Molecular analysis of the sheep cathelin family reveals a novel antimicrobial peptide

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Abstract Cathelin-related genes are characterized by the presence of a prepro sequence which is highly conserved both within and between species. 3′ RACE analysis on sheep bone marrow RNA, using a primer based on a conserved cathelin family coding region, demonstrated the presence of at least three ovine cathelin-related cDNAs. One of these encodes a novel prepropeptide with a predicted C-terminal cleavage product RGLRRLGRKIAHGVKKYGPTVLRIIRIAG. The chemically synthesized form of this 29 amino acid peptide is shown to be a thermostable, broad spectrum, bactericidal agent.

Key words: Ovine; Cathelin; 3′ RACE; Antimicrobial peptide

1. Introduction

Mammalian leukocytes play an important role in host defense against a broad spectrum of microbial pathogens. In this role they demonstrate multiple pathways for the uptake and neutralization of invading microorganisms. For example, leukocytes synthesize a variety of granule-associated peptides which may act individually or in concert to kill phagocytized microbes (for reviews see [1–3]). The combined presence of antimicrobial peptides as diverse in structure as cyclic dodecapeptide [4], cysteine-rich protegrins and defensins [2], and bactericidal/permeability-increasing peptide [5] may represent the successful evolution of in vivo antimicrobial multi-drug defense, which minimizes the chance of organisms developing resistance to individual peptides.

Our laboratory has chosen to focus on molecular mechanisms regulating developmental expression of myeloid antimicrobial peptides. Sheep are a traditional animal model correlate to humans for studying biological changes occurring during development. For this reason, we have identified cDNA sequences representing antimicrobial peptide gene families expressed in ovine bone marrow. One such family, the cathelins, is characterized by the presence of mRNAs encoding a highly conserved N-terminal prepro sequence attached to one of a diverse group of C-terminal cleavage peptides [6–12]. We report here the identification of ovine bone marrow-derived, cathelin-related cDNA sequences. One of these sequences encodes a novel prepropeptide with a putative C-terminal 29 amino acid peptide we have termed SC5. Chemical synthesis of SC5 and subsequent testing in vitro shows it to be a potent, broad-spectrum antimicrobial peptide.

2. Materials and methods

2.1. Bone marrow RNA isolation and 3′ RACE analysis

Ovine bone marrow RNA was isolated from an adult iliac crest sample by the procedure of Chomczynski and Sacchi [13]. 3′ rapid amplification of cDNA ends (3′ RACE) was performed on 5 μg of bone marrow total RNA using the 3′ RACE System (Gibco BRL, Grand Island, NY). The gene specific primer (SH1: 5′ ATGGAGACCCGGGCCAGCCT 3′) was based on the conserved initial coding sequences of cathelin family cDNAs. 3′ RACE PCR products were blunt end ligated into the pCR-Script SK(+) blunt-end vector (Stratagene, La Jolla, CA) and transfected into *E. coli* Sure cells (Stratagene). Ampicillin-resistant colonies containing plasmids with cathelin-related inserts were identified by hybridization with a second oligonucleotide (Sh2: 5′ GCACGGCCTCCCTGTAGCTGAGGGC 3′) whose sequence was based on coding nucleotides 91–115 of PR39 [14].

2.2. Sequencing of cathelin-related cDNAs

Colonies hybridizing to the Sh2 probe were grown as 3 ml minipreps, plasmid DNA isolated, and inserts sequenced using the T3 and T7 primers flanking the cloning site as well as internal primers. In determining the initial coding sequences for cDNA SC5-1 we sequenced the corresponding region of the SC5-1 gene (data not shown). Sequences were compared to the GenBank database using the GCG version 7 program (Genetics Computer Group Inc., Madison, WI).

2.3. Synthesis of SC5 peptide

The 29 amino acid peptide SC5 was synthesized commercially by Quality Controlled Biochemicals (Hopkinton, MA) using solid phase Fmoc chemistry, and purified by RP-HPLC on a YMC ODS, 12 μm particle size, and a 120 Å pore size (YMC, Wilmington, NC). The mass of 3256 Da was verified by mass spectroscopy using a Voyager MALDI-TOF (Perseptive Biosys., Framingham, MA).

2.4. Analysis of SC5 antimicrobial activity

The chemically synthesized SC5 peptide was resuspended in sterile water and used directly for antimicrobial assays as described in the text. Initial antimicrobial assays were performed using the phoP mutant of *Salmonella typhimurium* [15] due to its increased sensitivity to antimicrobial peptides.

3. Results

3′ RACE analysis on adult sheep bone marrow RNA, using a primer based on conserved cathelin sequences, produced PCR products of approximately 600 base pairs in length. The products were gel-purified and blunt-end ligated into the pCR-Script vector. Plasmids containing a cathelin-related insert were identified by hybridization with a nested second oligonucleotide, representing additional sequences conserved within the cathelin gene family. Among the twelve hybridizing colonies characterized the 5′ sequences of all cDNA inserts demonstrated a high degree of conservation (data not shown), while the 3′ sequences demonstrated significant diversity. Three of

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The nucleotide sequence data reported in this paper have been registered in the EMBL Nucleotide Sequence Database under accession numbers X92758 for SC5-1 and X92757 for SC5-2.

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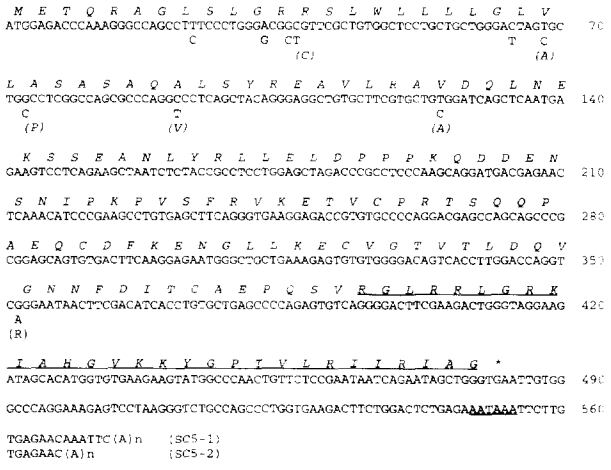

Fig. 1. Nucleotide and deduced amino acid sequences for the SC5-encoding 3′ RACE products SC5-1 and -2. Nucleotide numbering is along the right margin. SC5-1 cDNA sequence is presented, with single letter amino acid codes directly above in italics. Nucleotide substitutions and subsequent amino acid changes present in the SC5-2 sequence are indicated below the SC5-1 sequence. The initial 23 nucleotides of coding sequence could not be determined from the 3′ RACE analysis as the primer Sh1 spanned that region. Therefore, we determined the sequence of this region from the cloned SC5-1 gene. Predicted C-terminal cleavage product SC5 is underlined, as is the polyA addition signal sequence.

twelve inserts encoded a peptide homologous to the bovine BAC5 [9], four encoded a peptide homologous to the bovine cyclic dodecapeptide [4], and the remaining five inserts encoded a unique peptide labeled SC5. Two SC5-encoding cDNA inserts (SC5-1 and -2) were sequenced in their entirety on both strands (Fig. 1). SC5-2 differed from SC5-1 by 10 nucleotide substitutions resulting in six amino acid substitutions.

Analysis of the prepropeptide encoded by the SC5 cDNAs demonstrated a classical cathelin-like prepro region (Fig. 2). Based on the known valine processing site for such cathelin family members as the protegrins [16], PM23 [10], PR39 [14], BAC7 [11], and indolicidin [12] we predicted that the C-terminal cleavage product SC5 would be a 29 amino acid peptide with the sequence RGLRRLGRKIAHGVKKYGPTVLRIIRIAG. Preliminary analysis suggested that this peptide could assume

Table 1
Antibacterial activity of SC5

Organism (ATCC no.)	Minimal inhibitory concentration (μg/ml)
Staphylococcus aureus (25923)	5 –10
Pseudomonas aeruginosa (27853)	5 –10
Klebsiella pneumoniae (13883)	2.5–5
Candida albicans (14053)	10 –25

Single colonies of the indicated microorganisms were incubated in TSB at 37°C overnight. 2.5×10^4 cells were added to 0.2 ml of 0.25% TSB in individual wells of a 96 well microtiter plate. SC5 was added at concentrations of 2.5–50 μg/ml. Cells grown in the absence of SC5 served as a control. After incubation for 16 h at 37°C, O.D. 600 absorbance was used to determine relative cell numbers.

an amphipathic α-helical structure, similar to that of the linear magainin [17] and cecropin [18] peptides, and therefore might demonstrate antimicrobial properties.

Chemical synthesis of the SC5 peptide allowed us to address the following questions: (1) was it an antibacterial agent; (2) was it thermostable; (3) was it bactericidal; and (4) did it demonstrate a broad spectrum of antimicrobial activity?

The phoP mutant strain of *S. typhimurium* was chosen for our initial assay bacterium due to its relative sensitivity to antimicrobial peptides [15]. Fig. 3 shows that this strain is sensitive to SC5 in a plate assay and that preincubation of the peptide at 65°C does not alter the antibacterial effect. Figure 4 presents the results of a standard assay for bactericidal activity again using the phoP *Salmonella* mutant strain. SC5 showed significant cidal activity at concentrations as low as 1 μg/ml (0.3 μM/l).

Finally, a panel of Gram (+), Gram (−), and fungal organisms all demonstrated sensitivity to SC5 (Table 1) at minimal inhibitory concentrations comparable to those shown for the magainin and β-defensin peptides [19].

4. Discussion

A wealth of cathelin gene family cDNA and peptide data has contributed to our understanding of the complexity of myeloid-specific antimicrobial agents. A remarkable feature of this gene family is the degree of amino acid and nucleic acid conservation

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Cathelin                                  Z-LRYREAVLRAVDRLNEQSSEANLYRLLELDQPP-KADEDPGTPKPVSFTVKETVCPRP
Bovdode  METPRASLSLGRWSLWLLLLGLALPSASAQALSYREAVLRAVDQLNEQSSEPNIYRLLELDQPP-QDDEDPDSPKRVSFRVKETVCSRT
Bovbac5  ...Q........C.........V.....................F..R...A.L.......PT.-N..L..GTR..........D.P..
Bovind   .Q.Q................GLVV.....................L...A.L.......P..-K.N..LGTR..........V.P..
SC5-1    ...Q..G.....R.........V.A.....................K...A.L.......P..K....NSNI..P.........V.P..
SC5-2            ....C.................V..........A.....K...A.L.......P..K....NSNI..P.........V.P..

Cathelin TRQPPELCDFKE----KQCVGTVTLNPSIHSLDISCNEIQSV
Bovdode  TQQPPEQCDFKENGLLKRCEGTVTLDQVRGNFDITCNNHQSI RITKQPWAPPQAARLCRIVVIRVCR*
Bovbac5  S...L..........V.Q.V......PSNDQ...NC.EL..V RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPFPGRR*
Bovind   I...A.......K.RV.Q.V......PSNDQ..LN..EL..V ILPWKWPWWPWRRG*
SC5-1    S...A............E.V........GN......AEP..V RGLRRLGRKIAHGVKKYGPTVLRIIRIAG*
SC5-2    S...A............E.V.........N......AEP..V RGLRRLGRKIAHGVKKYGPTVLRIIRIAG*
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Fig. 2. The SC5 prepropeptide is a member of the cathelin gene family. Amino acid sequence alignment of the pig cathelin peptide [10], bovine cyclic dodecapeptide [4], bovine BAC5 [9], bovine indolicidin [12], and SC5 prepropeptides demonstrates the inter-species conservation present in the prepro piece. The predicted hydrophobic signal peptide includes the first 29 residues, while the anionic pro piece includes 101 amino acids in the bovine sequences and 102 amino acids in the sheep sequence. Mature peptide regions are presented in italics along with the predicted SC5 sequence. Initial eight amino acids of the SC5-1 prepropeptide were derived from the gene sequence.

present in the prepro region of genes both within a species and between species (pig, human, bovine, and rabbit) (reviewed [2]). We and others [7,10] have utilized this sequence conservation to construct oligonucleotide primers useful in isolating cDNAs encoding novel family members. The identification of a cDNA encoding sheep peptide SC5, a new and potent broad spectrum antimicrobial agent, attests to the valuable information which can be obtained via this approach.

The predicted SC5 29 amino acid peptide belongs to the family of cationic, cysteine-free, amphipathic peptides which have been characterized extensively in the case of the *Xenopus* magainins and the insect cecropins [3]. The presence of a terminal glycine could indicate that C-terminal amidation is involved in the final peptide product. The antimicrobial properties of SC5 demonstrated in this work are consistent with this agent acting as a broad-spectrum, bactericidal agent in vivo. In evaluating the amino acid structure of SC5, the presence of potential dibasic amino acid endopeptidase sites at positions 4–5, 8–9, and 15–16 of SC5 suggests that cleavage at one or more of these positions may be involved either in the production of a smaller mature peptide or as a mechanism for terminating SC5 activity. Antimicrobial assays involving shortened versions of SC5 will be of interest in this regard. A future direction of our research will be the documentation of possible SC5 processing in vivo utilizing SC5-specific antisera.

The presence of highly conserved prepro sequences in genes encoding markedly differing mature peptides predicts that this prepro piece plays an important functional role in the biology of the expressing cell type. Mutational analyses, such as those reported for the human defensin prepro sequence [20], should contribute to our knowledge of the functional significance of the conserved region. Additionally, the results of cathelin family chromosomal mapping studies as well as gene structure analysis may aid in suggesting the nature of the genetic mechanisms responsible for the derivation of this multigene family [14,16].

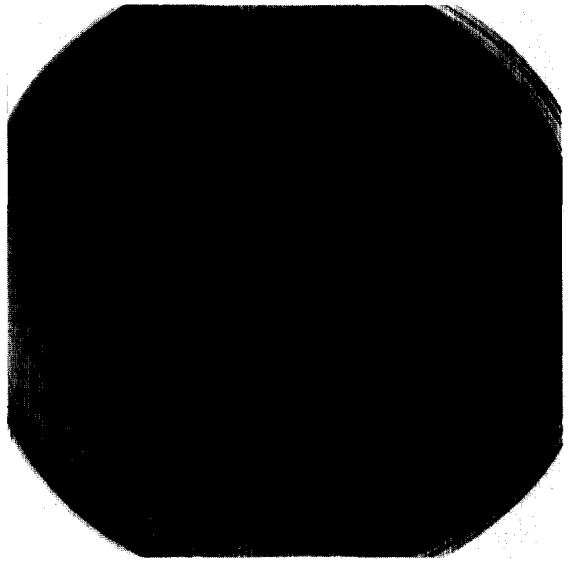

Fig. 3. SC5 activity detected using a well diffusion assay. Thin (1 mm) plates (0.5% tryptone, 0.75% agarose) were seeded with mid-logarithmic cells of the phoP mutant *S. typhimurium*. 2 mm diameter wells were created in the agarose and 2 μl of SC5 samples dissolved in sterile water were pipetted. Plates were incubated for 14–18 h at 37°C and activity was measured as the radius of the clearing zone. The single top well is a water control; middle and bottom wells are 2 μg/μl SC5 peptide samples incubated for 30 min at 65 and 37°C, respectively.

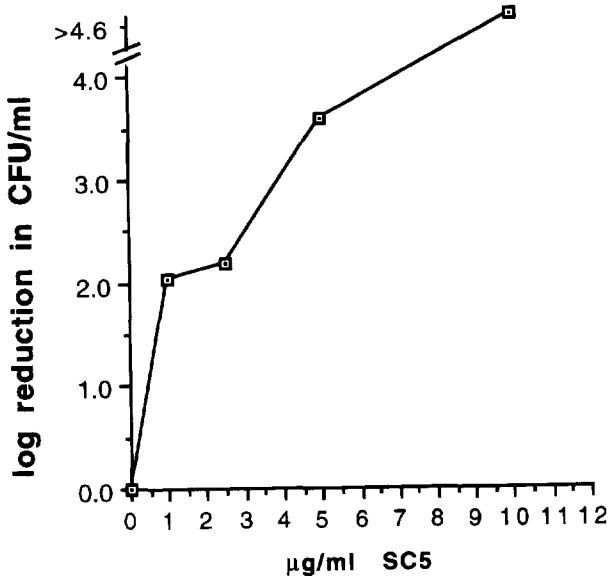

Fig. 4. Bactericidal effect of SC5. A single colony of the phoP mutant of *S. typhimurium* was grown overnight in trypticase soy broth (TSB) at 37°C. Mid-logarithmic cells were obtained by subculturing and incubating for an additional 4 h at 37°C. 1×10^6 cells were added to 100 μl of 10 mM sodium phosphate buffer, pH 7.4, and incubated for 2 h at 37°C in the presence of SC5. Cells incubated in the absence of SC5 served as an internal control. Dilutions were made of each sample and were plated on LB agar overnight at 37°C. Activity is expressed as $\log_{10}$ reduction in CFU/ml.

Investigating the structure of genes encoding myeloid antimicrobial peptides such as SC5 may lead to the identification of mechanisms for pharmacological regulation of their expression. Augmentation of endogenous host defense mechanisms, especially in immature or immune-compromised individuals, may prove to be beneficial in combating multi-drug resistant microorganisms.

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