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O. Shamova (✉) · D. Orlov · V. Kokryakov
Department of General Pathology and Pathophysiology, Institute for Experimental Medicine of the Russian Academy of Medical Sciences, St-Petersburg, Russia
e-mail: oshamova@yandex.ru

C. Stegemann · P. Czihal · R. Hoffmann
Department of Chemistry and Mineralogy, Institute of Bioanalytical Chemistry, Leipzig University, Leipzig, Germany

K. Brogden
Department of Periodontics, Dows Institute for Dental Research, College of Dentistry, University of Iowa, Iowa City, IA, USA

N. Kolodkin
Research Institute of Pure Biochemicals, St-Petersburg, Russia

G. Sakuta
Institute of Cytology of the Russian Academy of Science, St-Petersburg, Russia

A. Tossi
Department of Biochemistry, Biophysics and Macromolecular Chemistry, University of Trieste, Trieste, Italy

H.-G. Sahl
Institute of Medical Microbiology, Immunology and Parasitology – Pharmaceutical Microbiology Section, University of Bonn, Bonn, Germany

R. I. Lehrer
David Geffen School of Medicine at University of California, Los Angeles, CA, USA

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Abstract We isolated a new proline-rich peptide, ChBac3.4, from leukocytes of the goat (*Capra hirca*) and determined its amino acid sequence by Edman degradation and mass spectrometry. ChBac3.4 (RFRLPFRRPPIRIHP PPFYPPFRRFL–NH2) had over 50% sequence identity to the Bac5 peptides found in the leukocytes of goats, sheep and cattle. ChBac3.4 exhibited broadspectrum antimicrobial activity, especially under low salt conditions. Since *E. coli* ML35p treated with ChBac3.4 manifested increased outer and inner membrane permeability and a rapid and extensive loss of cytoplasmic potassium, the antimicrobial properties of this peptide may depend, in part, on its ability to damage microbial membranes. Nevertheless, even high concentrations of ChBac3.4 were not significantly hemolytic for human erythrocytes. In vitro, ChBac3.4 was selectively cytotoxic, damaging human K562 erythroleukemia cells and human U937 hystiocytic lymphoma cells, but not other human target cells. ChBac3.4 appears to differ from other proline-rich cathelicidins in virtue of its increased ability to damage microbial membranes. This novel antimicrobial peptide warrants further study, especially with respect to its various effects on microbial and mammalian cells.

Keywords Proline-rich antimicrobial peptide · Goat leukocytes · Innate immunity

O. Shamova (✉) · D. Orlov · V. Kokryakov
Department of General Pathology and Pathophysiology, Institute for Experimental Medicine of the Russian Academy of Medical Sciences, St-Petersburg, Russia
e-mail: oshamova@yandex.ru

C. Stegemann · P. Czihal · R. Hoffmann
Department of Chemistry and Mineralogy, Institute of Bioanalytical Chemistry, Leipzig University, Leipzig, Germany

K. Brogden
Department of Periodontics, Dows Institute for Dental Research, College of Dentistry, University of Iowa, Iowa City, IA, USA

N. Kolodkin
Research Institute of Pure Biochemicals, St-Petersburg, Russia

G. Sakuta
Institute of Cytology of the Russian Academy of Science, St-Petersburg, Russia

A. Tossi
Department of Biochemistry, Biophysics and Macromolecular Chemistry, University of Trieste, Trieste, Italy

H.-G. Sahl
Institute of Medical Microbiology, Immunology and Parasitology – Pharmaceutical Microbiology Section, University of Bonn, Bonn, Germany

R. I. Lehrer
David Geffen School of Medicine at University of California, Los Angeles, CA, USA

Abbreviations

AU	Acid-urea
CEE	Continuous elution electrophoresis
MHB	Mueller-Hinton broth
MIC	Minimal inhibitory concentration
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
TSB	Tryptic soy broth

Introduction

Antimicrobial peptides (AMPs) are effector molecules of the innate immune system. They are widely distributed in animal tissues and cells that frequently encounter

microorganisms, including phagocytes, cytotoxic lymphocytes, NK cells, and epithelial cells. AMPs act against bacteria, including many that resist conventional antibiotics, and some are also effective against certain fungi and viruses (Lehrer and Ganz 1999; Hancock and Sahl 2006; Peschel 2002). Over the past 20 years structurally diverse antimicrobial peptides have been discovered in bacteria, protozoa, invertebrates and vertebrate animals, and plants (Sahl and Bierbaum 1998; Boman 2003; Lehrer and Ganz 2002; Zanetti 2004; Zasloff 2002). Nearly all endogenous AMPs of animals and plants are cationic, amphipathic molecules. Some AMPs are intrinsically alpha-helical, others contain beta-sheet elements that are stabilized by intramolecular cystine-disulphide bonds; and others still are unusually rich in certain amino acids, such as proline, tryptophan or histidine residues. Many AMPs kill microorganisms by causing membrane disruption; while others act by affecting intracellular processes (Shai 2002; Brogden 2005; Brötz and Sahl 2000).

Proline-rich peptides constitute a group of AMPs with distinct antimicrobial activity predominantly towards Gram-negative bacteria. Their antimicrobial action is believed to occur via non-lytic mechanism and directed to intracellular targets (Gennaro et al. 2002; Otvos 2002), and a putative transporter has been recently identified in *E. coli* as the membrane protein sbmA (Mattiuzzo et al. 2007). However, at higher concentrations some of them may also act by damaging bacterial membranes (Podda et al. 2006). Pro-rich AMPs have been found in many invertebrate animals, including insects (Casteels et al. 1989; Bulet et al. 1993; Cociancich et al. 1994), crustaceans (Destoumieux et al. 1997; Schnapp et al. 1996), and annelids (Cho et al. 1998). In vertebrate animals, proline-rich AMPs were discovered in cattle (Gennaro et al.1989), sheep (Huttner et al. 1998; Shamova et al. 1999; Anderson et al. 2003), goats (Shamova et al. 1999), deer (Treffers et al. 2005) and pigs (Agerberth et al. 1991; Harwig et al. 1995). Their high proline content and the presence of certain repeated proline motifs enables the peptides to interact with a variety of proteins that participate in signal transduction, transcription and other essential biological processes. In particular, SH3, WW, and several other protein-interaction domains prefer ligand sequences that are proline-rich (Kay et al. 2000). Their ability to bind to and modulate key regulatory proteins may not only explain the antibacterial mechanisms of some proline-rich AMPs (Gennaro et al. 2002; Otvos 2002; Mattiuzzo et al. 2007), they may also explain why some of these AMPs possess anti-inflammatory (Shi et al. 1996), wound healing (Gallo et al. 1994), and angiogenic properties (Li et al. 2000) that render them multifunctional defense molecules.

Several proline-rich AMPs have been isolated previously from the cells of vertebrate animals or deduced from their cDNA, all of which belong to the cathelicidin family of vertebrate AMPs (Tomasinsig and Zanetti 2005). This report describes the purification, composition, and antimicrobial properties of ChBac3.4, a novel proline-rich AMP from leukocytes of the goat. The peptide's name embodies its species of origin (Ch = *Capra hirca*), its approximate mass (3.4 kDa) and the term "Bac" an abbreviation for "bactenecin", created from the words bacterium and *necare* (Latin, to kill) (Gennaro et al. 1989).

Materials and Methods

Preparation of Leukocytes

Venous blood was obtained from healthy goats and anticoagulated with citrate. Leukocytes were prepared by lysing the erythrocytes with 0.83% ammonium chloride (2 cycles), followed by brief exposure to cold 0.22% saline. The leukocyte preparations contained about 80% neutrophils.

Purification of ChBac3.4

The leukocytes were treated as previously described (Shamova et al. 1999). Briefly, leukocytes were centrifuged at 225 × *g* for 10 min, resuspended in 10% acetic acid, sonicated, and extracted overnight at 0–4°C. The extracts were clarified at 3,000 × *g* for 30 min at 4°C, and the supernatants were lyophilized. This material was dissolved in 0.1 M Tris-0.15 M NaCl buffer (pH 7.5) and treated with 1.5–2.0 μg of human neutrophil elastase (ART Biochemicals, Athens, USA)/mg of protein for 30 min at 37°C. Proteolysis was stopped by adding acetic acid to a final concentration of 5%. After passage through a YM-10 filter (Amicon, Beverly, USA), the ultrafiltrates were concentrated by vacuum centrifugation and desalted on a Sep-Pak light C_{18} cartridge (Waters Millipore, Milford, USA). The recovered material was dried, resuspended in 1 ml of 5% acetic acid containing 3 M urea, and subjected to preparative continuous electrophoresis (Harwig et al. 1993). Fractions containing 3- to 5-kDa peptides were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pooled, and purified by reversed-phase high-pressure liquid chromatography (RP-HPLC) on a Vydac C_{18}-column by using linear gradients of 0–60% acetonitrile in 0.1% trifluoroacetic acid or 0.13% hexafluorobutyric acid. The purity was confirmed by mass spectrometry and by SDS-PAGE and acid-urea-PAGE. An analogous procedure was used to purify goat

ChBac5 which served as a reference peptide in this study of ChBac3.4.

Sequence Analysis

Purified peptides were analyzed on a matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometer (MALDI-TOF/TOF-MS, Proteomics Analyzer 4700, Applied Biosystems GmbH) and a hybrid MS consisting of a quadrupole and an orthogonal TOF analyzer (QqTOF-MS, QStar Pulsar I, Applied Biosystems), which was equipped with a static nano electrospray ionization (ESI) source (Proxeon, Odense, Denmark). Typically 0.5 µl purified peptide solution and 0.5 µl of CHCA matrix solution (4 mg/ml α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile containing 0.1% TFA) were mixed on a stainless steel target and air-dried at room temperature. The mass analysis was performed in positive ion reflectron mode using an acceleration voltage of 20 kV, 70% grid voltage and a delay of 1.277 ns. Alternatively, the peptides were dissolved in 50% aqueous acetonitrile containing 5% formic acid and the product ion spectra recorded on the ESI-QqTOF-MS in positive ion mode using a collision voltage of 20–50 V along the collision cell to obtain fragment ions for all parts of the peptide sequence. The peptide sequences were retrieved from all tandem mass spectra required on both instruments manually by de novo sequencing.

Alternatively, the partial N-terminal peptide sequence was determined by gas-phase Edman degradation using 300–500 pmols of purified peptide loaded on a Porton Model 2090E instrument. Amino acid analysis of the peptide was performed with an Alpha-Plus analyzer (LKB, Sweden).

The protein sequence data reported in this paper will appear in the UniProt Knowledgebase under the accession number P85170.

Protein concentrations in leukocyte extracts were measured by the bicinchoninic acid procedure (Pierce, Rockford, USA). The concentrations of the purified peptides as well as the synthetic ones were determined using tyrosine absorption ($\varepsilon_{280} = 1290\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$) for ChBac3.4 and PG1 or phenylalanine absorption ($\varepsilon_{257} = 200\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$) for ChBac5, and confirmed by the Bradford assay.

Peptide Synthesis

Peptide ChBac3.4 was synthesized by the solid-phase Fmoc/^{t}Bu-strategy (Fields and Noble 1990) using a Syro2000 multiple peptide synthesizer (MultiSynTech GmbH, Witten, Germany) (Singer et al. 2006). After completion of the peptide synthesis, the peptides were cleaved with 5% water, 4% *m*-cresol, 5% thioanisole and 2% ethanedithiol in trifluoroacetic acid (TFA) at room temperature for 4 h and precipitated with cold diethyl ether. The crude peptides were purified on an Äkta HPLC System (Amersham Bioscience GmbH, Freiburg, Germany) using a Jupiter C_{18}-column (20 mm × 250 mm, Phenomenex Inc., Torrance, USA) and a linear acetonitrile gradient in the presence of 0.1% TFA. The molecular mass of the peptide was confirmed by MALDI-TOF MS and its purity determined by analytical RP-HPLC.

Synthetic ChBac5 was produced by Boc-chemistry (Merrifield and Barany 1980) on a *p*-methylbenzhydrylamine resin (Sigma, USA) on a Vega Coupler 250 peptide synthesizer (USA) using 1-hydroxybenzotriazole ester in situ neutralization (Schnolzer et al. 1992). Peptide purification was carried out by RP-HPLC (Gilson; France) on a Waters Prep-NovaPak 6 µm C_{18} (19 × 300 mm) column. Purity, as assessed by reverse phase analytical chromatography on DeltaPak 5 µm C18 100A (3.9 × 150 mm^2) column, was about 99%. The molecular mass was confirmed by MALDI-TOF MS.

Antimicrobial Assays

The peptides were tested for antimicrobial activity against *Escherichia coli* ML-35p, *E. coli* ATCC 25922, *E. coli* M15, *Pseudomonas aeruginosa* ATCC 25782, *Listeria monocytogenes* EGD, *Staphylococcus aureus* 710A, *S. aureus* ATCC 25923, Methicillin Resistant *S. aureus* (MRSA) ATCC 33591 and *C. albicans* 820 by microdilution broth assay and a two-stage radial diffusion technique, done as previously described (Lehrer et al. 1991). Briefly, bacteria were grown to mid-logarithmic phase at 37°C in trypticase soy broth (Difco, Detroit, USA) and *C. albicans* was grown overnight in Sabouraud dextrose broth (NIFC, St-Petersburg, Russia). Approximately 4 × 10^6 CFU of washed in 10 mM sodium phosphate buffer pH 7.4 organisms were dispersed in a 10-ml volume of underlay gel that contained 10 mM sodium phosphate, 0.3 mg of trypticase soy broth powder per ml, and 1% (wt/vol) agarose (A6013 Sigma, La Jolla, USA) with or without 100 mM NaCl. Sample wells, each 2.5 mm in diameter, were punched in the underlay gel. Serial peptide dilutions were prepared in 0.01% acetic acid containing 0.1% human serum albumin, and 5-µl peptide samples were applied. Overlay gels (10 ml of 1% agarose, 60 mg of trypticase soy broth powder per ml or Sabouraud dextrose broth powder in the case of Candida albicans) were poured 3 h after the peptide samples were added. The clear zones were measured to the nearest 0.1 mm after overnight incubation and were expressed in units (1 mm = 10 U) after subtracting the well diameter. The minimal inhibitory concentration (MIC) was defined by the x intercept of a regression line through zone diameters obtained from a series of serially diluted peptide samples.

Broth microdilution assay was performed according to the guidelines of the National Committee for Clinical

Laboratory Standards (National Committee for Clinical Laboratory Standards 1993), using Mueller Hinton (MH) Broth, except that the 10 × stock peptides were prepared and serially diluted in acidified water (0.01% acetic acid) with 0.1% BSA (sterilized by filtration through 22 μm membrane) instead of in Mueller Hinton Broth (Steinberg and Lehrer 1997). The overnight cultures of each strain were diluted with MH broth and their absorbance was measured at 620 nm. Then the suspensions were diluted to approximately 2×10^5 CFU/ml and 90 μl of the suspensions were mixed with 10 μl of the peptide dilutions in the wells of a microtitre plate. After incubation for 18 h at 37°C the MIC was read as the lowest concentration of antimicrobial agent resulting in the complete inhibition of visible growth and results given are mean values of 3–6 independent determinations.

Permeability of *E. coli* Membranes

To examine the ability of antimicrobial peptides to permeabilize the inner and outer membranes of gram-negative bacteria, we used a previously described procedure that uses *E. coli* ML-35p (Lehrer et al. 1988). The parental strain of the assay organism, *E. coli* ML-35, expressed cytoplasmic *β*-galactosidase activity constitutively but lacked lactose permease and could not transport *β*-galactoside substrates through its inner membrane. The assay strain also expressed periplasmic *β*-lactamase activity. The ML35p construct allowed outer membrane permeability to be assessed by monitoring the hydrolysis of a chromogenic *β*-lactamase substrate nitrocefin [3-(2,4-dinitrostyryl)-(6R,7R)-7-(2-thienylacetamido)ceph-3-em-4-carboxylic acid); Calbiochem-Novabiochem, San-Diego, USA] by detection of the hydrolysis product at 486 nm. Inner membrane permeability was monitored by measuring the hydrolysis of *o*-nitrophenyl-*β*-D-galactoside (ONPG, Sigma, La Jolla, USA) at 420 nm. The assays were performed in 96-well microtiter plates that were monitored every minute with SpectraMax 250 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, USA) using SOFTmax PRO software supplied by the manufacturer. The final incubation medium contained 10 mM sodium phosphate buffer, 100 mM NaCl, pH 7.4. Incubation wells (final volume of 100 μl) also contained of either 2.5 mM ONPG or 20 μM nitrocefin; 2.5×10^7 CFU/ml of washed, stationary-phase *E. coli* ML-35p cells; and indicated concentrations of the peptide of interest or an equivalent volume of acidified water (negative controls). Assays were run at 37°C, with 5 s of shaking every minute. Reactions were started by adding the bacteria. The time to maximal permeabilization was considered to occur when the tangent to the linear portion of the OD/time curve first diverged from the preceding and relatively flat curve that preceded it.

E. coli ML-35p was maintained on trypticase soy agar plates containing 100 mg of ampicillin per ml. Organisms used for antimicrobial testing or membrane permeability assays were picked from a single colony, incubated in 50 ml of sterile Trypticase soy broth for 16 h at 37°C, washed three times with 10 mM sodium phosphate buffer (pH 7.4), adjusted to an optical density at 620 nm of 1 (2.5×10^8 CFU/ml), and kept on ice until used.

Kinetics of Antimicrobial Activity

Quantitative bacterial counts were performed to monitor bactericidal activity. Stationary phase bacteria were prepared as described above and incubated with antimicrobial peptides at 37°C in an agarose-free liquid medium containing 2.5×10^7 CFU/ml in 10 mM sodium phosphate buffer pH 7.4 with 100 mM NaCl. Aliquots (5 μl) were removed at defined intervals, diluted appropriately in 10 mM sodium phosphate buffer, and 40 μl of the dilutions were transferred to nutrient agar plates. Colonies were counted after overnight incubation at 37°C.

Potassium Release

Test organisms were prepared as described for the membrane permeability study. Experiments were carried out at 37°C in stirred polypropylene tubes surrounded by a 50-ml water-jacketed reaction vessel (Kimble/Kontes, Vineland, USA). The tube contained 2.5×10^7 CFU of washed, stationary phase bacteria in 10 mM sodium phosphate buffer, pH 7.4, 100 mM NaCl, at a final volume of 250 μl. An Orion SensorLink PCM-700 pH/ISE meter, fitted with a MI-442 potassium electrode (Microelectrodes, Bedford, USA) and an SDR-2 reference electrode (World Precision Instruments, Sarasota, USA), was used as reported (Orlov et al. 2002). Relative voltage data were collected every 5 s. The electrode was calibrated with standard solution of 100 mM NaCl containing 0.01–1 mM KCl (Sigma, La Jolla, USA). Over this entire range, the measured electrode voltage (V_{meas}) and the $\log_{10}[K^+]$ showed a linear relationship (Eq. 1), whose m and z coefficients were obtained by a least squares analysis.

$$V_{meas} = m \log_{10}[K^+] + z. \quad (1)$$

We simulated complete potassium release from bacteria by disrupting them with prolonged sonication (3 min at 85% power) using a USD sonicator (St-Petersburg, Russia). The initial (K_{init}), post-sonication (K_{total}) and interval $[K^+]$ concentrations of potassium in the medium were calculated from measured voltages (V_{meas}) by applying Eq. 2.

$$[K+] = 10^{(Vmeas-z)/m} \quad (2)$$

where z and m were the linear regression equation coefficients from Eq. 1. These calculations were performed

using the Sigma Plot graphics program (SPSS Science, Chicago, USA). Finally data were converted to percent potassium release with Eq. 3.

$$\% \text{ Release} = ([K^+]_{meas} - [K^+]_{init} / ([K+]_{total} - [K+]_{init}) \times 100. \quad (3)$$

Hemolytic activity was tested by incubating various concentrations of peptide with a suspension (2.5% v/v) of washed human red blood cells in phosphate-buffered saline (PBS). After 30 min at 37°C, the tubes were centrifuged for 3 min at 10,000 × g, and the absorbance (*A*) of the supernatants was measured at 540 nm. The percentage of hemolysis was calculated by Eq. 1, where A_{exper} and $A_{control}$ signify the absorbance values of supernatants from treated to untreated red cells, and A_{total} is the supernatant of red cells treated with 1% Triton X-100.

$$\% \text{ Hemolysis} = ([A]_{exper} - [A]_{control} / ([A]_{total} - [A]_{control}) \times 100 \quad (4)$$

Cytotoxicity Assay

A conventional MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test was used to examine the cytotoxic activity of AMPs (Mosmann 1983). Target cells included human erythroleukemia cells K-562, human hystiocytic lymphoma U-937, human lung carcinoma A-549, human embryonic lung fibroblasts MRC-5 and normal human skin fibroblasts. Adherent cells (A-549, MRC-5, human skin fibroblasts) were cultured in DMEM (Biolot, St-Petersburg, Russia) supplemented with glutamine and 10% fetal calf serum (FCS). Before the experiments 10^5 cells were plated in 96-well microplates and incubated overnight in this medium to allow them to adhere. After replacing this medium with serum-free DMEM, serial dilutions of the peptides were added to the wells. K-562 and U-937 cells were grown in suspension in RPMI 1640 medium (Biolot, St-Petersburg, Russia) supplemented with glutamine and 10% FCS, and were dispensed to microplates (10^5 cells/well in RPMI 1640) just before adding the peptides. After adding the AMPs the plates were incubated for 24 h at 37°C under 5% CO_2. Cell-free media and cells incubated without peptides served as controls. Four hours before the incubation ended, MTT in PBS (5 mg/ml) was added to each well. After the incubation was stopped by adding isopropanol/ 0.04 M HCl, the optical density was measured at 540 nm, subtracting absorbance at 690 nm as a background. Toxicity was determined by nonlinear regression analysis of the corresponding dose response curves using the Sigma Plot 9 program to calculate IC_{50} values (the concentration of the test substance that reduced the OD_{540} capacity by 50%.

Results

Peptide Purification

Similarly to the reported procedure used for the purification of caprine Bac5 (ChBac5), we treated goat (*Capra hirca*) leukocyte extracts with elastase (Shamova et al. 1999) to liberate the mature AMPs from their cathelin-linked precursors. Figure 1a, b illustrates stages in the purification. Since the molecular mass of the new peptide was 3375 Da, the peptide was named ChBac3.4. Figure 2a, b shows SDS-PAGE and AU-PAGE of acetic acid extracts of untreated and elastase-processed components of goat neutrophils and the purified ChBac3.4. In parallel we purified the previously described ChBac5 (Shamova et al. 1999). A crude

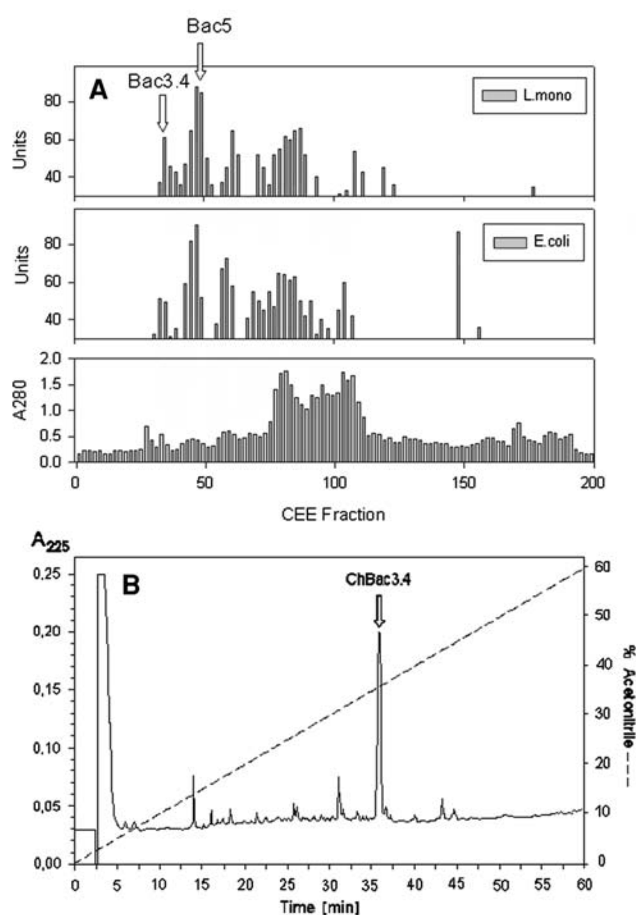

Fig. 1 Purification of ChBac3.4. **a** Preparative continuous elution electrophoresis (CEE) of a YM10 ultrafiltrate of the elastase-treated goat leukocyte extract. CEE fractions were tested for antimicrobial activity against *Listeria monocytogenes* EGD (*upper panel*) and *E. coli* ML35p (*middle panel*) in radial diffusion assays. The *lower panel* indicates the absorbance of CEE fraction at the wavelength 280 nm. ChBac3.4 was present in Fractions 28–36 and ChBac5 eluted in fractions 42–50. **b** RP-HPLC of CEE Fraction 31 using a linear gradient of water-acetonitrile (0–60% at 1%/min) on a Vydac C_{18}-column. The ChBac3.4 peak is marked

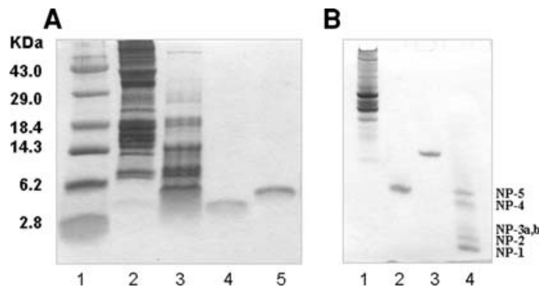

Fig. 2 Panel **a** shows an SDS-PAGE gel. Lane contents: 1, mass standards; 2, acid extract of goat leukocytes (40 μg of protein); 3, goat leukocyte extract, post-elastase treatment (20 μg of protein); 4, purified ChBac3.4 (1 μg of protein); 5, purified ChBac5 (1 μg of protein). Panel **b** shows an acid-urea PAGE gel. Lane contents: 1, acid extract of goat leukocytes (30 μg of protein); 2, purified ChBac3.4 (1 μg of protein); 3, purified ChBac5 (1 μg of protein). 4, a mixture of rabbit defensins containing NP-1, 2, 3a, 3b, 4 and 5 (5 μg of protein). The gels were stained with Coomassie blue

acetic acid extract from 1 × 10^9 goat leukocytes contained approximately 10 mg of total protein. Subjecting this material to elastase cleavage followed by the purification procedure yielded approximately 10 μg of highly purified ChBac3.4 and 20–25 μg of ChBac5.

Structural Characterization

N-terminal sequencing of the obtained peptide by gas-phase Edman degradation unambiguously identified the first 23 residues of ChBac3.4 as: RFRLPFRRPPIRIHPPPFYPP FR_XXX, and this was independently confirmed by tandem mass spectrometry of a tryptic digest. The C-terminal residues were retrieved from the signal m/z 434.34 using collision induced dissociation. Among the four possible sequences matching the recorded fragment ion spectrum, i.e., H_2N-RFI/L-$CONH_2$ or H_2N-RI/LF-$CONH_2$, the first sequence was confirmed by synthesizing both peptide families and comparing their fragmentation pattern with the native tryptic peptide. However, it was not possible to distinguish the two isomers leucine and isoleucine by their fragment ion spectra (Fig. 3a, b). Thus, ChBac3.4 was hydrolyzed and the amino acid composition determined by

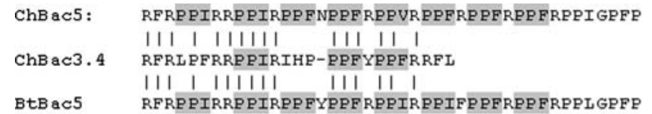

Fig. 4 Aligned amino acid sequences of ChBac3.4 and the previously reported sequences of goat (*Capra hircus*) Bac5 (Lehrer et al. 1991) and bovine (*Bos taurus*) Bac5 (ChBac5 and BtBac5, respectively) (Kokryakov et al. 1993). Identical residues are connected with lines. Repeated PPX motifs, wherein X is an apolar hydrophobic amino acid (I, F or P), are highlighted with *grey*. A single gap was introduced in ChBac3.4 to enhance the alignment

amino acid analysis. The obtained amino acid ratios confirmed the sequence and showed that two Leu and two Ile residues are present, i.e. the last residue is leucine. Considering the obtained data we established the complete primary structure of ChBac3.4 (Fig. 4) which is compared with caprine and bovine Bac5 (ChBac5 and BtBac5, respectively). ChBac3.4 shows structural similarity to both BtBac5 and ChBac5. The principal difference is the presence of a three residue (Ile-His-Pro) insertion in the middle part of the molecule and several replacements at the C-terminus. The IHP insertion interrupts the periodicity of the X_1PPX_2 tetramer motif which occurs nine times in Bac5 (Fig. 4). In this motif, X_1 is usually arginine and X_2 is a hydrophobic residue such as Leu, Ile or Phe. The insertion also results in a lack of the RxxPxxP motif, which is present in Bac5 as well as in porcine Pro-rich peptide PR-39 and which has been shown to be responsible for its binding to SH3 domains in a variety of proteins and to mediate a range of biological effects on mammalian cells (Shi et al. 1996).

Antimicrobial Activity

We used two techniques to examine the antimicrobial effects of ChBac3.4: a radial diffusion assay and broth microdilution assay. The radial diffusion assay was used in the first stages of our research when we worked with limited amounts of the purified peptide, and was later used to study their synthetic counterparts. The activity of ChBac3.4 was tested in comparison with two reference

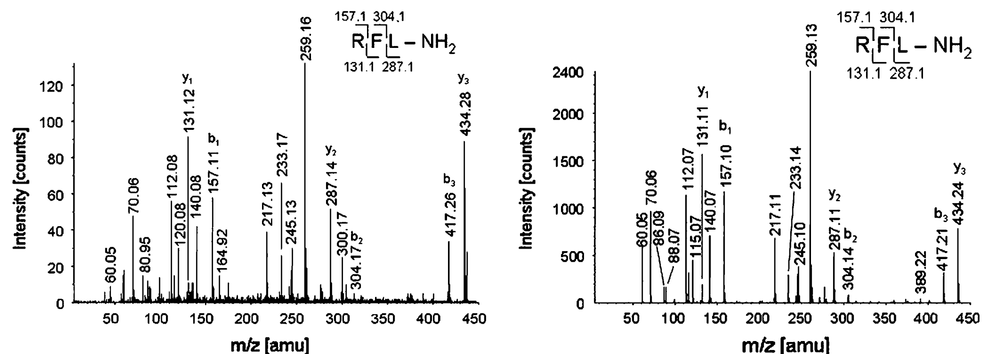

Fig. 3 Tandem mass spectra recorded on an ESI-QqTOF-MS for m/z 434.2 detected in the tryptic digest of the native ChBac3.4 peptide (*left*) and the synthetic H-RFL-NH_2 (*right*)

Table 1 Antimicrobial activity of native and synthetic ChBac3.4

	ChBac3.4		ChBac5		PG1	
	Low salt	High salt	Low salt	High salt	Low salt	High salt
E. coli ML35p	0.4 ± 0.1 (0.8 ± 0.2)	0.5 ± 0.2 (0.9 ± 0.3)	0.3 ± 0.1 (0.6 ± 0.2)	0.2 ± 0.1 (0.4 ± 0.1)	0.2 ± 0.1	0.2 ± 0.1
E. coli ATCC 25922	0.5 ± 0.1	1.0 ± 0.3	0.2 ± 0.1	0.5 ± 0.2	0.2 ± 0.1	0.3 ± 0.1
E. coli M15	0.4 ± 0.1	0.5 ± 0.2	0.6 ± 0.3	0.5 ± 0.2	0.2 ± 0.05	0.2 ± 0.1
P. aeruginosa ATCC 27853	0.3 ± 0.1	1.3 ± 0.5	0.2 ± 0.1	3.1 ± 1.1	0.1 ± 0.05	0.8 ± 0.3
L. monocytogenes EGD	0.4 ± 0.1 (0.9 ± 0.3)	2.2 ± 1.6 (1.4 ± 0.4)	0.5 ± 0.3 (0.6 ± 0.1)	2.0 ± 0.6 (1.5 ± 0.7)	0.3 ± 0.05	0.3 ± 0.1
S. aureus 710A	0.9 ± 0.2	5.3 ± 2.3	0.6 ± 0.2	20.4 ± 5.2	0.2 ± 0.05	0.2 ± 0.1
MRSA ATCC 33591	0.7 ± 0.3 (0.9 ± 0.1)	>50 (>40)	1.5 ± 0.5 (0.5 ± 0.1)	>50 (>40)	0.4 ± 0.1	0.4 ± 0.2
Candida albicans 820	2.4 ± 1.1 (1.1 ± 0.2)	>50 (>40)	1.6 ± 0.7 (0.8 ± 0.1)	>50 (>40)	0.4 ± 0.1	1.2 ± 0.4

Data show the minimal effective concentrations (MEC), in μM. All values are means ± S.D., each derived from 5 to 7 radial diffusion assays which were performed under two conditions: low salt (10 mM phosphate buffer), and high salt (10 mM phosphate buffer + 100 mM NaCl). ChBac5 and porcine protegrin PG1 were tested in parallel. The MECs in parentheses were performed with native, leukocyte-derived peptides

antimicrobial peptides: caprine Bac5 and the structurally different, cathelicidin-derived, porcine AMP protegrin 1 (PG1) (Kokryakov et al. 1993). Like ChBac5 and PG-1, ChBac3.4 exhibited potent, broad-spectrum antimicrobial activity in radial diffusion assays performed under low salt conditions (Table 1). ChBac3.4 remained highly active against *E. coli*, *P. aeruginosa* and *L. monocytogenes* in the presence of 100 mM NaCl, but showed much reduced potency against *S. aureus*, MRSA, and *C. albicans* under these conditions. Native and synthetic ChBac3.4 and ChBac5 peptides showed very similar behavior.

When the peptides were studied by conventional microbroth dilution assays in Mueller-Hinton Broth (Table 2), their respective MICs were higher than the MECs obtained in radial diffusion assays, most likely due to inactivation by medium components, as described elsewhere in detail (Turner et al. 1998). ChBac3.4 was active against Gram-negative bacteria (*E. coli* and *P. aeruginosa*), and the Gram-positive organism, *L. monocytogenes*. It demonstrated some activity towards Staphylococci, while ChBac5 was inactive in this range of applied concentration.

Action of Chbac3.4 on the Integrity of Bacterial Membranes

Considering that Pro-rich peptides are suggested to act via a non-lytic antimicrobial mechanism (Gennaro et al. 2002; Otvos 2002; Mattiuzzo et al. 2007), we assessed the effects of ChBac3.4 on bacterial membranes. For this study we used *E. coli* ML35p strain which expresses a plasmid-encoded periplasmic β-lactamase, is constitutive for cytoplasmic β-galactosidase, and lacks lactose permease (Lehrer et al. 1988). This indicator strain allowed us to monitor the permeabilization of its outer and inner membranes in real-time assays by monitoring the exposure of an impermeant, extracellular substrate to the action of the periplasmic or cytoplasmic hydrolases. We also examined the peptide's effect on potassium release and membrane potential under identical experimental conditions (10 mM sodium phosphate buffer; 100 mM NaCl, pH 7.4). Porcine PG1, a peptide with remarkable membrano-lytic activity, was used as a positive control. Figure 5 shows that the outer membrane of *E. coli* became fully permeable to

Table 2 Broth microdilution assays

	Minimal inhibitory concentrations (MIC), μM*		
	ChBac3.4	ChBac5	PG-1
E. coli ML35p	2.3 ± 0.5	1.6 ± 0.5	2.2 ± 0.1
E. coli ATCC 25922	3.8 ± 1.5	3.1 ± 2.4	3.2 ± 2.1
E. coli M15	6.0 ± 2.0	4.7 ± 2.3	2.2 ± 1.2
Pseudomonas aeruginosa ATCC 27853	6.3 ± 2.9	8.2 ± 3.6	2.1 ± 1.1
Listeria monocytogenes EGD	2.8 ± 2.2	2.8 ± 1.0	0.5 ± 0.2
Staphylococcus aureus 710A	8.0	>16	1.2 ± 0.7
MRSA ATCC 33591	10.0 ± 3.7	>16	2.3 ± 1.3
Candida albicans 820	>16	>16	10.7 ± 7.7

* All values are represented as means ± S.D., each derived from 3 to 5 experiments which were performed in triplicates

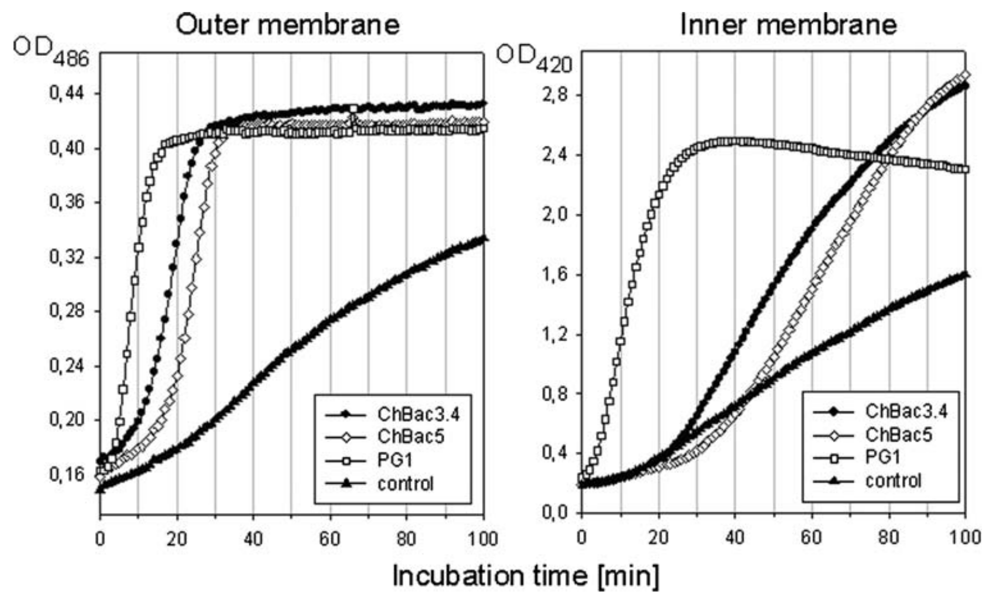

Fig. 5 Membrane permeabilization. We tested the ability of ChBac3.4 to permeabilize the outer (*left panel*) and inner (*right panel*) membranes of *E. coli* ML-35p and compared its action with effects of caprine ChBac5 and porcine PG1. The peptides at final concentrations of 5 μM were incubated with bacteria (2.5×10^7 CFU/ml) at 37°C in 10 mM sodium phosphate buffer with 100 mM NaCl, pH 7.4. The extent of membrane permeabilization is proportional to the slope of each curve, and the time to maximal permeabilization. The time to maximal permeabilization can be equated to the time that the tangent of the linear portion of the curves diverges from the preceding portion of the curve

nitrocefin, a β-lactamase substrate, about 3 min after adding PG1, 10 minutes after adding ChBac3.4 and 20 min after adding ChBac5. The inner membrane became permeable to ONPG about 3 min after adding PG-1, 23 min after adding ChBac3.4, and 40 min after adding ChBac5. In this experiment, all of the peptides were applied at 5 μM, which is above both the MIC (by broth microdilution) and MEC (by radial diffusion). At MIC concentrations (1 μM), ChBac3.4 and ChBac5 lowered the bacteria viability, but affected only the outer membrane without any appreciable influence on *E. coli* inner membrane permeabilization (data not shown).

In parallel to membrane permeabilization, we also measured the kinetics of bactericidal action of the peptides by carrying out colony count assays (Fig. 6). The killing action of both ChBac3.4 and ChBac5 was slower than that of PG1. About 40 min of incubation of the peptides with *E. coli* resulted in a 2 $\log_{10}$ (99%) reduction in viability for treated bacteria, even though the influence of the peptides on the inner membrane permeabilization within this period of time was moderate. However, since viability is assessed after an overnight (18 h) incubation in colony count assays and permeability is assessed in real time by the nitrocefin/ONPG hydrolysis assay these assays are not temporally equivalent.

Figure 7 shows that adding 5 μM PG1 induced a rapid and extensive efflux of K^+ from treated *E. coli*, and that Bac3.4 had a similar, but less pronounced effect. In contrast, bacteria treated with ChBac5 sustained more minor K^+ efflux. Taking these two assays into account, the data indicate that the ability of ChBac3.4 to impair the integrity of the outer and inner bacterial membranes is inferior to that of PG1 but superior to that of ChBac5.

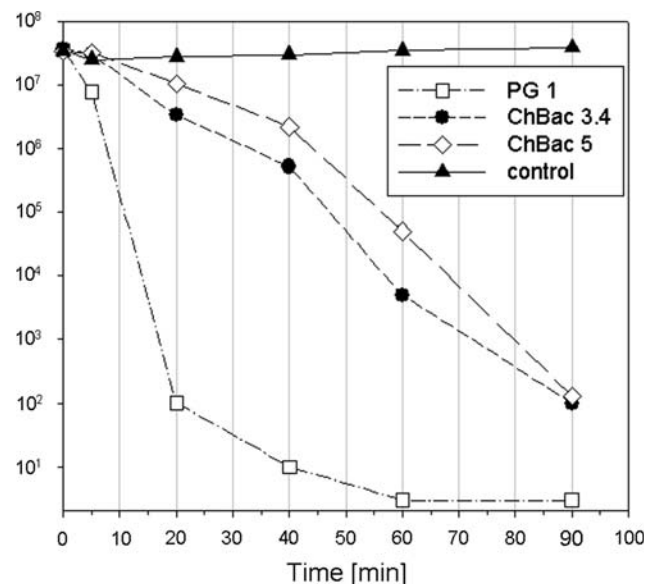

Fig. 6 Kinetics of a bactericidal activity of ChBac3.4 in comparison with caprine Bac5 and porcine PG1. The peptides were incubated with *E. coli* ML35p (2.5×10^7 CFU/ml) at 37°C in 10 mM sodium phosphate buffer with 100 mM NaCl, pH 7.4

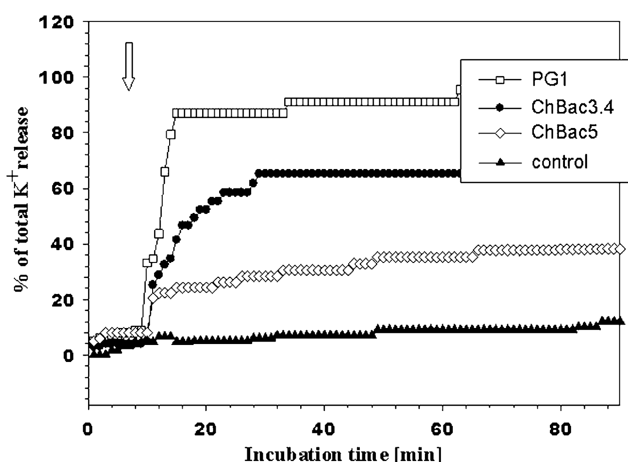

Fig. 7 Effect of ChBac3.4, ChBac5 and PG1 on K^+ release by *E. coli* ML35p. In 10 min after the beginning of the experiment the bacteria were exposed to 5 µM of either ChBac3.4 or ChBac5, or PG1. Untreated bacteria served as a control showed a loss of intracellular potassium. An arrow indicates the moment of addition of the peptides

Cytotoxic Effects on Mammalian Cells

Table 3 shows that ChBac3.4 and ChBac5 have low hemolytic activity at concentrations from 1 to 100 µM. However their influence on viability of nucleated targets was diverse. While ChBac5 did not show any toxic activity towards all other tested mammalian cells up to the concentration of 25 µM (Table 3), ChBac3.4 exerted selective toxic effects towards two types of cultural mammalian cells: human erithroleukemia K-562 cells and human hystiocytic lymphoma U-937. Other cells—human lung carcinoma A-549, human embryonic lung fibroblasts MRC-5 and normal human skin fibroblasts, were resistant to the peptide treatment.

Discussion

Pro-rich antimicrobial peptides are a family of host defense peptides with a special mechanism of antibiotic action and an array of intriguing effects on eukaryotic cells. Whereas numerous proline-rich AMPs have been found in invertebrates, only a few such peptides are known in vertebrates. Here we have described a novel Pro-rich peptide, ChBac3.4, that was purified from goat leukocytes, and which presumably is a member of the Pro-rich cathelicidins (Tomasinsig and Zanetti 2005). It consisted of 26 residues and evidenced appreciable homology to the N-terminal region of caprine ChBac5 and of ovine and bovine Bac5. However, whereas the ChBac5 and ovine and bovine Bac5 peptides contain repeated tetrameric RPPX motifs along nearly their entire length, the repetitive sequences of ChBac3.4 are primarily trimeric, as shown in Fig. 4. ChBac3.4 also lacks the RxxPxxP sequence found in porcine PR-39 (and in Bac5) that allows PR-39 to bind to SH3 domains in different proteins (Shi et al. 1996).

The N-terminal region of Bac5 was previously demonstrated to be responsible for the antimicrobial activity of this peptide (Tokunaga et al. 2001; Raj and Edgerton 1995), similarly to that of the other bovine Pro-rich peptide Bac7 (Benincasa et al. 2004). Since our ChBac3.4 comprises the naturally occurring truncated version of Bac5, we expected to find an activity similar or more potent than in Bac5. Radial diffusion assays revealed that both ChBac3.4 and ChBac5 were active against gram-negative and gram-positive bacteria and *C. albicans*, a fungus, under low salt conditions. In the presence of a more physiological NaCl concentration, the peptides retained activity against the gram-negative bacteria in our panel, but showed reduced effectiveness against *C. albicans*, *S. aureus* and MRSA. Their decreased

Table 3 Cytotoxic activity of ChBac3.4 towards human red blood cells (RBC); human erithroleukemia cells K-562, human hystiocytic lymphoma U-937, human lung carcinoma A-549, human embryonic lung fibroblasts MRC-5 and normal human skin fibroblasts

	% Hemolysis of human RBC in the presence of AMPs applied in the indicated concentrations[a]			IC_{50}, µM (MTT test)[b]				
	25 µM	50 µM	100 µM	K-562	U-937	A-549	MRC-5	Human skin fibroblasts
ChBac3.4	2.1 ± 0.3	3.2 ± 0.4	5.6 ± 0.8	4.3 ± 0.7	10.8 ± 2.8	>25*	>25*	>25*
	1.1* ± 0.1	2.2* ± 0.3			10.0* ± 2.2			
ChBac5	1.9 ± 0.4	2.9 ± 0.4	4.8 ± 1.2	>25	>25	>25*	>25*	>25*
	1.2* ± 0.3	2.2* ± 0.3			>25*			
PG1	58.6 ± 7.4	67.4 ± 9.2	95.7 ± 5.4	24.0 ± 4.5	5.1 ± 1.2	2.9 ± 0.5	23 ± 6.3	>25

All values are means ± S.D. The values marked with asterisk were performed with native, leukocyte-derived peptides. ChBac5 and porcine protegrin PG1 were tested in parallel

[a] The values are derived from three experiments which were performed in triplicates or in duplicates in the case of synthetic or native peptides, respectively

[b] The values are derived from three experiments which were performed in quadruplicates or in triplicates in the case of synthetic or native peptides, respectively

activity with increasing ionic strength may result from interference with their electrostatic binding to negatively charged bacterial surface molecules, such as (lipo)teichoic acids. Alternatively, it could reflect a failure to bind intracellular targets in Gram-positive bacteria, as postulated for other proline-rich AMPs (Gennaro et al. 2002).

The results obtained by the broth microdilution assay allowed to compare the activity of ChBac5 with previously described bovine Bac5 and Bac7 (Gennaro et al. 1989) and ovine Bac5 (Shamova et al. 1999) which were tested in similar conditions. The activity of ChBac3.4 against gram-negative bacteria closely matched to that of ChBac5 and that described for bovine Bac5, Bac7 and their N-terminal fragments (Gennaro et al. 1989; Podda et al. 2006; Benincasa et al. 2004); and to the activity of Bac5, OaBac5mini and OaBac7.5mini of sheep (Anderson and Yu 2003; Anderson et al. 2004). Conversely, ChBac3.4 was significantly more active against gram-positive bacteria than ChBac5 as well as than bovine Bac5, Bac7 and somewhat more active than a 24-residues N-terminal fragment of ovine Bac5 (OaBac5mini) and OaBac7.5mini, which also demonstrated some effects in respect with Staphylococci (Anderson et al. 2004). A relatively higher positive charge of ChBac3.4, indicated also by its more rapid electrophoretic elution profiles (Figs. 1, 3), may be among the possible explanations of its higher activity with respect to other bactenecins, or alternatively this peptide presents special features in its mode of action.

To further evaluate the antimicrobial effect of ChBac5 we examined its influence on bacterial membranes. We expected to find, as was previously described for pyrrhocoricin and some other pro-rich peptides of invertebrates, that it inactivated bacteria without affecting bacterial membrane integrity, via stereospecific interactions with intracellular targets that inhibited crucial biochemical reactions such as chaperonin-assisted protein folding (Otvos 2002). We were therefore surprised to find that ChBac5 could permeabilize both the inner and outer membranes of *E. coli*, albeit acting relatively slowly compared to porcine protegrin PG1. Although this observation does not exclude the possibility that intracellular effects also contribute to the bactericidal activity of ChBac5, at least for *E. coli* ML-35p, these may be additional to a membrane-attack mechanism.

Just such a dual mechanism has been described for another mammalian proline-rich peptide called Bac7 (1–35). At submicromolar concentrations, this peptide rapidly killed bacteria by a non-lytic, energy-dependent mechanism, whereas its D-enantiomer was inactive. At higher concentrations (>32 μM), both L- and D-Bac7 (1–35) killed bacteria via a lytic mechanism. This suggested the involvement of a stereospecific mechanism of uptake for the natural peptide, recently identified as the membrane protein sbmA in *E. coli* (Mattiuzzo et al. 2007), which is supplemented by a second, non-stereoselective mechanism, based on membranolytic effect at concentrations several times the MIC value (Podda et al. 2006).

Our experiments demonstrated that ChBac3.4 acted in a similar manner to ChBac5, although its membrane-disrupting properties were relatively greater. Using a set of bacterial membrane permeability assays we found, that in general the effects of both ChBac3.4 and ChBac5 were quite different from the action of the distinctly membranolytic porcine antimicrobial peptide PG1. But nevertheless we observed that at a concentration above the MIC, both caprine bactenecins caused release of potassium by bacterial cells. ChBac3.4 and ChBac5 also increased permeabilization of the outer membrane of *E. coli* ML35p and caused delayed permeabilization of the inner membrane for molecules larger than potassium ions—the chromogenic substrates for β-lactamase and β-galactosidase. In comparison with PG1 the bactenecins were significantly less active, but our data support the idea that bacterial membranes could be one of the cell targets of these peptides when applied at higher concentrations (Podda et al. 2006).

ChBac3.4, in contrast to ChBac5, exerted toxic effects towards some mammalian cells; however further investigation is needed to elucidate if this is connected with inhibiting respiration or other vital functions of the target cells.

Concluding Remarks

We isolated a new proline-rich peptide from goat leukocytes and explored its antimicrobial and cytotoxic properties. ChBac3.4 has a structure that resembles, but is distinct from, the N-terminal region of ChBac5 and the Bac5 homologs found in cattle and sheep. ChBac3.4 appears to differ from ChBac5 in virtue of its increased ability to damage microbial membranes and its toxicity towards some mammalian cells. Since caprine phagocytes lack defensin-like peptides (Zhao et al. 1999), ChBac3.4 may play an important host defense role in goats.

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