin (10); and peptides with one or two disulfide bonds, *e.g.* bovine cyclic dodecapeptide (11) and pig protegrins (12).

A characteristic feature of cathelicidins is the extensive conservation of their mRNAs in the 5′ region corresponding to the 5′ noncoding region, the signal peptide and the prosequence. This feature has allowed identification of the mRNAs of several novel congeners in various mammalian species. At present, more than 20 cathelicidins with molecular masses of 16–26 kDa have been identified in bovine (13–16), ovine (17, 18), porcine (8, 19–24), rabbit (25, 26), and human (5, 6, 27) myeloid cells. In addition several mouse sequences have appeared in the GenBank/EBI Data Bank.

In this paper, we report the sequences of two novel bovine myeloid cathelicidins, as deduced from their cDNAs. Their C-terminal 27 and 28 residues show structural features consistent with antimicrobial activity. Peptides corresponding to these sequences have been chemically synthesized and named BMAP-27[1] and BMAP-28 (bovine myeloid antimicrobial peptides of 27 and 28 residues). Both show a potent and broad spectrum antimicrobial activity *in vitro*, that includes bacterial and fungal species, and are cytotoxic to human erythrocytes and neutrophils, although at concentrations much higher than those antimicrobial. Cytotoxicity to mammalian, but not microbial cells, is greatly reduced by shortening the two molecules at the C terminus. The cytotoxic effects on mammalian cells are largely mediated by the hydrophobic C-terminal residues, as shown by using a BMAP-28 analogue identical to the parent molecule except in the C-terminal region. We have thus identified two novel and potent antimicrobial peptides, and shown that removal of the C-terminal residues significantly increases their target specificity for microbial cells. These truncated BMAP analogues virtually devoid of cytotoxic activity are good candidates as leads to develop antibacterial and antifungal drugs.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X97608 and X97609.

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[1] The abbreviations used are: BMAP, bovine myeloid antimicrobial peptide; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; TFE, trifluoroethanol; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl.

EXPERIMENTAL PROCEDURES

Materials—Fmoc-amino acids and reagents for peptide synthesis were obtained from PerSeptive Biosystems (Framingham, MA) and Novabiochem (Laufelfingen, Switzerland). HPLC-grade acetonitrile, *N*-methyl-2-pyrrolidone, dimethyl sulfoxide, dichloromethane and *N,N*-dimethylformamide were from Lab-Scan (Dublin, Ireland). Trifluoroacetic acid, *N*-methylmorpholine, and trifluoroethanol (TFE) were from Janssen Chimica (Beerse, Belgium). *o*-Nitrophenyl-β-D-galactopyranoside and 1,8-diazabicyclo(5.4.0)undec-7-ene were from Sigma and Aldrich, respectively. Mueller-Hinton broth, Bacto-agar, dextrose, mycological peptone, and yeast extract powder were purchased from Unipath Ltd (Basingstoke, United Kingdom). All other chemicals were of analytical grade.

cDNA Cloning and Sequencing, and Northern Analysis—Total RNA was extracted from bovine bone marrow cells with guanidinium thiocyanate (28). The experimental conditions to obtain the 3′ and 5′ cDNA ends of BMAP-27 and BMAP-28 were as described previously (20). For the 3′ end amplification, bovine bone marrow mRNA was first reverse-transcribed using the antisense primer adaptor 5′-TCGGATCCCTCGAGAAGC(T_{18})-3′. The 3′ cDNA ends of BMAP-27 and BMAP-28 were then amplified at once by PCR, using the antisense primer adaptor 5′-CGAGCTCGGATCCCTCGAGAAGCTT-3′ and a sense oligonucleotide 5′-CGCGAATTCTGTGAGCTTCAGGGTG-3′ derived from the conserved 5′ sequence of cathelicidin cDNAs. To obtain the 5′ cDNA ends, the sequence-specific antisense oligonucleotide primers 5′-ACAGGATTCTTCCATGGGCT-3′ and 5′-AATTGGGCCATACTTCTTCC-3′ were used for reverse transcription of BMAP-27 and BMAP-28, respectively. The two cDNAs were then amplified by using the sense primer 5′-CAAGAATTCGGAGACTGGGGACCATG-3′ derived from the conserved 5′ region of bovine cathelicidins, with the sequence-specific antisense primer 5′-CAAGAATTCCCCAAATGGAGTAGCG-3′ (BMAP-27) or 5′-AATGAATTCCTACCCAGGCTTCGAA-3′ (BMAP-28). Amplified cDNA was cloned in Bluescript SK^+ vector (Stratagene, San Diego, CA) and sequenced on both strands with deazaguanosine and automated fluorescent DNA sequencing (EMBL fluorescent DNA sequencer, Heidelberg, Germany) as described previously (15). Northern analysis of bovine bone marrow total RNA was performed as described (15).

Sequence Analyses—cDNA sequence analysis was conducted with the IG suite, version 5.4 (IntelliGenetics Inc., Mountain View, CA, U.S.A.). Similarity searches were carried out on the Swiss-Prot data base using the FastDB and Genalign programs. The secondary structure of the peptides was predicted with programs using the Chou and Fasman (29) and Garnier *et al.* (30) algorithms.

Peptide Synthesis and Purification—Solid phase peptide synthesis of BMAP-27, BMAP-28, and their analogues was carried out on a Milligen 9050 synthesizer (Milligen, Bedford, MA). The synthesis was performed using the PAL-PEG-PS resin (0.2 mmol/g), and *N*-methyl-2-pyrrolidone containing 20% dimethyl sulfoxide as the general solvent. Couplings were carried out with a 6-fold excess of an equimolar mixture of Fmoc-amino acid, *N*-hydroxybenzotriazole, and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate in the presence of *N*-methylmorpholine. Deprotection from the Fmoc group was performed with a solution of piperidine, *N*-methyl-2-pyrrolidone, *N,N*-dimethylformamide (1:2:2, v/v) in the presence of 0.7% (v/v) 1,8-diazabicyclo(5.4.0)-undec-7-ene. To improve difficult couplings (as predicted by the Peptide Companion program from CSPS (Tucson, AZ)), an 8-fold excess of Fmoc-amino acid and of *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate as a coupling agent were used. As the first synthesis of BMAP-28 gave a very poor yield, this peptide and its analogues were synthesized increasing the column temperature to 45 °C and washing the resin with the so-called "magic mixture" (*N*-methyl-2-pyrrolidone/*N,N*-dimethylformamide/dichloromethane (1:1:1, v/v) with 1% Triton X-100 and 2 M ethylenecarbonate) (31) immediately before each coupling step. Finally, capping with acetic anhydride 20% (v/v) was performed after each predicted difficult coupling. Side-chain protecting groups were as follows: trityl (His), *t*-butyl (Ser, Tyr), *t*-butyloxycarbonyl (Lys and Trp), and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Arg). Deprotection and cleavage from the resin were carried out using a mixture of trifluoroacetic acid/phenol/water/ethane dithiol/thioanisole/triisopropylsilane (89:2.5:2.5:2.5:2.5:1, v/v) for 2–3 h at room temperature. After cleavage, each peptide was repeatedly extracted with ethyl ether and purified by reverse phase high performance liquid chromatography on a C18 Delta-Pak column (Waters, Bedford, MA), using an appropriate 0–60% water/acetonitrile gradient in the presence of 0.1% trifluoroacetic acid.

Analytical Assays—Peptide concentration was determined by measuring the absorbance of Trp and Tyr at 280 nm in 6 M guanidine hydrocloride (BMAP-28 and its analogues) or Phe at 257 nm in 0.1% trifluoroacetic acid (BMAP-27 and BMAP-27(1–18)). Molar extinction coefficients of 5690, 1280, and 195 were used for Trp, Tyr, and Phe, respectively (32, 33). The molecular mass of each peptide was determined with an API I ion spray mass spectrometer (PE SCIEX, Toronto, Canada). Circular dichroism spectroscopy was carried out on a Jasco J-600 spectropolarimeter (Jasco Corp., Tokyo, Japan) with a cell path length of 2 mm. Peptide samples (10–20 μM) were dissolved in 5 mM sodium phosphate buffer, pH 7.0, in the absence or presence of TFE up to 60% (v/v). The α-helical content was estimated by the method of Chen *et al.* (34) from the $[\theta]_{222}$ measurements, using −1500 and −39,500(1–2.5/*n*) deg·cm^2·dmol^{-1}, as the values for 0% and 100% of helix, respectively; *n* is the number of residues in the amidated peptide. The possible error in the determination of the helical content of BMAP-28 and of its analogues, as introduced by the interaction of aromatic side chains of Tyr and Trp with the helix, was evaluated as reported by Doig and Baldwin (35).

Antibodies to the synthetic BMAP-27 and BMAP-28 were raised in New Zealand White rabbits by repeated injections of 150 μg of peptide. Western blot analysis was performed as described (36).

Bacterial Growth Suppression and Membrane Permeabilization—Antibacterial and antifungal activities of the purified peptides were determined as the minimum inhibitory concentration (MIC) by a microdilution susceptibility test in 96-well microdilution plates. The following bacterial strains were tested, under the assay conditions previously described (37): *Escherichia coli* ATCC 25922, ML-35, and D21; *Salmonella typhimurium* ATCC 14028; *Pseudomonas aeruginosa* ATCC 27853; *Serratia marcescens* ATCC 8100; *Staphylococcus aureus* ATCC 25923, Cowan 1, and two methicillin-resistant clinical isolates carrying the *mecA* gene (provided by L. Dolzani, Dept. of Biomedical Sciences, University of Trieste, Italy); *Staphylococcus epidermidis* ATCC 12228; and *Bacillus megaterium* Bm 11. The antifungal activity was determined using clinical isolates of *Candida albicans* and *Cryptococcus neoformans*. The assay conditions were similar to those used for bacteria (37), except that the fungal species were grown and tested in Sabouraud liquid medium and the MIC was determined after incubation at 30 °C for 36–48 h. Inner membrane permeabilization of the *E. coli* ML-35 strain was evaluated as described previously (38).

Cytotoxic Activity—Hemolysis of human and bovine red blood cells was evaluated as described (38). Polymorphonuclear cells were isolated from human peripheral blood by the dextran standard procedure. The cell population was at least 92% neutrophils as evaluated by Giemsa staining and expression of CD66. Isolated neutrophils (4×10^6 cells/ml) were incubated for 30 min at 37 °C with 1, 6, and 30 μM peptide, and then 10 min at 4 °C with propidium iodide (5 μg/ml final concentration). The percentage of permeabilized cells was evaluated by cytofluorimetric analysis using a FACScan (Becton-Dickinson, San Jose, CA) equipped with the Lysis 2 software. Cells within the channels of fluorescence intensity 1–150 were regarded as non-permeabilized, by comparison with cells incubated without peptides or with the fibronectin fragment CS-5 of 20 residues, used as negative control. Cell debris were excluded from analysis by appropriately raising the FSC threshold.

RESULTS AND DISCUSSION

cDNA Cloning of the Precursors of BMAP-27 and BMAP-28—A reverse transcription-polymerase chain reaction-based approach that allows the immediate amplification of cDNAs containing cathelicidin-related sequences was used to obtain the 3′ cDNA ends of bovine myeloid congeners. Amplification was carried out using a sense primer 5′-CGCGAATTCTGTGAGCTTCAGGGTG-3′ derived from the highly conserved prosequence, which is upstream of the diverse antimicrobial domains of cathelicidins, and an antisense adaptor priming in the polyadenylated tail region. All the amplified products were sequenced and shown to include the previously described Bac5 (15), indolicidin (13), cyclic dodecapeptide (14), and two other cathelicidin cDNAs, with novel 3′ sequences corresponding to putative antimicrobial domains of 27 and 28 amino acid residues (Fig. 1). Following a nomenclature used for other cathelicidins, the deduced polypeptides were designated preproBMAP-27 and preproBMAP-28, for "precursors of bovine myeloid antimicrobial peptides of 27 and 28 residues" to emphasize the presence of C-terminal putative antimicrobial domains. Northern analysis of bovine bone marrow total RNA using sequence-

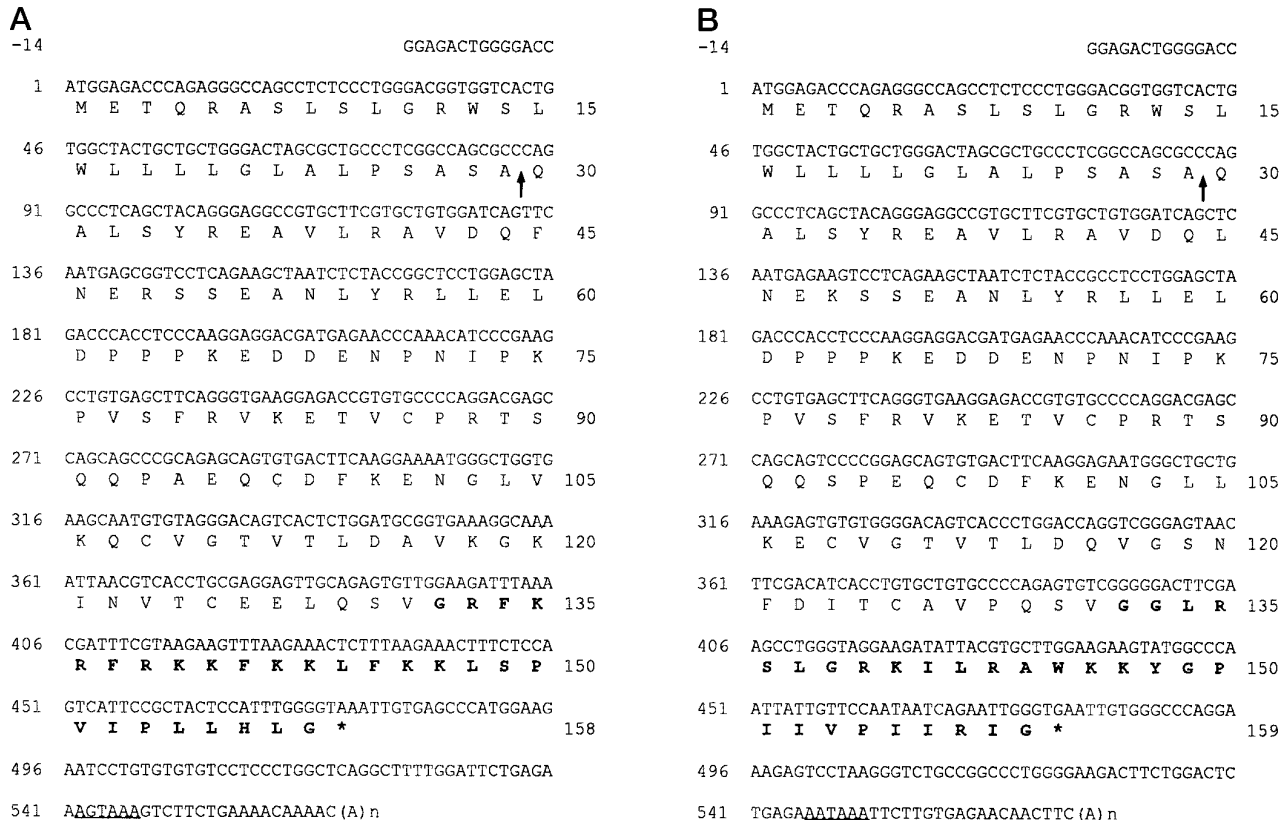

FIG. 1. **Nucleotide and deduced amino acid sequences of the precursors of BMAP-27 (*A*) and BMAP-28 (*B*).** *Numbering* is on the *left* for nucleotide and on the *right* for amino acid sequences. The putative cleavage site of the signal sequence is indicated by an *arrow*, the stop codon by an *asterisk*, and the polyadenylation signal is *underlined*. The sequences of the putative antimicrobial peptides BMAP-27 and BMAP-28 are shown in *boldface*.

specific antisense oligonucleotides revealed corresponding transcripts of approximately 0.65 kilobases (data not shown). The full-length cDNA sequences of the two novel cathelicidins were obtained by generating 5′ cDNA ends that partially overlap the amplified 3′ regions, using, respectively, a BMAP-27 (5′-CAAGAATTCCCCAAATGGAGTAGCG-3′) or a BMAP-28 (5′-AATGAATTCCTACCCAGGCTTCGAA-3′) sequence-specific antisense oligonucleotide primer, and a sense oligonucleotide primer 5′-CAAGAATTCGGAGACTGGGGACCATG-3′ derived from the conserved 5′ noncoding region of bovine cathelicidins.

Features of the Predicted Sequences—The full-length cDNA sequences of the novel cathelicidins show open reading frames of 158 and 159 codons, the former corresponding to a 17,853-Da polypeptide with a calculated pI of 10 (preproBMAP-27, Fig. 1*A*), the latter to a polypeptide of 17,617 Da with a pI of 8.3 (preproBMAP-28, Fig. 1*B*). Both sequences show the characteristic features of cathelicidins, including a conserved N-terminal preproregion and a diverse C-terminal sequence (4). The preproregions include a 29-residue signal peptide and a 102-residue propiece, are 88% identical, and share 78–83% identity to the preproregions of the other bovine congeners (13–16), from which they also differ for an additional Glu residue at position 66. The C-terminal sequences (corresponding to residues 132–158 in Fig. 1*A* and 132–159 in Fig. 1*B*), share a 28.5% identity and an additional 50% similarity. These sequences were predicted to correspond to antimicrobial peptides, based on the following structural features, which are common to many cathelicidin-derived antimicrobial peptides: (i) they are cationic, due to a high content of Arg and Lys residues; (ii) both sequences are preceded by a valyl residue at position 131, which is conserved in most congeners and is a common processing site for the maturation of cathelicidin-derived antimicrobial peptides (8, 13, 15–24, 39); (iii) helical wheel projection of both peptides shows that residues 1–18 arrange in an amphipathic α-helical conformation (Fig. 2). Such a conformation is also predicted in this region for BMAP-27 by both the Chou-Fasman (29) and the Garnier *et al.* (30) methods, but only by the former method for the segment 8–16 of BMAP-28. The presence of a Pro residue at position 19 likely interrupts the helical segment, which is followed by a highly hydrophobic C-terminal tail. Both peptides end with a Gly residue that is a likely amide donor in a post-translational amidation of the C terminus (2, 10, 13, 15, 19, 21, 24). These features suggest that BMAP-27 and BMAP-28 may add to the bovine repertoire of antimicrobial peptides. This includes cathelicidin-derived peptides (9–11) and β-defensins (40–42). Among these, they would be the first ones identified with an α-helical conformation. Helical antimicrobial peptides are not unusual in the animal kingdom and include a number of recently identified mammalian peptides (5, 8, 17, 22, 43, 44), in addition to the well known insect cecropins (1, 2) and amphibian magainins (1, 2). Apart from pig cecropin P1 (44), those identified in mammals all derive from cathelicidins (4), and SMAP-29 from sheep (17, 18) is the one showing the highest similarity to BMAP-28 (61% identity in the peptide sequence, and 92% in the preprosequence). The identity between SMAP-29 and BMAP-27 is lower than 20% in the peptide sequence and 85% in the preprosequence. From an evolutionary point of view, these observations suggest that (i) the ovine SMAP and the bovine BMAPs originated from duplication of a common ancestor gene, and (ii) BMAP-27 and BMAP-28 genes arose before the ovine and bovine species diverged.

Biological Properties of Synthetic BMAP-27 and BMAP-28—That BMAP-27 and BMAP-28 exert antimicrobial activity has been proved by using synthetic peptides of corresponding se-

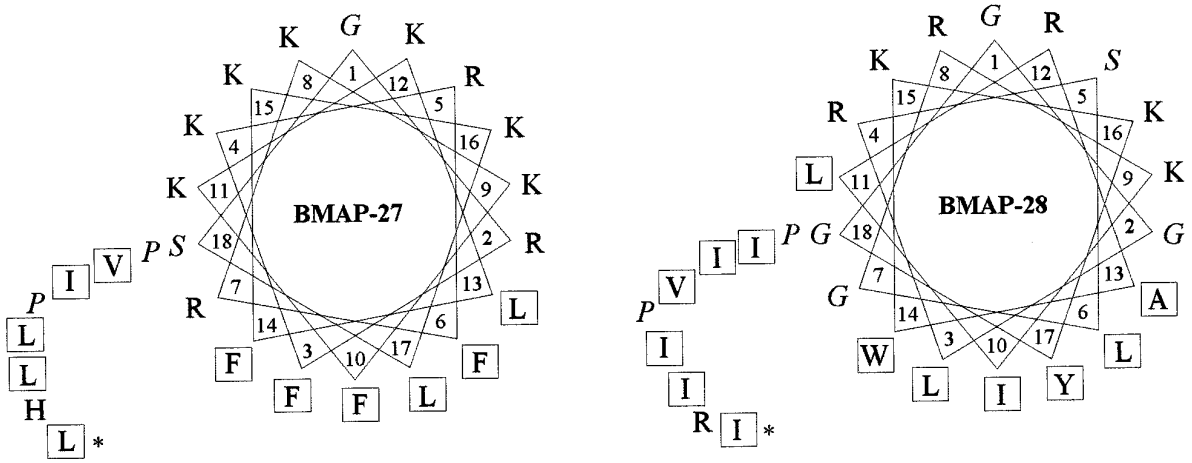

FIG. 2. **Helical wheel projections of the sequences of BMAP-27 and BMAP-28.** The representation shows the amphipathic structure of the 1–18 region. Charged residues are indicated in *boldface*, hydrophobic residues are *boxed*, and all other residues are in *italics*. The *asterisk* indicates amidation of the C terminus.

quence. These were synthesized as C-terminal amides of 26 and 27 residues, respectively, as suggested by a C-terminal Gly residue inferred from cDNA sequences. The purified peptides were shown to be the correct ones by ion spray mass spectrometry (3225.5 Da *versus* a calculated mass of 3225.1 Da for BMAP-27 and 3073.1 Da *versus* 3073.9 Da for BMAP-28).

The synthetic peptides were used to raise rabbit antibodies that were used for Western analysis of bovine neutrophil granule extracts. They recognized molecules with an approximate molecular mass of 14 kDa, corresponding to the proforms of BMAP-27 and BMAP-28 (data not shown). The presence of BMAP-28 in peripheral neutrophils was also confirmed by the isolation of its proform (45).

The secondary structure of both peptides was investigated by circular dichroism spectroscopy. CD spectra showed that they are unordered in aqueous solution and undergo a conformational transition in the presence of TFE, with a 35–40% helical content at 45% TFE, in good agreement with the structure prediction analysis (Fig. 3*A* for BMAP-28).

The effects of BMAP-27 and BMAP-28 against a panel of bacterial and fungal species were evaluated as the minimal concentration capable of inhibiting visible microbial growth (MIC). Both peptides showed a remarkable *in vitro* antibacterial activity against several Gram-negative and Gram-positive bacteria, with MIC values of 0.25–4 μM (Table I). Interestingly, both peptides are active against two clinical isolates of methicillin-resistant *S. aureus* (MRSA) with MIC values comparable with those of the methicillin-susceptible strains ATCC 25923 and Cowan 1 (Table I). They also inhibit the growth of the fungal species *C. albicans* and *C. neoformans*, with MIC values of 4–8 μM (Table I).

The ability of the two peptides to damage prokaryotic membranes was also assessed, using the β-galactosidase-constitutive and lactose permease-deficient *E. coli* ML35, a strain previously used to determine the membrane-perturbing activity of several other antimicrobial peptides (8, 22, 23, 38, 43). BMAP-27 and BMAP-28 both induce a rapid permeabilization of the inner bacterial membrane at 0.2–1 μM, a steady state for the β-galactosidase activity being reached within few minutes from their addition (Fig. 4, *panels A* and *B*). BMAP-28 appears to be more effective than BMAP-27, with a reaction rate equal to that of sonicated bacteria at 0.5 μM peptide concentration. While these membrane-perturbing effects were expected, in view of the amphipathic character of the two molecules, this activity was found to be considerably higher than that of other membrane-active peptides evaluated under the same conditions, including PMAP-23 (23), the Pro- and Arg-rich Bac5 and Bac7 (38), and also the α-helical peptides PMAP-36(1–20) (22), PMAP-37 (8), and CAP18(106–125) (43).

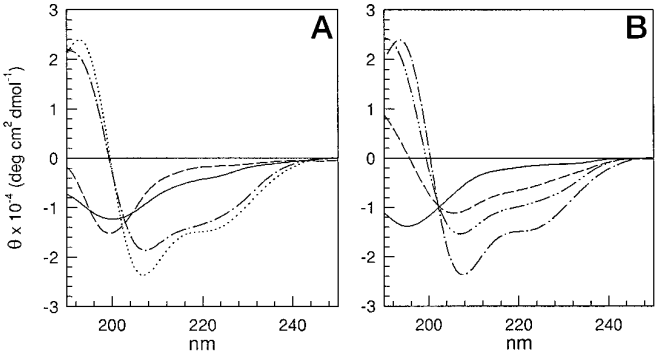

FIG. 3. **Circular dichroism spectra of BMAP-27, BMAP-28 and of their analogues.** The spectra were measured at 10–20 μM peptide in 5 mM sodium phosphate buffer, pH 7.0, in the absence or the presence of increasing concentrations of TFE. *A*, BMAP-28, no TFE (——) and 45% TFE (···); BMAP-28(1–18), no TFE (- - -) and 45% TFE (–·–·). *B*, *m*BMAP-28 in the absence (——) and the presence of 15% (- - -), 30% (–··–··), and 45% (–·–·) TFE.

Compared with these peptides, BMAP-27 and BMAP-28 also show a broader and more potent antimicrobial activity. Taken together, these features may denote a high cytotoxic potential for these molecules. This prompted us to investigate further their target cell specificity by extending the analysis to mammalian cells. Their ability to permeabilize both nucleated and non-nucleated cells was investigated by measuring the exclusion of propidium iodide by neutrophils and hemolysis of red blood cells, respectively.

At BMAP concentrations of 1–6 μM, comparable with or higher than the MIC values, human neutrophils were not significantly permeabilized, as compared with untreated controls or to controls treated with an irrelevant peptide. Conversely, at the experimental conditions used, these cells are almost completely permeabilized ($\geq$94%) by both peptides at 30 μM concentration (Fig. 5). The effects of the two peptides on human and bovine red blood cells were evaluated with a standard hemolysis assay. Both caused some lysis of human red blood cells at concentrations that are approximately 1 order of magnitude higher than those antimicrobial (Fig. 6). In particular, 14.5% of the cells were lysed at 3 μM, and over 90% at 100 μM by BMAP-28, whereas BMAP-27, at the same concentrations caused a cell lysis of 3.5% and 32.7%, respectively. Among the

TABLE I
Antimicrobial activity of BMAP-27, BMAP-28, and of their analogues

MIC was defined as the lowest concentration of peptide preventing visible microbial growth after incubation for 18 h at 37 °C (bacteria) or 36–48 h at 30 °C (fungi). All the strains were grown in Mueller-Hinton broth, except *B. megaterium*, grown in LB medium, and *C. albicans* and *C. neoformans* grown in Sabouraud medium. Results were determined with approximately 1.0–2.0 10^5 (bacteria) and 0.25–0.50 $\times$ 10^5 (fungi) colony forming units/ml and are the mean of at least four independent determinations with a divergence of not more than 1 MIC value. ND, not determined.

Organism and strain	MIC (μM)				
	BMAP-27	BMAP-27-(1–18)	BMAP-28	BMAP-28-(1–18)	*m*BMAP-28
E. coli ATCC 25922	1	2	2	1	1
E. coli ML35	1	4	2	0.5	0.5
E. coli D21	0.25	4	0.5	0.5	ND
S. typhimurium ATCC 14028	1	4	1	2	1
P. aeruginosa ATCC 27853	1	1	1	1	0.5
S. marcescens ATCC 8100	2	2	2	2	4
S. aureus ATCC 25923	2	2	2	2	8
S. aureus Cowan 1	2	2	1	1	ND
S. aureus (MRSA)	4	4	4	4	≥64
S. aureus (MRSA)	4	4	2	4	≥64
S. epidermidis ATCC 12228	1	1	1	1	2
B. megaterium Bm11	2	2	2	2	2
C. albicans	8	16	8	16	8
C. neoformans	4	4	4	2	4

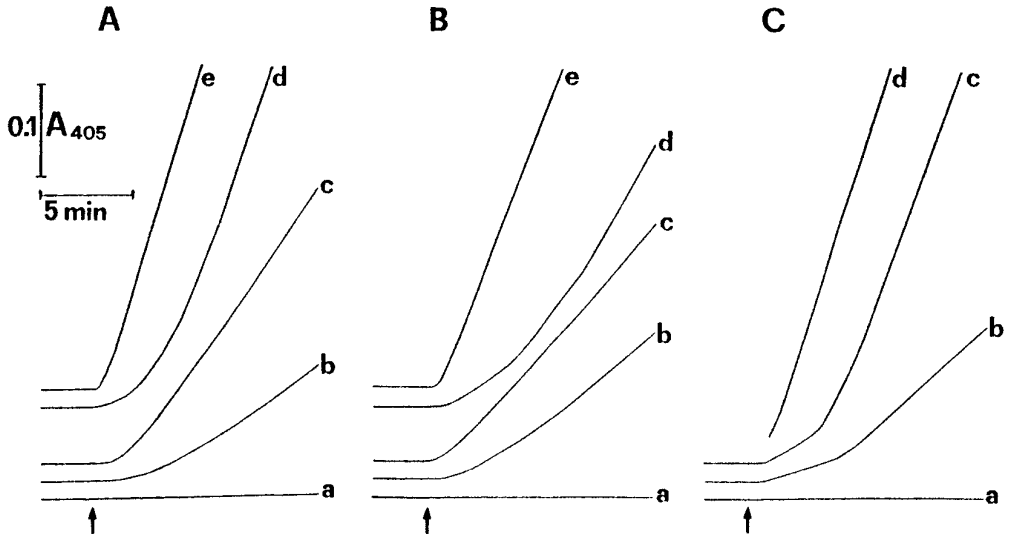

FIG. 4. **Kinetics of permeabilization of *E. coli* ML-35 inner membrane by BMAP-27, BMAP-28, and by their analogues.** Permeabilization was determined spectrofotometrically at 405 nm by following the unmasking of cytoplasmic β-galactosidase activity. Each assay was performed with approximately 10^7 colony-forming units/ml in 10 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 1.5 mM substrate. *A*, untreated bacteria (*trace a*); BMAP-28(1–18), 0.2 μM (*trace b*) and 5 μM (*trace d*); BMAP-28, 0.2 μM (*trace c*) and 0.5 μM (*trace e*). *B*, untreated bacteria (*trace a*); BMAP-27(1–18), 1 μM (*trace b*) and 5 μM (*trace c*); BMAP-27, 0.2 μM (*trace d*) and 1 μM (*trace e*). *C*, untreated bacteria (*trace a*); *m*BMAP-28, 0.2 μM (*trace b*), 1 μM (*trace c*), and sonicated bacteria (*trace d*). The *arrows* indicate addition of peptides. Results are representative of two to three very similar and independent experiments.

cathelicidin-derived α-helical antimicrobial peptides, only PMAP-37 (8) and SMAP-29[2] display hemolytic activity on human cells comparable with that of the BMAP peptides. Other helical peptides, such as insect cecropins (2) and amphibian magainins (2), are inactive even at concentrations that are 1–2 orders of magnitude higher than those antimicrobial.

The hemolytic activity of the two BMAPs was much lower, when measured on bovine erythrocytes (data not shown). These cells were 5–10-fold less susceptible than human red blood cells to BMAP-28 (3, 6, and 22% hemolysis at 10, 30, and 100 μM peptide), and virtually unaffected by BMAP-27 even at 100 μM. We speculate that the different susceptibility of human and bovine red blood cells to these molecules may be accounted for by species-specific differences in the erythrocyte membrane composition, and may reflect the need to protect the host cells from undesired cytotoxic effects. We have also noted that the effects of BMAP-28 on the cell membranes are consistently higher than BMAP-27, in spite of the structural similarity of the two molecules, thus suggesting that minor structural differences can modulate the activity of these peptides.

Biological Properties of the Synthetic BMAP-27(1–18), BMAP-28(1–18), and mBMAP-28—The results described above indicate that BMAP-27 and -28 are indeed potent, but not highly selective antimicrobial agents, due to their toxic effects on human cells that may restrict their potential therapeutic applications. In an attempt to improve the toxicity index of these peptides, we looked for structural analogues with increased selectivity toward microorganisms. It was decided in particular to remove the hydrophobic C-terminal tail, while maintaining the complete N-terminal helix (residues 1–18). This modification was suggested by previous studies indicating that several peptides made of a highly cationic α-helix and lacking a hydrophobic tail are antimicrobial, but not cytotoxic (22, 43). By contrast, gross modifications of the helix may lead to loss of antimicrobial activity (43). Accordingly, BMAP-derived peptides corresponding to residues 1–18 of both BMAPs,

[2] R. Gennaro, manuscript in preparation.

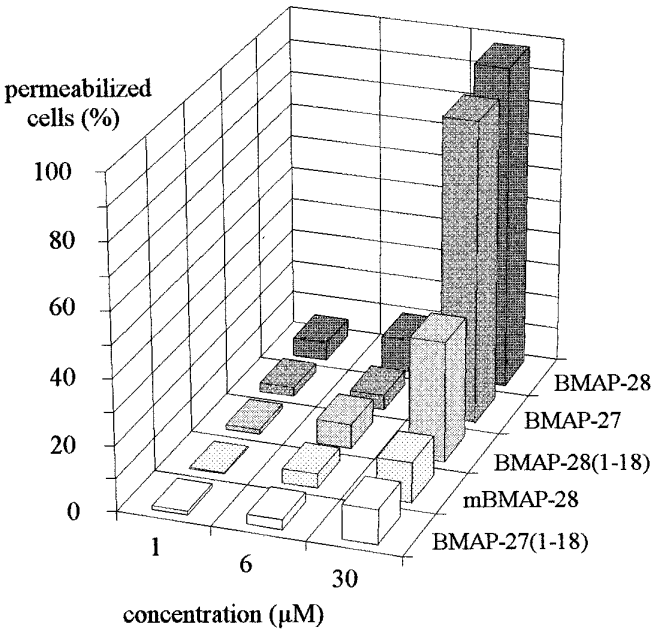

FIG. 5. **Cytotoxic activity of BMAP-27, BMAP-28 and of their analogues on human neutrophils.** Peripheral blood neutrophils in RPMI medium supplemented with 10% fetal calf serum were incubated with peptides at the indicated concentrations for 30 min at 37 °C. Results were obtained by fluorescence-activated cell sorting analysis, are expressed as percentage of cells stained with propidium iodide, and are the mean of three independent experiments with S.E. $< \pm 2.1$. Controls were run either in the absence of peptide or in the presence of a fibronectin fragment, and the percentage of permeabilization ranged from 2 to 6.

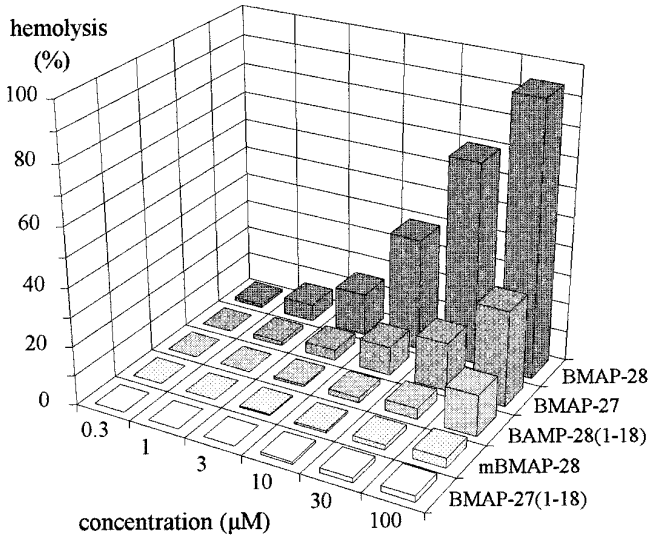

FIG. 6. **Lytic activity of BMAP-27, BMAP-28 and of their analogues on human erythrocytes.** Hemolysis was performed by incubating a 10% (v/v) cell suspension in phosphate buffered saline (pH 7.4) with the indicated peptide concentrations for 15 min at 37 °C. After stopping the reaction, samples were centrifuged and the supernatant absorbance was read at 415 nm. Total hemolysis was obtained by adding 0.2% Triton X-100 to the cell suspension. Results are the mean of three to six independent experiments with S.E. values ranging from ± 0.1 to ± 1.8.

and lacking the C-terminal hydrophobic tail, were chemically synthesized with an amidated C terminus. These peptides were named BMAP-27(1–18) and BMAP-28(1–18). Their helical content, as determined by CD spectroscopy in the presence of 45% TFE, is respectively 55% and 45%, and is higher than that of the parent molecules (Fig. 3*A* for BMAP-28(1–18)), consistent with the prediction that the helix is confined to the N-terminal region of the parent peptides. The biological activity of both peptides was tested toward microbial and mammalian targets. Their spectrum of activity and MIC values are comparable with those of the parent molecules, including the MRSA strains (Table I). These results indicate that the hydrophobic tail is not essential for the antibacterial nor for the antifungal activity. Conversely, the lytic effects on human red blood cells are lost in the case of BMAP-27(1–18) and greatly reduced in the case of BMAP-28(1–18) (Fig. 6). Also, permeabilization of human neutrophils is decreased to approximately 11% and 37% at 30 μM BMAP-27(1–18) and BMAP-28(1–18), as compared with the near complete lysis induced by the parent molecules at the same concentration (Fig. 5). These observations indicate that shortening of the sequences at the C terminus increases the target selectivity toward microbial cells. Removal of the C-terminal sequence also affects the kinetics of permeabilization of the inner membrane of *E. coli* ML-35 (Fig. 4, *panels A* and *B*). A 5–10-fold higher concentration of the truncated analogues is required to achieve a kinetics of permeabilization comparable with that of the respective parent peptides, likely indicating a somewhat less effective initial interaction with target bacterial membranes. Bacterial growth inhibition, however, does not appear to be affected, as indicated by the MIC values for *E. coli* ML-35, which are comparable with those of BMAP-27 and BMAP-28.

We asked whether the effects shown with the truncated BMAP analogues may depend on specific removal of the hydrophobic residues at the C terminus, rather than on simple shortening of the peptides. We addressed this question and evaluated the involvement of the C-terminal hydrophobic residues in the lytic effects on mammalian cells by using a synthetic BMAP-28 analogue of the same length as the parent molecule and named *m*BMAP-28 (where *m* stands for modified). The N-terminal 19 residues of this peptide are unchanged, while the sequence extending outside the helix (residues 20–27) and forming a hydrophobic cluster at the C terminus was changed from Ile-Ile-Val-Pro-Ile-Ile-Arg-Ile-NH_2 to Gln-Ala-Thr-Pro-Ala-Thr-Arg-Gln-NH_2, by replacing highly hydrophobic residues, but not Pro-23 and Arg-26, with more hydrophilic ones, selected according to the Eisenberg hydrophilicity scale (46), and so as to maintain a comparable conformational propensity. CD spectra of this analogue are very similar to those of the parent molecule, with a calculated α-helical content of about 15%, 25%, and 40% at 15%, 30%, and 45% TFE, respectively (Fig. 3*B*), indicating that the secondary structure content of BMAP-28 is preserved.

When compared with the parent peptide, *m*BMAP-28 does not show any significant lytic activity on human red blood cells (3.3% *versus* 94.1% lysis at 100 μM peptides, Fig. 6). In addition, neutrophil permeabilization, as determined by the propidium iodide exclusion, is greatly reduced (Fig. 5), indicating that the lytic effects on these cells are deeply influenced by the changes made in the sequence. Conversely, *m*BMAP-28 efficiently permeabilizes the inner membrane of *E. coli* ML-35 (Fig. 4*C*). However, the antimicrobial activity, at variance with that of the truncated analogue, does not overlap exactly with that of the parent molecule. In particular, while the MIC values are comparable or slightly lower than those of BMAP-28 against *E. coli*, *P. aeruginosa*, and *S. typhimurium*, and only slightly higher against *S. marcescens* and *S. aureus* ATCC 25923 (Table I), they are significantly higher against the MRSA strains, which thus appear to be much less susceptible to this analogue (Table I). Whether this is dependent on the methicillin resistance trait or on other features of these strains is not clear, but interestingly, a similar difference in susceptibility for methicillin-susceptible and -resistant *S. aureus* strains was

also observed with synthetic antimicrobial peptides derived from a core undecapeptide of sapecin B (47).

We have thus identified two novel cathelicidin-derived peptides with a wide spectrum and very potent antimicrobial activity, indeed among the highest reported for α-helical type antimicrobial peptides. This activity has also been investigated with respect to the presence of a hydrophobic tail, which is outside the helical region. A major result of these experiments is the dramatic reduction of the lytic activity on mammalian cells, shown by analogues in which the tail is removed or modified to decrease its hydrophobicity.

The considerable reduction of cytotoxic activity that is achieved with shortened BMAP analogues, while maintaining a potent antimicrobial activity also against antibiotic-resistant micro-organisms, provides a significant improvement in terms of their potential therapeutic utility. This encourages further experiments aimed at the evaluation of these peptides as drug leads for the treatment of bacterial and fungal infections.

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