CMLS, Cell. Mol. Life Sci. 60 (2003) 536–549
1420-682X/03/030536-14

CMLS Cellular and Molecular Life Sciences

Research Article

Phylogeny, processing and expression of the rat Cathelicidin rCRAMP: a model for innate antimicrobial peptides

S. Termén [a,*], M. Tollin [b], B. Olsson [a], T. Svenberg [c], B. Agerberth [b] and G. H. Gudmundsson [a,d]

[a] Microbiology and Tumor Biology Center, Nobels väg 16, Karolinska Institutet, 17177 Stockholm (Sweden), Fax: +46 8 328878, e-mail: stefan.termen@mtc.ki.se.
[b] Medical Biochemistry and Biophysics, Karolinska Institutet, 17177 Stockholm (Sweden)
[c] Department of Surgical Sciences, Karolinska Hospital, 17176 Stockholm (Sweden)
[d] Institute of Biology, University of Iceland, 101 Reykjavík (Iceland)

Received 4 December 2002; received after revision 17 January 2003; accepted 20 January 2003

Abstract. A database search identified a rat cDNA clone which phylogenetic analysis revealed to encode a cathelicidin most similar to mouse cathelicidin CRAMP. The analysis also showed that the evolutionary pattern of the cathelicidin family is lineage specific. The rat cathelicidin is called rCRAMP. Its peptide was isolated from granulocytes, and determined to be 43 amino acids long by mass spectrometry and N-terminal sequencing. Synthetic rCRAMP had antimicrobial activity. The expression of rCRAMP was investigated by reverse-transcriptase polymerase chain reaction followed by Southern hybridization and by Western blot analysis. rCRAMP was identified in granulocytes, thymus, testis, lung, mouth mucosa, tongue, oesophagus, colon, caecum and small intestine, a distribution similar to cathelicidins of mouse and human. The rat is a small laboratory animal with additional disease models available compared to the mouse. Our results open up the possibility to use the rat as a model system to study responses connected to cathelicidin expression in health and disease.

Key words. rCRAMP; rat; cathelicidin; antimicrobial peptide; phylogeny; processing; expression.

Cationic antimicrobial peptides kill bacteria by disrupting their cell membranes. They are part of our innate immunity and can be considered as endogenous antibiotics. The peptides are present on surfaces in contact with the outside environment, like the epithelia of the skin and the gut, and in phagocytic neutrophilic granulocytes, the first cells to be recruited from the bloodstream to sites of infection [1, 2].

The cathelicidins constitute a class of cationic antimicrobial peptides in mammals. They are synthesized as preproproteins and have a well-conserved cathelin-like proregion. The C-terminal domain of cathelicidins corresponds to the mature antimicrobial peptide [3, 4]. The other major class of cationic antimicrobial peptides in mammals is the defensins. The number of defensins and cathelicidins can vary between mammalian species, e.g. in pig, 11 cathelicidins are known, but only one defensin [5, 6]. In humans, there are ten known defensins, six of the α type and four of the β type, but only one cathelicidin, called LL-37 [7–9]. The cathelicidins in general have a widespread tissue distribution and are expressed in both epithelial cells and neutrophilic granulocytes.

The active peptides from different cathelicidins vary greatly in structure, and this probably reflects a need to keep up with their rapidly evolving microbial targets. They are non-homologous, and the proregion seems to be a handle onto which several different tools can be at-

* Corresponding author.

tached [10]. The peptides are released from the proregions through cleavage by proteases [11, 12].

To date, only a few studies on mammalian antimicrobial peptides have been done in vivo. LL-37 is upregulated in keratinocytes during inflammatory disorders [13] and downregulated by *Shigella* spp. in the colon epithelium [14]. For future studies, a murine model would be convenient. A mouse with the cathelicidin CRAMP knocked out is available through the work of Nizet et al. [15] who reported that the knock-out is more sensitive to skin infection than wild-type mice. A few other studies have been published on mouse cathelin-related antimicrobial peptide (CRAMP) [16–19]. However, the rat is also widely used in medical research and provides additional disease models. This work was conducted with the aim of setting a platform for the rat as a model system for cathelicidin research.

Other cationic antimicrobial peptides in the rat belong to the defensin family. Six are known, and include α-defensins RatNP 1–4 [20] and β-defensins RBD 1–2 [21]. Interestingly, while the rat has neutrophil defensins, the mouse lacks them [22].

We identified a rat cDNA clone through a database search using the cDNA sequence of LL-37 as a template. Here we provide evidence that the preproregion of the protein encoded by this rat cDNA is homologous to known cathelicidins, and hence establish the fact that it is a cathelicidin. We also display a phylogenetic tree to demonstrate its place in the cathelicidin family and the general structure of the family. The rat cathelicidin is called rCRAMP [23], a name analogous to CRAMP of the mouse. By isolating the rCRAMP peptide from granulocytes and subjecting it to mass spectrometry and N-terminal sequencing, we determined that the peptide released from the proprotein is 43 amino acids in length, and therefore longer than the mouse CRAMP peptide. We predict the structure of the rCRAMP peptide and examine its antimicrobial and cytotoxic properties. RNA expression and peptide/protein expression of rCRAMP are examined.

Materials and methods

Identification and sequencing of a rat clone with homology to cathelicidins

A rat cDNA sequence with homology to known cathelicidins was identified through a BLAST search (http://www.ncbi.nlm.nih.gov/blast/index.html) and the plasmid was obtained from Research Genetics (Huntsville, Ala.). Sequence confirmation of the cloned insert was performed using the ABIPrism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif.) and an ABI Prism 377 sequencer (Applied Biosystems).

Comparison of different cathelicidins by alignment of their preproregions and construction of a phylogenetic tree

Cathelicidin sequences available in the databases were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) and the Antimicrobial Sequences Database (http://www.bbcm.univ.trieste.it/~tossi/pag1.htm). The program ClustalX 1.8 [24] run on a Windows98 platform was used to make a multiple alignment of their preproregions, and the program package Phylip Version 3.5c (available at http://evolution.genetics.washington.edu/phylip.html) and Treeview 1.6.5 [25] were used to create phylogenetic trees, both neighbour joining and maximum parsimony, bootstrapped 1000 times. For a list of cathelicidin sequences used for the analyses see table 1.

Animals

Germ-free rats and their controls, i.e. rats born germ free but raised outside the germ-free environment, were adult Agus rats obtained from the facility for gnotobiology at the Karolinska Institute. Conventional adult Sprague-Dawley rats were used for all other experiments. The Stockholm North Ethical Committee on Animal Experi-

Table 1. Cathelicidin sequences used in analyses reported in this paper. All sequences are protein unless otherwise indicated.

Rat (*Rattus norvegicus*) rCRAMP (AF484553)
Human (*Homo sapiens*) LL-37 (P49913)
Rhesus monkey (*Macaca mulatta*) rhLL-37 (AAG40802.1)
Pig (*Sus scrofa*) PR-39 (P80054), PMAP-23 (P49930), prophenin-2 (P51525), PMAP-37 (P49932), PMAP-36 (P49931), protegrin-1 (S57607), protegrin-5 (S57609)
Cow (*Bos taurus*) BMAP-34 (P56425), BMAP-28 (P54229), BMAP-27 (P54228), indolicidin (P33046), Bac7 (P19661), Bac5 (P19660), dodecapeptide (P22226)
Mouse (*Mus musculus*) CRAMP (P51437)
Rabbit (*Oryctolagus cuniculus*) CAP18 (P25230)
Sheep (*Ovis aries*) Dodecapeptide (P54230), 7.5-kDa bactenecin (L46852.1) (mRNA), sheep 5-kDa bactenecin (AAB49716.1), SMAP-29 (S68228), bactinecin 6 (AAB61999.1), bactinecin 11 (AAB62000.1)
Horse (*Equus caballus*) eCATH-3 (CAA12228.1), eCATH-2 (CAA12227.1), eCATH-1 (CAA12226.1)
Goat (*Capra hircus*) MAP34-A (CAB45525.1), MAP28 (CAB45524.1), Bac7.5 (CAB45523.1)

ments granted ethical approval for the use of animals in this study.

Peptide synthesis

The full-length mature rCRAMP peptide, and two shorter versions denoted rCRAMP(10–43) and rCRAMP (13–37) were purchased from Innovagen (Lund, Sweden). Synthetic LL-37 was made in our laboratory as described previously [26]. The peptides had the following amino acid sequences: NH_2-RFKKISRL-AGLLRKGGEKFGEKLRKIGQKIKDFFQKLAPEIEQ-COOH (rCRAMP peptide), NH_2-GLLRKGGEKFGEK-LRKIGQKIKDFFQKLAPEIEQ-COOH [rCRAMP (10– 43)], NH_2-RKGGEKFGEKLRKIGQKIKDFFQKL-COOH [rCRAMP(13–37)] and NH_2-LLGDFFRKSKE-KIGKEFKRIVQRIKDFLRNLVPRTES-COOH (LL-37). The molecular weights of the peptides were confirmed by mass spectrometry, and high-performance liquid chromatography analysis (HPLC) showed one distinct peak for each peptide.

Preparation of antibodies against the rCRAMP peptide

Antiserum against synthetic rCRAMP(10–43) was obtained by a standard immunization scheme. Peptide (100 µg) was mixed with Freund's complete adjuvant and injected intramuscularly into a rabbit. A second dose was injected after 3 weeks and a third after an additional 10 days. Antiserum was collected and assessed by enzyme-linked immunosorbent assay. An IgG fraction was enriched on a protein-G-Sepharose column (Amersham Pharmacia Biotech, Piscataway, N. J.,), and affinity purified against rCRAMP(10–43) by Innovagen.

Isolation and characterization of granulocytic rCRAMP peptide

Granulocytes were isolated by peritoneal lavage of four Sprague Dawley rats. Intraperitoneal injection of 25 ml of a 1% glycogen solution was performed, and 4 h later the rats were anaesthetized intramuscularly and lavage was performed by washing the peritoneal cavity with 25 ml phosphate buffered saline (PBS) (pH 7.2). The lavage was cell filtered and centrifuged. The pellet was treated with distilled water for 1 min to lyse contaminating erythrocytes, centrifuged again and washed with PBS. Granulocyte purity was assessed by microscopy. The cells were degranulated by treatment with 20 µl of 0.2 mM 12-O-tetradecanoylphorbol-13-acetate in 20 ml NaCl/P_i at 37 °C for 30 min, centrifuged, and the supernatant was collected.

To enrich peptides and proteins, the supernatant was applied to OASIS columns (60 mg; Waters, Milford, Mass.). The columns had been activated with acetonitrile and then equilibrated in 0.1% trifluoroacetic acid (TFA). Unbound material was washed away with 0.1% TFA, followed by 10% acetonitrile in 0.1% TFA. The peptides/proteins were then eluted with 80% acetonitrile in 0.1% TFA and lyophilized.

To isolate the native rCRAMP peptide by reverse-phase HPLC, an ÄKTA purifier system (Amersham Pharmacia Biotech) was utilized and detection of the column effluent was performed at 214 and 280 nm. About 0.5 mg of lyophilized granulocyte granule eluate from OASIS was loaded onto a Vydac C18 (4.6 × 250 mm) column (The Separations Group, Hesperia, Calif.). Elution was performed with a gradient of 0–40% acetonitrile in 0.1% heptafluorobutyric acid in 20 column volumes (CV), followed by an isocratic gradient at 40% for 3 CV and finally 40–70% in 15 CV. Fractions (1 ml) were collected and lyophilized, redissolved in water, lyophilized again and dissolved in 50 µl 0.1% TFA. The rCRAMP peptide was then located by dot blot analysis where 1 µl of each fraction was spotted onto a Hybond C Super membrane (Amersham Pharmacia Biotech). rCRAMP peptide was detected on the membrane using the α-rCRAMP(10–43) antibody. Immunoreactive fractions were analysed by gel electrophoresis using 10–20% tricine gels and visualized by silver staining using SilverXpress (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions. One fraction was further analysed by mass spectrometry using a matrix-assisted laser-desorption/ionization instrument (Voyager-DEPRO; Applied Biosystems). The N-terminal sequence was determined by Edman degradation utilizing a PE-ABI Prosice 5HT 494 protein sequencer (Applied Biosystems).

Extraction of peptides and proteins from tissues

The tissues used were thymus, mouth mucosa, lung, tongue, testis, colon and oesophagus. Frozen tissue was ground into small pieces in a mortar with liquid nitrogen and then homogenized in 60% acetonitrile with 1% TFA using a Polytron PT 1200 CL (Kinematica, Cincinatti, Ohio). Peptides/proteins were extracted out in the same solution at 4 °C overnight. Undissolved material was removed by centrifugation and the supernatants were frozen and lyophilized. The dried material was dissolved in 0.1% TFA, and centrifuged. Peptides and proteins were enriched utilizing OASIS columns as described above.

Western blot analysis

Lyophilized peptide/protein extracts from tissues and granulocytes described above were incubated at 56 °C for 1 h followed by 95 °C for 5 min. The following steps were carried out at room temperature. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE) and blotting were performed using 10–20% tricine gels and polyvinylidene difluoride (PVDF) membranes according to the manufacturer's instructions (Invitrogen). The membrane was blocked for 1 h in 5% fat-free milk in PBS with 0.25% Tween and the antibodies used were the antibody against rCRAMP(10–43) described above

(1 : 1000 dilution) and a horseradish peroxidase-conjugated monkey anti-rabbit Ig (Amersham Pharmacia Biotech) (1 : 1000 dilution). To visualize the results, an enhanced chemiluminescence detection system was used (Amersham Pharmacia Biotech).

Inhibition zone assay

Thin 1% agarose plates (1 mm) of Luria Bertani medium (LB) containing 6×10^4 microbial cells/ml were poured and 3-mm wells were punched. Three types of LB medium were used: standard LB medium, LB without salt and LB supplemented with medium E [27]. The composition of medium E is 0.8 mM $MgSO_4$, 9.5 mM citric acid, 57.5 mM K_2HPO_4 and 16.7 mM $NaNH_4HPO_4$. The test microbes used were the bacteria *Bacillus megaterium* strain Bm11 and *Escherichia coli* strain D21, and the fungus *Candida albicans* (ATCC 14053). Of each peptide, 1.35 nmol were dissolved in 2.8 µl of 0.1% TFA and applied to the wells. After an overnight incubation at 30 °C, the diameters of the inhibition zones were measured.

Chromium release assay

The cytolytical effect of the synthesized peptides was determined by their ability to lyse ^{51}Cr- labelled YB2/0 rat cells (a gift from K. Andersson, MTC, Karolinska Institutet, Stockholm, Sweden). The cells were cultured in RPMI 1640 supplemented with 10% inactivated fetal calf serum, penicillin/streptomycin, 2 mM L-glutamine, 0.5 × Mem amino acids, 1 mM sodium pyruvate, 10 mM Hepes buffer (all from Sigma, St. Louis, Mo.) and 50 µM β-mercaptoethanol (Invitrogen). A triplicate twofold dilution of the peptides in 10 µl water and 140 µl cell culture medium containing 10^4 cells was made from 100 to 0.78 µM in 96-well microdilution plates, and the plates were then incubated for 4 h at 37 °C. Negative controls were cells in 140 µl medium and 10 µl of water without peptide, as well as cells subjected to a dilution series with bovine serum albumin (Pierce Biotechnology, Rockford, Ill.) with amounts ranging from 20 µg to 150 ng. As a positive control, the cells were lysed completely with 6.7% SDS. The amount of γ radiation released was measured in a CliniGamma 1272 or a 1470 Automatic Gamma Counter (Wallac Oy., Turku, Finland).

Reverse transcriptase-polymerase chain reaction followed by Southern blot hybridization

mRNA was isolated utilizing the kit MicroPoly(A)Pure (Ambion, Austin, Tex.), according to the manufacturer's instructions. Different rat organs (100 mg of each) were homogenized with a rotor-stator Polytron homogenizer in the first buffer of the kit (100 mg tissue/ml buffer). The RNA was DNase treated according to the DNA Free kit (Ambion) for 55–75 min.

The RNA samples (1.15 µl of each) were diluted to 10 µl and denatured for 5 min at 94 °C, and then chilled to 4 °C. Random hexamer primers and 200 units of Moloney leukaemia virus reverse transcriptase (RT) (Invitrogen) and other reactants recommended by the manufacturer were added to a total volume of 20 µl. First-strand synthesis was performed at 40 °C for 45 min, and the samples were then denatured at 94 °C for 5 min, followed by cooling to 4 °C. Controls without RT were included for all samples. In addition, negative controls without RNA were included.

The synthesized cDNA was used as template in separate polymerase chain reactions (PCRs). For amplification of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), the following commercial primer pair was used: 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ (Clontech, Palo Alto, Calif.). For rCRAMP, 5′-TGTGAGCCCCAAGGGGATGAGGA-3′ (spans the border between exon 1 and 2) and 5′-CCAAGGCAGGCCTACTGCTCTAT-3' (spans the border between exon 4 and the 3′ untranslated region) were used. cDNA template concentrations were adjusted to yield similar signal strength for the housekeeping gene G3PDH. PCR amplifications were performed with the following thermal-cycle profile: denaturation at 94 °C for 3 min, 30 cycles of amplification including denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min, followed by an extra extension step at 72 °C for 7 min.

The reaction mixtures were analysed in 1.5% agarose gels, and the DNA was blotted onto a Hybond N+ nylon membrane (Amersham Pharmacia Biotech) according to standard procedure [28]. The filters were then prehybridized for 4 h in 6 × standard saline citrate (SSC) and 5 × Denhardt's solution at 60–65 °C. Hybridizations were done overnight with radioactive probes under the same conditions as the prehybridization. The G3PDH probe was purchased from Clontech, and the rCRAMP cDNA was used as a probe for rCRAMP. The probes were labelled with ^{32}P using the Rediprime II Labelling System (Amersham Pharmacia Biotech) with random hexamer primers. After the hybridizations, the filters were washed at 60–65 °C, twice with 2 × SSC and 0.1% SDS for 15 min, and once with 0.1 × SSC for 10–15 min. The results were analyzed with a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, Calif.).

Results

Identification of an rCRAMP clone

A rat cDNA clone (GenBank accession number AA998531) with homology to LL-37 was identified through a database search and sequenced. Sequencing revealed several errors in the database sequence. The cDNA sequence coded for a preproprotein very similar to

mouse CRAMP, and the rat preproprotein was called rCRAMP. The annotated and corrected cDNA sequence has been submitted to GenBank and assigned the accession number AF484553 (fig. 1). The entire sequence of the preproprotein was not in the cDNA clone, so a few codons were lacking at the 5′ end, but could be identified in a large rat genomic sequence with the accession number AC121624 later entered into the database. The entire rCRAMP gene sequence is available in the genomic sequence. It differs from our cDNA sequence by three nucleotides, one in exon 1 and two in exon 3, possibly due to a polymorphism. The mature peptide sequences are identical.

Purification and N-terminal sequence of the mature peptide

To determine the processing site where cleavage occurs in order to liberate the active peptide, we isolated and characterized the mature peptide. Since cathelicidins are highly expressed in neutrophilic granulocytes, we isolated rat granulocytes and stimulated them to release their granule contents. Granule peptides and proteins were enriched on an OASIS column and separated by reverse-phase HPLC (fig. 2A). Fractions 133–138 reacted to the α-rCRAMP(10–43) antibody and were further analysed by SDS/PAGE and stained with silver. Material in fraction 135 gave rise to a band of about 5 kDa correspond-

```
1      ATGCAACCCCATAGGGACGTCCCTTCCCTGTGGCGCTCACTGTCA
        M  Q  P  H  R  D  V  P  S  L  W  R  S  L  S      15

46     CTGCTATTGCTCCTGGGCCTTGGGTTGCCTCTAaCCGTTTCCCAG
        L  L  L  L  L  G  L  G  L  P  L  T  V  S  Q      30

91     ACCCTCAGCTACAGGGAGGCTGTACTCCGTGCTGTGGATGACTTC
        T  L  S  Y  R  E  A  V  L  R  A  V  D  D  F      45

136    AACCAGCAGTCTTTGGACACCAATCTCTACCGTCTCCTGGACCTG
        N  Q  Q  S  L  D  T  N  L  Y  R  L  L  D  L      60

181    GATTCTGAGCCCCAAGGGGATGAGGACCCAGATACTCCCAAGTAT
        D  S  E  P  Q  G  D  E  D  P  D  T  P  K  Y      75

226    GTGAGGTTCCGAGTGAAGGAGACTGTCTGTAGCAAGGCATCACAG
        V  R  F  R  V  K  E  T  V  C  S  K  A  S  Q      90

271    CAGTTACCTGAGCAATGTGCCTTCAAGGAACAGGGGGTAGTGAAG
        Q  L  P  E  Q  C  A  F  K  E  Q  G  V  V  K     105

316    CAGTGTATGGGGACAGTCACCCTGAACCggGGCTGCAGAGTCTTTT
        Q  C  M  G  T  V  T  L  N  R  A  A  E  S  F     120

361    GACATCAGctGTGATGCGCCTGGTATACAGCCTTTCCGGTTCAAG
        D  I  S  C  D  A  P  G  I  Q  P  F  R  F  K     135

406    AAAATTTCCCGGCTGGCTGGACTCCTCCGAAAAGGTGGAGAGAAG
        K  I  S  R  L  A  G  L  L  R  K  G  G  E  K     150

451    TTTGGTGAAAAGCTTAGGAAAATCGGCCAGAAAATTAAGGATTTT
        F  G  E  K  L  R  K  I  G  Q  K  I  K  D  F     165

496    TTCCAGAAACTTGCACCTGAAATAGAGCAGTAGGCCTGCCTTGGC
        F  Q  K  L  A  P  E  I  E  Q  *                 175

541    CTGTTTTTGGATTCCCAAAATAATAAACTTGGTAAAAGAAA(A)n
```

Figure 1. The cDNA and translated sequence of rCRAMP. The sequence of the antimicrobial peptide is underlined. The boldface nucleotides and residues at the 5′ end were not in the clone we worked with and this information comes from GenBank sequence AC121624. In the rest of the sequence, lowercase nucleotides are different in AC121624.

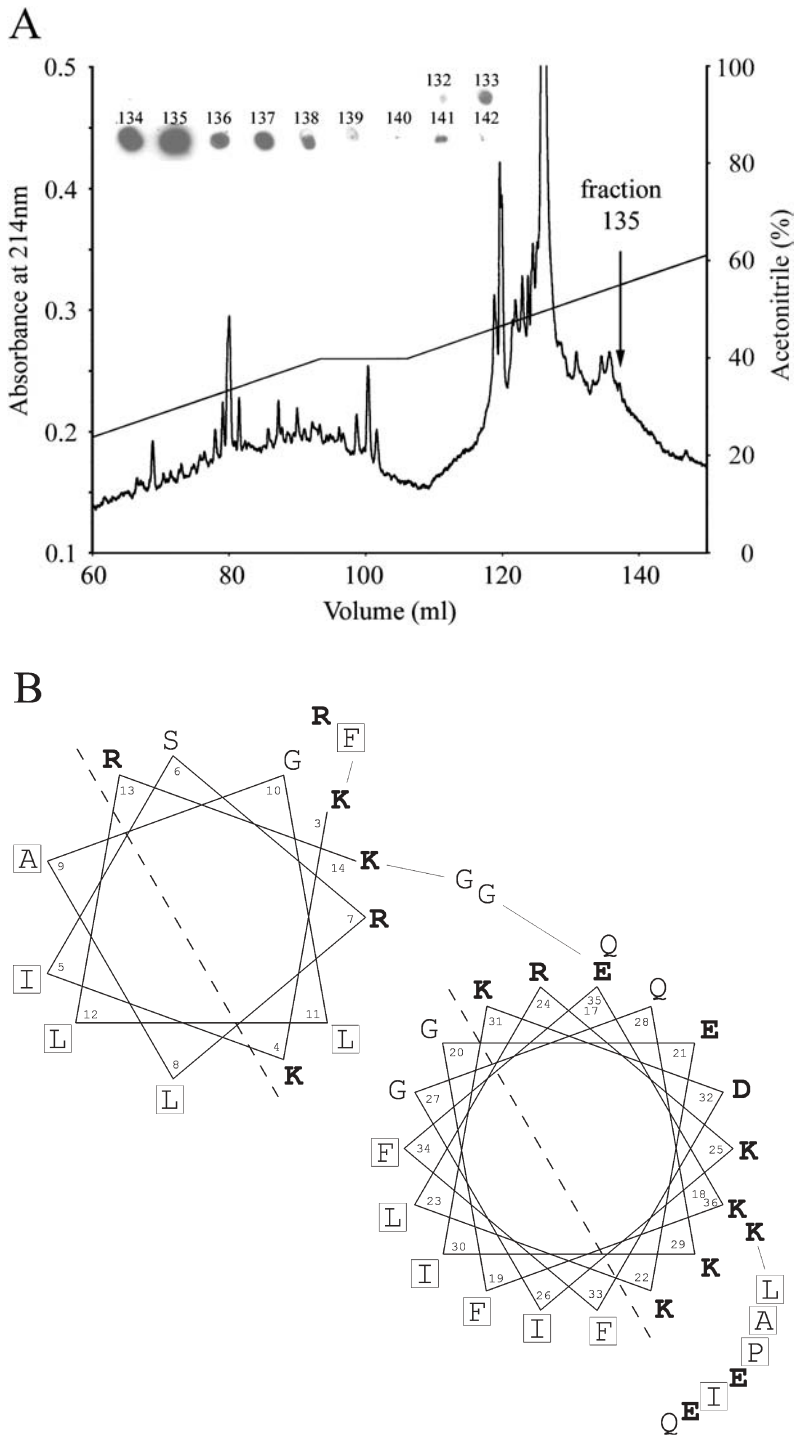

Figure 2. (*A*) Reverse-phase HPLC of granulocyte granule proteins/peptides. The result of a dot blot analysis of indicated fractions for rCRAMP peptide is inset. (*B*) Edmundson wheel presentation of rCRAMP peptide. Charged residues are indicated in boldface, and hydrophobic residues are boxed. The amphipathic α helices are shown as predicted with the program Jpred2.

ing to a signal detected with Western blot analysis (see below). Mass spectrometry of the material in this fraction gave a mass value ($M+H^+$) of 5033 Da. This correlates well with a peptide of 43 amino acids starting at position 133 in the full-length protein, which would theoretically give a mass value ($M+H^+$) of 5032 Da. N-terminal sequencing of the peptide identified the amino acids in positions 1–15 as RFKKISRLAGLLRKG. The determined sequence corresponds to positions 133–147 in the predicted full-length preproprotein and confirms the mass spectrometric data. We thereby demonstrated that the mature rCRAMP peptide liberated from granulocytes is 43 amino acids in length and has the sequence NH_2-RFKKISRLAGLLRKGGEKFGEKLRKIGQKIKDFFQ-KLAPEIEQ-COOH.

We used the program Jpred2 (http://www.compbio.dundee.ac.uk/~www-jpred) to predict the secondary structure of the rCRAMP peptide. The program is trained on globular proteins that have been solved by X-ray crystallography and takes the results of several structure prediction programs and makes a consensus structure. It suggested a structure of two amphipathic α helices connected by a glycine-glycine hinge, followed by a linear part (fig. 2B). This correlates with the results of Yu et al. [19] who used nuclear magnetic resonance spectroscopy to determine that mouse CRAMP peptide forms two similarly hinged α helices in 50% trifluoroethanol (TFE). The helix-hinge-helix feature is also shared with several other antimicrobial peptides. However, circular dichroism analysis had revealed that CRAMP forms a random structure in aqueous solution [19]. Our preliminary circular dichroism data (not shown) indicate that rCRAMP similarly forms an α-helical structure in 40% TFE and a random structure in water.

Alignment and phylogenetic tree

Cathelicidins have a conserved preproregion but the sequences of the peptides are highly variable. To investigate the relationship between rCRAMP and known cathelicidins, we aligned its preproregion to that of known cathelicidins (fig. 3A) and from the alignment we constructed a neighbour-joining phylogenetic tree (Fig. 3B). A maximum-parsimony phylogenetic tree was also constructed and the branches placed themselves in a comparable manner (data not shown). The alignment illustrates how well conserved the preproregions of this protein family are. The tree further illustrates how the preproregions of the cathelicidins have followed expected patterns in evolution [29] when considering the amino acid sequence. The mature peptides of the cathelicidins, on the other hand, are highly variable in structure and non-homologous, and therefore do not lend themselves easily to phylogenetic analysis. This analysis was done before we knew the correct processing site of rCRAMP and assumed it to be the same as has been described for mouse CRAMP [17, 18]. However, this makes little difference to the appearance of the tree.

Tissue distribution of rCRAMP

The expression of rCRAMP was examined with conventional non-quantitative RT-PCR followed by Southern hybridization (fig. 4). In an untreated rat, we detected expression in colon, lung, testis and thymus. Interestingly, in contrast to human, there was no expression in the epididymis.

To confirm the results of the rCRAMP expression in tissues obtained on the mRNA level, we also studied expression at the peptide level with Western blot analysis. The expression of rCRAMP peptide was lower in tissues than in granulocytes, but we could detect it in thymus, mouth mucosa, lung, tongue, colon and oesophagus (fig. 5). The peptides detected in the tissues were larger than synthetic rCRAMP(10–43) that we originally used as a reference, indicating that the 43-amino-acid peptide is the main variant in tissues. In colon and oesophagus, a second peptide signal was detected, possibly corresponding to an oligomer or a differentially processed form. In addition, a signal approximately corresponding to the size of the precursor was detected in granulocytes, thymus and lung. In lung, the blood content is high, so the rCRAMP peptide expression there could originate from granulocytes.

rCRAMP expression in organs from fasted and germ-free rats

We hypothesized that rCRAMP expression would be turned off by fasting, since we had seen that there was less total antimicrobial activity in protein extracts from the intestine of fasted rats compared to controls (unpublished data). Fasted rats are used to study translocation of bacteria across the intestinal wall, something that often occurs in patients following surgery, and which can lead to sepsis. However, the mRNA expression of rCRAMP was not turned off in rats fasted for 24 h (fig. 6A).

In addition, we examined germ-free rats and detected expression of rCRAMP in the lung and gastrointestinal tract (fig. 6B). Consequently, contact with live bacteria is not required for rCRAMP expression.

Structure and function

To study the structure-function relationship of rCRAMP, the full-length peptide, and two truncated versions, rCRAMP(10–43) and rCRAMP(13–37), were synthesized. rCRAMP(10–43) lacks two-thirds of the α helix on the N-terminal side of the hinge. rCRAMP(13–37) lacks almost all of the α helix on the N-terminal side of the hinge and also the C-terminal linear part of the peptide.

The antimicrobial activity of the three peptides was assessed in inhibition zone assays (fig. 7A). The antibacte-

A

```
Cow_Bac5                    1   METQRASLS--LGRCSLWLLLLGLVLPSAS-AQALSYREAVLRAVDQFNERSSEANLYRLLELDPTP-NDDLDPGTRKPVSFRVKETDCPRTSQQPLEQCDFKENGLVKQCVGTVTLDPSNDQFDINCNE--LQSV---------- 130
Sheep_5_kDa_bactinecin      1   ....G.........W.............................G.L.................A....EV..............V....T...P.E.......................................... 130
Cow_Indolicidin             1   .Q............W..........V.....................L..L.............P..K.NE.L........T....V....I...A.......K.R.................L.......................... 130
Sheep_bactinecin_6          1   ....M.N...................L.....................G.L..K...V..........P..K.VE.W.A...........V...MT...P.....................LS....D...GLD.....F.................. 130
Sheep_bactinecin_11         1   ....M.N...................L.....................G.L..K...V..........P..K.VE.W.A...A.......V...MT...P.....................S.........L.......................... 130
Sheep_7.5_kDa_bactinecin    1   ....M..P..................L.....................G.L..K...V..........P..K.AE.Q.A...........V...M....P.....................S..T...E..L.......................... 130
Goat_Bac7.5                 1   ........................L.....................G.L..K...V..........P..K.VE.Q.AQ..........V........P.....................S.........L.......................... 130
Cow_Bac7                    1   ..............W.............................RI.................P..K.VE.R.A...T..T....V.....P..P.....................I...Q.D.L..L.......................... 130
Cow_BMAP-34                 1   .......F......S..........V.......D.............................P.EQ.VEH..A......T....V....TP..P.....................RYWIRGD...T..N..I..-.............. 130
Goat_MAP34-A                1   ......G.......W..C..............RPF.....................A.............P.EQ.AE.Q.A...........V.........V.....R..............RYWIRGD...TR.D..I..-.............. 130
Cow_BMAP-28                 1   ..............W.........A.....................L..K.................P.KE..EN.NIP..........V.........SP...........L.E........QVGSN...T.AV..P.................. 131
Sheep_SMAP-29               1   ..............R...........A..R.....................L..K.............P.KQ..ENSNIP..........V.........A...........L.E........QVGNN...T.A...P.................. 131
Cow_BMAP-27                 1   ..............W.........A.....................................P.KE..EN.NIP..........V.........A.....................AVKGKINVT.E..................... 131
Goat_MAP28                  1   ..................................G....H...RI..Q.............P.K-..EN.NIP..........V.....R..T.....................AVKGKMN.T.E.....-.............. 129
Cow_Dodecapeptide           1   ...P..........W.........A.....................L..Q...P.I.......QP.Q-..E..DSP.R........V.S..T...P...........L.R.E......QVRGN...T..N..H..IRITKQPWAPPQAA.. 143
Sheep_Dodecapeptide         1   ........................A........V.............L..Q...P.I.......QP.Q-..E..DSP.R........V....T...P...........L.R.E......QVRGN...T..N..H..IRITKQPWAPPQAA.. 143
Pig_PR-39                   1   ........C.....W..........V.....................RL..Q.............QP..KA.E....P.....T....V....PTR..P.L.......R..........N..IHSL..S.....I.................. 130
Pig_PMAP-36                 1   ........C.....W..........V.....................RL..Q.............QP..KA.E....P.....T....V...PTUR.P.L.......R..........N....PL....D...I..-.............. 129
Pig_Prophenin-2             1   ........C.....W........A..V.....................RL..Q.............QP..KA.E....P.....T....V...PTRR.P.L.......R..........QIK.PL..T...G.VRRFPWWWPFLRRPRLRRQ 146
Pig_PMAP-37                 1   ........C.....W........A..V.....................RL..Q.............QP..KA.E....P.....T....V...PTUR.P.L.......R..........QIK.PL..T.....I.................. 130
Pig_Protegrin-1             1   ........C.....W........A..V.....................RL..Q.............QP..KA.E....P.....T....V...PTR..P.L.......R..........QIK.PL..T.....V.G.............. 130
Pig_Protegrin-5             1   ........C.....W..........V.....................RL..Q.............QP..KA.E....P.....T....V...PTR..P.L.......R..........QIK.PL..T.....V.G.............. 130
Pig_PMAP-23                 1   ........C.....W..........V.....................RL..Q.............QP..KA.E....P.....T....V...PTR..P.L.......R..........KEIRGN...T..Q.................... 130
Horse_eCATH-3               1   .....NTRC.....W.PL.......I.P.T.T.....K........GL.Q...DE.........L..KG.K.SD.P.....M....V...IMK.TP..................I...VK.Y..AS.D...P.R.............. 130
Horse_eCATH-1               1   .....NTRC.....W.PL.......I.P.T.T.....K........GL.Q...DE.........L..KG.K.SD.P.....M....V...IMK.TP..................I.G.VK.M..VS.G...P.R.............. 130
Horse_eCATH-2               1   .....D.C......W..L.......I.L.T.T.T...K........GL.Q...DE.........L..KE.E..D.P.....T....V....T.....E..................V...AK.Y...S.DK..P.PI.............. 130
Rabbit_CAP18                1   ...HKHGP....AWW..L......LM.P.I...D.T..........A..QQ..........SM..QQ.LE.AK.Y.PQ....T....E....TWKLP.......D....R......RYQAW.S...R..RA.QE.PEPT............ 134
Human_LL-37/hCAP18          1   .K...DGH......W..V.......M.L.II..V...K......I.GI.Q...D.......D...R..TM.G..D.P.....T....V....T..SP.D....KD....R.M.....NQARGS...S.DKD.NKR-.............. 131
Rhesus_monkey_rhLL-37       1   .K...DGH......W..V.......M.L.II..V...K......I.GI.Q...D.......D...R..TM.G..D.P.....T....V....T..SP.D....KD....R.M.....NQARGS...S.DKD.NKR-.............. 131
Rat_rCRAMP                  1   ----.DVP....W.SLSL......G..LTV.S.T............D..QQ.LDT......D..SE..QG.E..D.P.Y.R......V.SKA...LP...A...Q.V....M.....NRAAES...S.DAPGI.PFRFKKISRLA...... 137
Mouse_CRAMP                 1   .QF..DVP.LW.W.SLSL......GF----.S.TP...D.......D..QQ.LDT......D...E..QG.E..D.P.S.R......V.GKAER.LP...A...Q.V....M.A...N.AA.S...S...PGA.PFRFKKISRLA...... 139
Clustal Consensus                       .  *      ****.* .     ::  :* :***:*:. :*: * : *:****.:*        .    : . * **** * :   :  * * *::.* :*.* *::        :.        .
```

Figure 3. Phylogenetic analysis of rCRAMP. (*A*) Alignment of cathelicidin preproregions with ClustalX 1.8, and presented using BioEdit 5.0.9. The preproregions of cathelicidins are remarkably well conserved. In the sequences, – indicates a gap and . indicates that the amino acid or gap in that position is the same as in the uppermost sequence. In the Clustal consensus line, * indicates a single fully conserved residue, : indicates conservation within a 'strong' group of amino acids and . indicates conservation within a 'weak' group of amino acids, as defined by the Gonnet Pam250 matrix. (*B*) Unrooted neighbour-joining phylogenetic tree made from the alignment, bootstrapped 1000 times. Many different trees can be constructed from the same alignment and the bootstrap value at each branch represents in how many out of 1000 constructed trees, the branch places itself there. The higher the value, the more probable that true phylogeny has been found. Neigbour-joining is a distance-based method, and with the given scale that indicates 0.1 amino acid substitutions per site, the close relationship of the cathelicidins can be seen. The tree was made with the Phylip package version 3.5c, and presented using TreeView 1.6.5. rCRAMP was the most closely related to CRAMP of mouse.

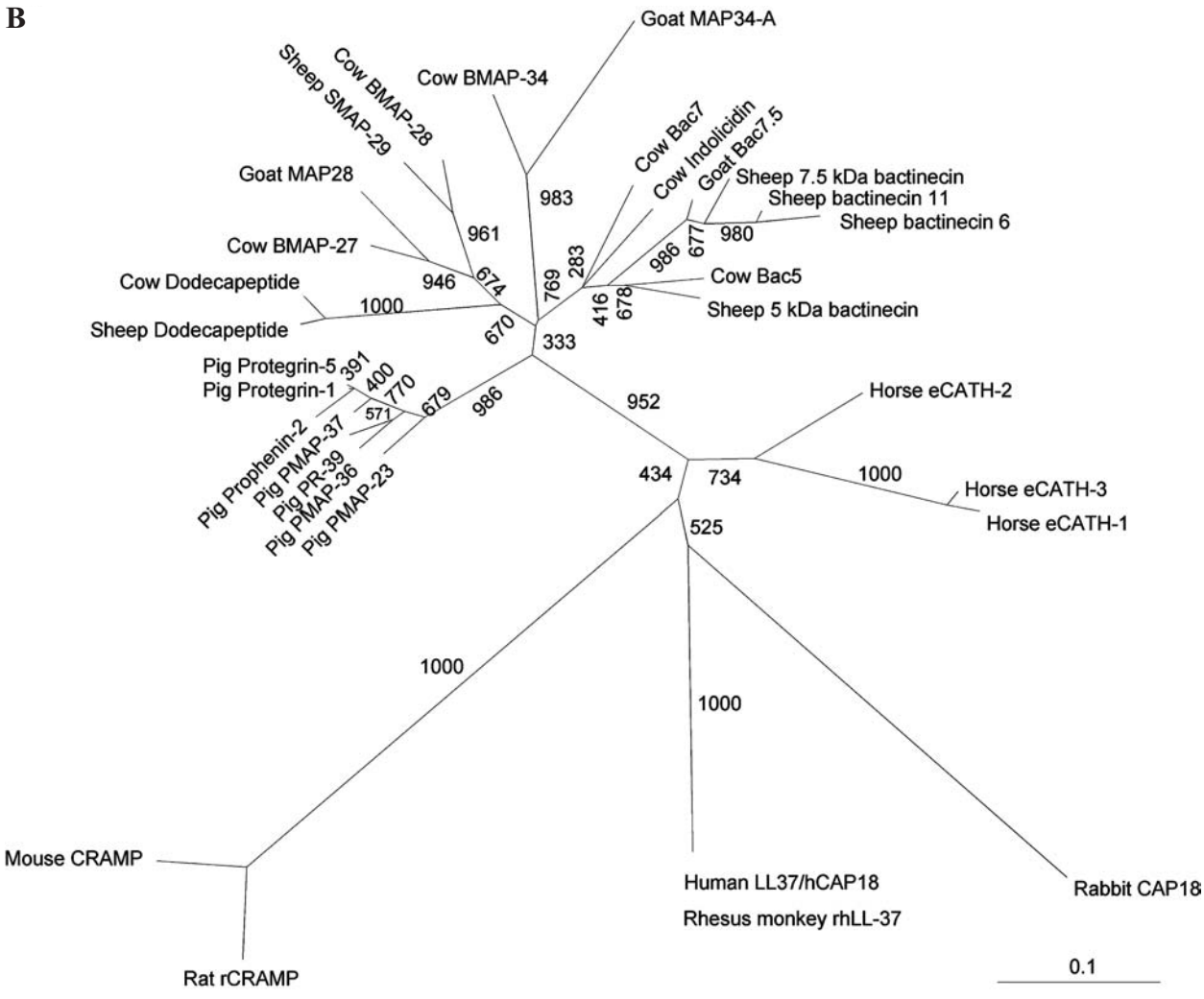

Figure 3 (continued)

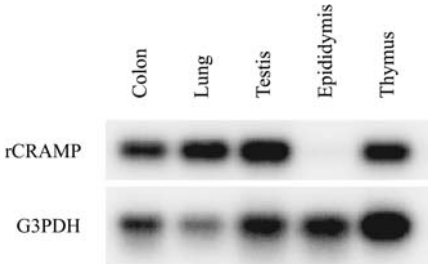

Figure 4. Southern hybridization of RT-PCR of rCRAMP for indicated rat organs. There was expression in colon, lung, testis and thymus.

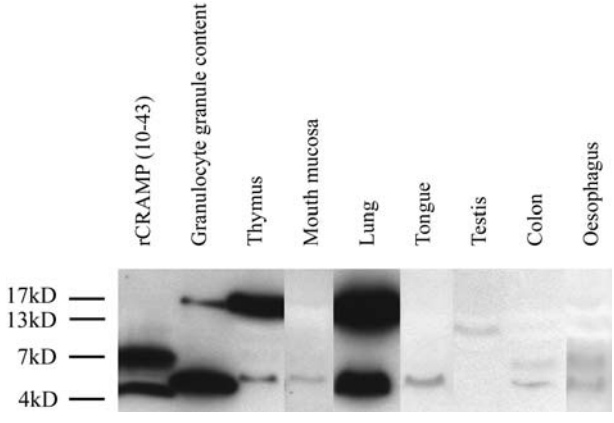

Figure 5. Western blot of the rCRAMP peptide. The rCRAMP peptide could be stained in extracts from granulocytes, thymus, mouth mucosa, lung, tongue, colon and oesophagus. We loaded 1.5 µg of granulocyte granule content, 100 μg of mouth mucosa, 150 µg of all other tissues and 5 ng of rCRAMP(10–43). The figure represents a combination of blots. Mouth mucosa, tongue and oesophagus have been pasted in. The upper band for rCRAMP(10–43) is possibly an oligomer.

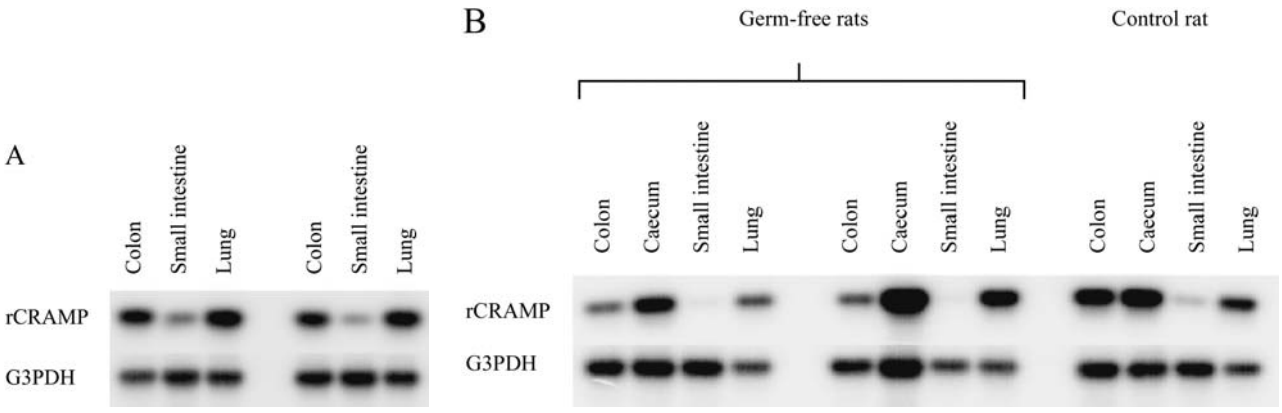

Figure 6. RT-PCR followed by Southern hybridization of organs from two fasted rats (*A*) and two germ-free rats (*B*) plus a control rat. The control rat was born germ free but raised outside the germ-free environment. These rats also expressed rCRAMP.

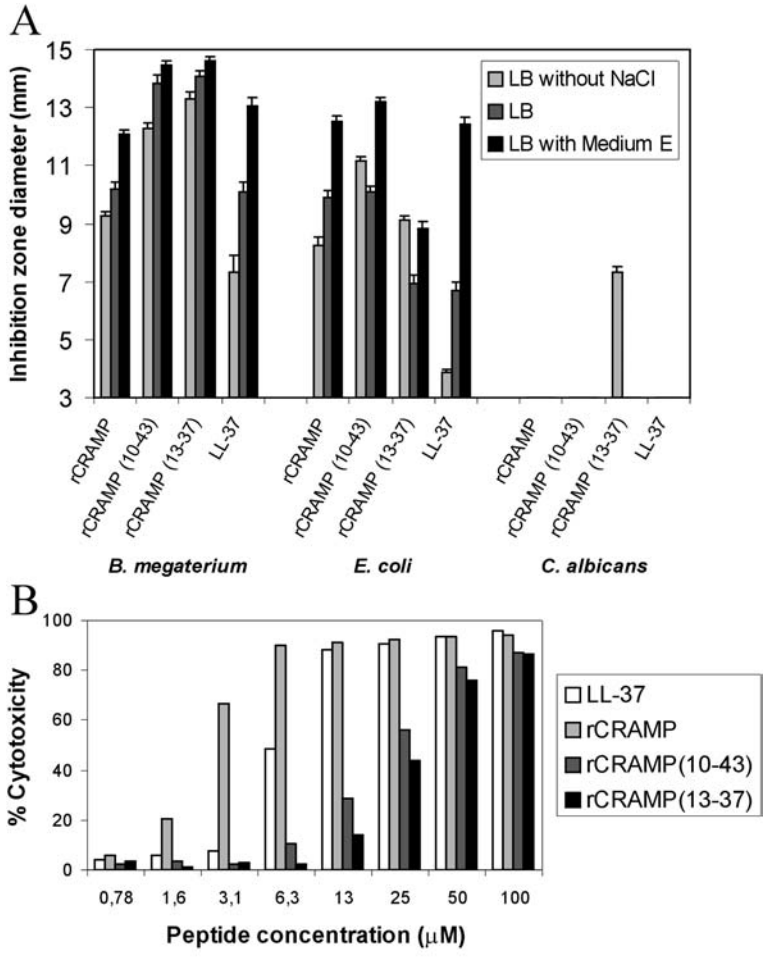

Figure 7. Activity of rCRAMP peptide, rCRAMP(10–43), rCRAMP(13–37) and LL-37. (*A*) Antimicrobial activity tested in an inhibition zone assay. The test organisms were a Gram-positive bacterium, a Gram-negative bacterium and a yeast. LB without NaCl indicates LB medium without salt (low salt), LB indicates standard LB (standard salt), and LB with Medium E indicates standard LB supplemented with medium E (high salt). The experiment was performed in quadruplicate and the zones were measured twice yielding eight values per experimental point. Standard deviations were calculated according to the n–1 method. All peptides were antibacterial, and rCRAMP(13–37) was antifungal in LB medium without salt. (*B*) Cytotoxicity test on YB2/0 rat cells. The 100% level of lysis was achieved with 6.7% SDS. rCRAMP peptide was the most cytotoxic followed by LL-37, rCRAMP(10–43) and then rCRAMP(13–37). The bars represent average values from two experiments.

rial activity was tested against the Gram-negative bacterium *E. coli* strain D21 and the Gram-positive bacterium *B. megaterium* strain Bm11. Antifungal activity was tested against the yeast *C. albicans*, a major opportunistic fungal pathogen [30]. The media used were standard LB medium, LB without salt and standard LB supplemented with medium E [27], a salt-containing medium known to promote helix formation of LL-37 and thereby its antimicrobial activity [26]. The only peptide that had antifungal activity was rCRAMP(13–37), and only in medium without salt.

For *B. megaterium*, more salt in the medium resulted in higher activity. The truncated peptides had similar activities and they were higher than the activity of the full-length rCRAMP peptide. The activity of rCRAMP peptide was comparable to the activity of LL-37.

For *E. coli*, rCRAMP peptide and rCRAMP(10–43) had similar activities, except that rCRAMP(10–43) had higher activity than rCRAMP peptide in LB without salt. The activity of rCRAMP(13–37) was similar to rCRAMP peptide in salt-free medium, but with salt the activity was lower. In the presence of medium E, the activities of rCRAMP peptide and LL-37 were equal, but without medium E, rCRAMP peptide was more potent than LL-37. For both bacteria, the activities of rCRAMP peptide and its derivatives were less dependent on salt concentration than that of LL-37.

To investigate whether rCRAMP peptide and its shorter derivatives are toxic to mammalian cells, we radioactively labelled cells of the rat cell line YB2/0 with the chromium isotope ^{51}Cr and exposed them to serial dilutions of each peptide (fig. 7B). The amount of radioactivity released compared to that of fully lysed cells resulted in the percentage of lysis. LL-37 was included for comparison. Exposure of the cells to bovine serum albumin up to 20 µg lysed the cells only slightly above background (data not shown). In repeated experiments, rCRAMP peptide was more cytotoxic than both rCRAMP(10–43) and rCRAMP(13–37). The shortest of the three rCRAMP peptides was also the least cytotoxic. rCRAMP peptide was more cytotoxic than LL-37 to rat cells.

Discussion

Phylogeny

The amino acid sequences of rat rCRAMP and mouse CRAMP [17] are very similar. Sequence identity of their entire proteins is 84% (calculated using BioEdit 5.0.9, http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The sequence identities of the preproregions of rat rCRAMP and mouse CRAMP compared to the preproregion of LL-37 are 60 and 52%, respectively.

The phylogenetic tree also shows that rCRAMP is indeed the most closely related to mouse CRAMP. Both are fairly closely related to the human LL-37 and its identical counterpart Rhesus monkey rhLL-37, with rabbit CAP18 being somewhat nearer (fig. 3B). The horse cathelicidins group together, as do the pig cathelicidins, while those of cow, sheep and goat cluster together in one big branch. This suggests that the evolution of the proteins in the cathelicidin family has been species or lineage specific. For cow, sheep and goat, the diversification into specific forms of cathelicidin seems to have occurred before the separation of these species. The antimicrobial peptides themselves were not included in the alignment because they are not homologous, albeit they are analogous. Variation among them may have arisen through exon shuffling.

In species where only one cathelicidin is known, its secondary structure has been reported to be an amphipathic α helix (e.g. in human [26]). This secondary structure seems to be important enough not to have been lost during the course of evolution and our secondary-structure prediction for rCRAMP peptide indicates that the rat cathelicidin also forms this structure. An amphipathic α helix is not crucial for antimicrobial activity [31], and one may speculate that this structure is connected to other activities such as receptor binding. Human LL-37 binds to the chemotactic receptor FPRL1 expressed by neutrophilic granulocytes, monocytes and T cells [32].

Activity of the processed rCRAMP peptide

The antibacterial activity of rCRAMP peptide is similar to that of LL-37 in high-salt medium. When tested in lower salt concentrations, the activity of LL-37 drops more than that of rCRAMP peptide. rCRAMP peptide seems to be less dependent on salt than LL-37 to form its α helix.

We hypothesized that rCRAMP(13–37) would be the most cytotoxic of the peptides since it was the only peptide active against the eukaryote *C. albicans*. That antifungal activity was, however, only detected in low-salt medium. rCRAMP(13–37) turned out to be the least cytotoxic of the tested peptides, probably due to the fact that cell culture media contain physiologic salt. rCRAMP (10–43) contains the extra N-terminal GLL compared to rCRAMP(13–37) and these residues could serve as a hydrophobic anchor, enhancing its cytotoxicity compared to rCRAMP(13–37). Full-length rCRAMP was the most cytotoxic of the three rCRAMP peptides, and it can form the longest α helix. Other studies have concluded that hydrophobic anchors and/or high α helicity are the major factors related to mammalian cytotoxicity [31].

The 43-residue rCRAMP peptide is cleaved off from the proprotein following a phenylalanine residue. The enzyme involved is likely cathepsin G. This enzyme is present in rat granulocyte granules and cleaves proteins C terminally of phenylalanine [33]. The mature peptide of mouse CRAMP extracted from bone marrow has been

shown to be 34 amino acids long, and mouse CRAMP has therefore been proposed to be cleaved off at an elastase site [17, 18]. Elastase and cathepsin G are both present in the azurophilic granules of neutrophilic granulocytes in mammals.

It is curious that the mouse CRAMP and rat rCRAMP should be processed differently when their primary structures are so similar (90% similarity calculated with BioEdit 5.0.9 using the Gonnet matrix). Cleavage of rCRAMP at the corresponding elastase site would result in the shorter rCRAMP(10–43). According to our results, this shorter peptide was actually more antibacterial and less cytotoxic than the full-length peptide. The shortest peptide, rCRAMP(13–37), was even less cytotoxic but also clearly less active against *E. coli*. The antifungal activity of rCRAMP(13–37) would be of little importance in the rat lumen since this activity is only seen under salt-free conditions. However, our results suggest that the cathepsin G cleavage site is the correct one. We have isolated rCRAMP peptide from degranulated granulocytes and our Western blot analyses of tissues support a long peptide. The peptide was in the same position in material from tissues from two different strains of rat, Sprague-Dawley (fig. 5) and Agus (data not shown). If the rat cathelicidin has two processing sites, the longer peptide predominates, since the short form does not turn up in Western blot analysis. If mouse CRAMP is isolated from the supernatant of degranulated mature granulocytes and not from bone marrow, it might prove to be processed at its cathepsin G site. Another possibility is that the differential processing better meets the potential threat of the particular pathogens each of the two murine species is likely to encounter.

During work on this paper, two studies have been published that compare the antimicrobial activities of CRAMP and rCRAMP, and rCRAMP was then synthesized based on the same unannotated cDNA clone that we identified. In one of the studies [23], 39-residue CRAMP and rCRAMP peptides were used, the ones that will result if they are cleaved off after potential dibasic cleavage sites. In the other report [34], 34-residue peptides were used, and CRAMP and rCRAMP were tested against the Gram-negative bacterium *Pseudomonas aeruginosa*, against which low activity (MIC_{50} 32 µg/ml) was detected. Notably, both of these studies used rCRAMP peptides with three substitutions according to our sequencing of the cDNA plasmid. Our sequence for rCRAMP (10–43) was NH_2-GLLRKGGEKFGEKLRKIGQKIK-DFFQKLAPEIEQ-COOH, while Saiman et al. [34] used NH_2-GLVRKGGEKFGEKLRKIGQKIKEFFQKLALE-IEQ-COOH, and the 39-residue peptide of Travis et al. [23] had the same substitutions. The change from a proline residue to a leucine residue could affect the function of the peptide.

Expression

The results of the rCRAMP expression mapping are summarized in table 2, where they are also compared to what is known about cathelicidin expression in humans [8, 9, 13, 14, 35–39], the rhesus monkey [40] and mouse [17,

Table 2. Detection of cathelicidin expression in humans, rhesus monkey, mouse and rat.

	Human	Rhesus monkey	Mouse	Rat
Bone marrow	✓		✓	
Granulocytes	✓		✓	✓
Lymphocytes	✓			
Monocytes	✓			
Thymus	✓			✓
Spleen			✓	
Skin	✓	✓ (epidermis)		
Tongue	✓	✓ (parotid gland)		✓
Mouth mucosa				✓
Airways	✓ (trachea)	✓		
Lung	✓			✓
Oesophagus	✓			✓
Stomach	✓	✓	✓	
Small intestine	✓	✓	✓	✓
Colon	✓	✓	✓	✓
Caecum				✓
Pancreas	✓	✓		
Kidney	✓	✓		
Bladder	✓	✓		
Mammae		✓		
Uterus	✓	✓		
Testis	✓*		✓	✓
Epididymis	✓			

* Expression in human testis could be contamination from epididymis.

18]. Not all organs in the table were investigated for all four species, but the overall tissue distribution appears to be similar in these species.

We detected expression in the thymus. Bals et al. [36] have previously reported expression of LL-37 in the human thymus detected by mRNA dot blot analysis. The thymus is an organ not directly exposed to potential pathogens, so the function of cathelicidin expression there is not obvious. The cathelicidins have other functions besides killing bacteria, e. g. they also exert chemotactic activity [32, 35], and so the function in the thymus does not have to be microbicidal.

Furthermore, we detected rCRAMP expression in the testis but not in the epididymis of the rat. Human testis was first reported to express LL-37 [8, 36]. However, in a more detailed study there was no expression in the testis, but very clear expression in the epididymis [38]. In our study, we could not detect the rCRAMP peptide in rat testis by Western blot analysis. Instead we detected a protein somewhat smaller than the precursor. Andersson et al. [41] indicate that LL-37 is not processed in the human male reproductive system. Part of the proregion may be functional in the rat.

Fasted and germ-free rats also expressed rCRAMP, and there seems to be a constitutive level of expression both in lung and gastrointestinal tract. The results from the germ-free rats revealed that contact with live bacteria was not required for rCRAMP expression, and similar results have been obtained for LL-37 [42]. Nevertheless, the germ-free rats are exposed to dead bacteria and bacterial components in their sterilized food, and these may induce rCRAMP expression. There may be different levels of rCRAMP expression when comparing the germ-free rats to their control, for example, regarding the small intestine where expression is near the detection limit, and to further investigate this one would need to utilize a quantitative method.

Conclusion

We have described a rat cathelicidin, rCRAMP, and demonstrated its homology to other members of the cathelicidin family. The tissue distribution of rCRAMP expression is similar to that of other cathelicidins. Thus, this is the rat counterpart to human LL-37. The 43-residue peptide generated from this cathelicidin was active against both Gram-negative and Gram-positive bacteria. For the mouse, there is a knock-out model, and the most complete overview would benefit from examining both mice and rats. The rat is also a small laboratory animal and provides additional disease models. Based on the data presented in this paper, the rat can be used as a model system to study cathelicidins.

Acknowledgements. We thank B. Heilborn for excellent help with the rats, B. Persson for advice regarding the bioinformatics, J. Michaëlsson for advice regarding the chromium release assay, and T. Bark for directing our interest towards the rat in the first place. This work was supported by The Swedish Research Council, Petrus and Augusta Hedlund's Foundation, The Swedish Foundation for Strategic Research (SSF), Åke Wiberg's Foundation, Magnus Bergvall's Foundation and Ruth and Richard Julin's Foundation.

1 Hancock R. E. and Scott M. G. (2000) The role of antimicrobial peptides in animal defenses. Proc. Natl. Acad. Sci. USA **97:** 8856–8861
2 Lehrer R. I. and Ganz T. (1999) Antimicrobial peptides in mammalian and insect host defence. Curr. Opin. Immunol. **11:** 23–27
3 Zanetti M., Gennaro R. and Romeo D. (1995) Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. FEBS Lett. **374:** 1–5
4 Lehrer R. I. and Ganz T. (2002) Cathelicidins: a family of endogenous antimicrobial peptides. Curr. Opin. Hematol. **9:** 18–22
5 Zhang G., Wu H., Shi J., Ganz T., Ross C. R. and Blecha F. (1998) Molecular cloning and tissue expression of porcine beta-defensin-1. FEBS Lett. **424:** 37–40
6 Zhang G., Ross C. R. and Blecha F. (2000) Porcine antimicrobial peptides: new prospects for ancient molecules of host defense. Vet. Res. **31:** 277–296
7 Lehrer R. I. and Ganz T. (2002) Defensins of vertebrate animals. Curr. Opin. Immunol. **14:** 96–102
8 Agerberth B., Gunne H., Odeberg J., Kogner P., Boman H. G. and Gudmundsson G. H. (1995) FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis. Proc. Natl. Acad. Sci. USA **92:** 195–199
9 Gudmundsson G. H., Agerberth B., Odeberg J., Bergman T., Olsson B. and Salcedo R. (1996) The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. Eur. J. Biochem. **238:** 325–332
10 Boman H. G. (1996) Peptide antibiotics: holy or heretic grails of innate immunity? Scand. J. Immunol. **43:** 475–482
11 Sorensen O. E., Follin P., Johnsen A. H., Calafat J., Tjabringa G. S., Hiemstra P. S. et al. (2001) Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. Blood **97:** 3951–3959
12 Scocchi M., Skerlavaj B., Romeo D. and Gennaro R. (1992) Proteolytic cleavage by neutrophil elastase converts inactive storage proforms to antibacterial bactenecins. Eur. J. Biochem. **209:** 589–595
13 Frohm M., Agerberth B., Ahangari G., Stahle-Backdahl M., Liden S., Wigzell H. et al. (1997) The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. J. Biol. Chem. **272:** 15258–15263
14 Islam D., Bandholtz L., Nilsson J., Wigzell H., Christensson B., Agerberth B. et al. (2001) Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. Nat. Med. **7:** 180–185
15 Nizet V., Ohtake T., Lauth X., Trowbridge J., Rudisill J., Dorschner R. A. et al. (2001) Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature **414:** 454–457
16 Dorschner R. A., Pestonjamasp V. K., Tamakuwala S., Ohtake T., Rudisill J., Nizet V. et al. (2001) Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A *Streptococcus*. J. Invest. Dermatol. **117:** 91–97
17 Gallo R. L., Kim K. J., Bernfield M., Kozak C. A., Zanetti M., Merluzzi L. et al. (1997) Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. J. Biol. Chem. **272:** 13088–13093

18 Pestonjamasp V. K., Huttner K. H. and Gallo R. L. (2001) Processing site and gene structure for the murine antimicrobial peptide CRAMP. Peptides **22:** 1643–1650

19 Yu K., Park K., Kim Y., Kang S. W., Shin S. Y. and Hahm K. S. (2002) Solution structure of a cathelicidin-derived antimicrobial peptide, CRAMP as determined by NMR spectroscopy. J. Pept. Res. **60:** 1–9

20 Yount N. Y., Wang M. S., Yuan J., Banaiee N., Ouellette A. J. and Selsted M. E. (1995) Rat neutrophil defensins: precursor structures and expression during neutrophilic myelopoiesis. J. Immunol. **155:** 4476–4484

21 Jia H. P., Mills J. N., Barahmand-Pour F., Nishimura D., Mallampali R. K., Wang G. et al. (1999) Molecular cloning and characterization of rat genes encoding homologues of human beta-defensins. Infect. Immun. **67:** 4827–4833

22 Eisenhauer P. B. and Lehrer R. I. (1992) Mouse neutrophils lack defensins. Infect. Immun. **60:** 3446–3447

23 Travis S. M., Anderson N. N., Forsyth W. R., Espiritu C., Conway B. D., Greenberg E. P. et al. (2000) Bactericidal activity of mammalian cathelicidin-derived peptides. Infect. Immun. **68:** 2748–2755

24 Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F. and Higgins D. G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids. Res. **25:** 4876–4882

25 Page R. D. (1996) TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12:** 357–358

26 Johansson J., Gudmundsson G. H., Rottenberg M. E., Berndt K. D. and Agerberth B. (1998) Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. J. Biol. Chem. **273:** 3718–3724

27 Vogel H. J. and Bonner D. M. (1956) Acetylornithinase of *Escherichia coli*: a partial purification and some properties. J. Biol. Chem. **218:** 97–106

28 Sambrook J. and Russell D. W. (2001) Molecular Cloning – A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.

29 O'Brien S. J., Eisenberg J. F., Miyamoto M., Hedges S. B., Kumar S., Wilson D. E. et al. (1999) Genome maps 10. Comparative genomics. Mammalian radiations. Wall chart. Science **286:** 463–478

30 Sternberg S. (1994) The emerging fungal threat. Science **266:** 1632–1634

31 Oren Z. and Shai Y. (1997) Selective lysis of bacteria but not mammalian cells by diastereomers of melittin: structure-function study. Biochemistry **36:** 1826–1835

32 Yang D., Chen Q., Schmidt A. P., Anderson G. M., Wang J. M., Wooters J. et al. (2000) LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J. Exp. Med. **192:** 1069–1074

33 Bjork P. and Ohlsson K. (1990) Purification and N-terminal amino-acid sequence analysis of rat polymorphonuclear leukocyte cathepsin G. Biol. Chem. Hoppe Seyler **371:** 595–601

34 Saiman L., Tabibi S., Starner T. D., San Gabriel P., Winokur P. L., Jia H. P. et al. (2001) Cathelicidin peptides inhibit multiply antibiotic-resistant pathogens from patients with cystic fibrosis. Antimicrob. Agents Chemother. **45:** 2838–2844

35 Agerberth B., Charo J., Werr J., Olsson B., Idali F., Lindbom L. et al. (2000) The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. Blood **96:** 3086–3093

36 Bals R., Wang X., Zasloff M. and Wilson J. M. (1998) The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. Proc. Natl. Acad. Sci. USA **95:** 9541–9546

37 Frohm Nilsson M., Sandstedt B., Sorensen O., Weber G., Borregaard N. and Stahle-Backdahl M. (1999) The human cationic antimicrobial protein (hCAP18), a peptide antibiotic, is widely expressed in human squamous epithelia and colocalizes with interleukin-6. Infect. Immun. **67:** 2561–2566

38 Malm J., Sorensen O., Persson T., Frohm-Nilsson M., Johansson B., Bjartell A. et al. (2000) The human cationic antimicrobial protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa. Infect. Immun. **68:** 4297–4302

39 Nagaoka I., Hirata M., Sugimoto K., Tsutsumi-Ishii Y., Someya A., Saionji K. et al. (1998) Evaluation of the expression of human CAP18 gene during neutrophil maturation in the bone marrow. J. Leukoc. Biol. **64:** 845–852

40 Bals R., Lang C., Weiner D. J., Vogelmeier C., Welsch U. and Wilson J. M. (2001) Rhesus monkey (*Macaca mulatta*) mucosal antimicrobial peptides are close homologues of human molecules. Clin. Diagn. Lab. Immunol. **8:** 370–375

41 Andersson E., Sorensen O. E., Frohm B., Borregaard N., Egesten A. and Malm J. (2002) Isolation of human cationic antimicrobial protein-18 from seminal plasma and its association with prostasomes. Hum. Reprod. **17:** 2529–2534

42 Hase K., Eckmann L., Leopard J. D., Varki N. and Kagnoff M. F. (2002) Cell differentiation is a key determinant of cathelicidin LL-37/human cationic antimicrobial protein 18 expression by human colon epithelium. Infect. Immun. **70:** 953–963