Volume 327, number 2, 231–236 FEBS 12768 July 1993

Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins

Vladimir N. Kokryakov[a,b], Sylvia S.L. Harwig[a], Elena A. Panyutich[a], Andrei A. Shevchenko[c], Galina M. Aleshina[b], Olga V. Shamova[b], Helen A. Korneva[b] and Robert I. Lehrer[a]

[a]*Department of Medicine, UCLA, Los Angeles, CA 90024, USA,* [b]*Department of General Pathology and Pathophysiology, Institute for Experimental Medicine, 197376 St. Petersburg, Russian Federation* and [c]*Laboratory of Biochemical Researches, Institute for Analytical Instrumentation of the Academy of Sciences of the Russian Academy of Science, 198103 St. Petersburg, Russian Federation*

Received 11 June 1993

Porcine leukocytes contained three homologous peptides, PG-1, 2 and 3, that manifested potent microbicidal activity against *Escherichia coli*, *Listeria monocytogenes* and *Candida albicans* in vitro. The peptides ('protegrins') were composed of 16 (PG-2) or 18 amino acid residues, and, like tachyplesins (broad-spectrum antibiotic peptides of horseshoe crab hemocytes), they contained two intramolecular cystine disulfide bonds. Considerably smaller than defensins, protegrins nevertheless showed substantial homology to them, especially to the 'corticostatic' rabbit defensin, NP-3a. The relatively simple structure of protegrins should provide useful prototypes for constructing congeners with selectively enhanced host defense activities.

Leukocyte; Phagocyte, Antibiotic peptide; Host–parasite relationship

1. INTRODUCTION

Mammalian phagocytes use antimicrobial oxidants [1] and an array of antimicrobial peptides and proteins [2,3] to kill ingested microorganisms. Unlike oxidants, these antimicrobial peptides and proteins show marked inter-species differences in structure, spectrum and potency. Among the best characterized cysteine-containing antimicrobial peptides are 'defensins' [4,5] and 'β-defensins' [6], peptides, M_r ≈3,500–4,800, the unique primary structural motifs of which are characterized by six invariant cysteines and three intramolecular cystine-disulfide bonds. Especially prominent in the polymorphonucleated neutrophils (PMN) of humans and several other mammals, defensins are also produced by rabbit pulmonary alveolar macrophages [5] and by specialized small intestinal epithelial (Paneth) cells in mice [7–9], and humans [10,11]. β-Defensins are found in bovine respiratory epithelial cells [12], bovine granulocytes [6] and avian leukocytes (Harwig et al., unpublished).

We have discovered a new family of small (M_r ≈2,000), cysteine-rich antimicrobial peptides ('protegrins') in porcine leukocytes. Their novel primary structure combines features of defensins with those of tachyplesins – an ancient family of antimicrobial peptides recently identified in horseshoe crab amebocytes [13]. The purification, primary structures and antibiotic properties of protegrins (<Latin *protegere*, to cover or protect) are described in this report.

Correspondence address R.I. Lehrer, Department of Medicine, UCLA Center for the Health Sciences, 10833 LeConte Avenue, Los Angeles, CA 90024, USA. Fax: (1) (310) 206-8766.

2. MATERIALS AND METHODS

2.1. *Leukocytes*

Fresh porcine blood was collected at an abattoir into 15 l vessels that contained an anticoagulant, 5% EDTA in normal saline, pH 7.4 (33 ml/l blood) After the blood cells had sedimented spontaneously for 90 min at room temperature, the leukocyte-rich supernatant was removed and centrifuged at 200 × *g* for 5–7 min. The sediments were pooled and suspended in 0.84% ammonium chloride to lyse the erythrocytes. The resulting leukocytes (70–75% PMN, 5–10% eosinophils, 15–25% lymphocytes and monocytes) were washed in normal saline, resuspended in ice cold 10% acetic acid at 10^8 cells/ml, homogenized and stirred overnight at 4°C. This preparation was centrifuged at 25,000 × *g* for 3 h at 4°C and its supernatant was lyophilized and weighed.

2.2 *Purification and antimicrobial testing*

950 mg (dry weight) of lyophilized extract, which contained 520 mg of protein by BCA analysis, was stirred overnight at 4°C in 100 ml of 10% acetic acid, and then centrifuged at 25,000 × *g* for 2h. The supernatant was removed and passed by pressure through a 50 ml stirred ultrafiltration cell (Amicon, Danvers MA) that contained a YM-5 filter The ultrafiltrate (24 5 mg of protein by BCA) was concentrated to 3 ml by vacuum centrifugation (Speed Vac Concentrator, Savant Instruments, Hicksville, NY), applied to a 2.5 × 117 cm column of BioGel P-10 (Bio-Rad, Hercules, CA), eluted at 4°C with 5% acetic acid, and collected in 6.6 ml fractions.

Aliquots (66 μl) of each fraction were dried by vacuum centrifugation, and resuspended in approximately 6.6 μl of 0.01% acetic acid. 5 μl samples of this concentrate were tested for antimicrobial activity against *Escherichia coli* ML-35, *Listeria monocytogenes* strain EGD and *Candida albicans* strain 820, by previously described radial diffu-

sion and gel overlay techniques [14]. The underlay agars used for all organisms had a final pH of 6.5 and contained 9 mM sodium phosphate, 1 mM sodium citrate buffer, 1% w/v agarose and 0. 30 mg/ml of trypticase soy broth powder (BBL, Cockeysville, MD). The units of activity in the radial diffusion assay were measured as previously described: 10 units correspond to a 1 mm diameter clear zone around the sample well. Active fractions were further examined by acid-urea (AU)-PAGE and SDS-PAGE, and the peptides of interest were purified by reverse-phase (RP)-HPLC.

2.3. *Molecular characterization*

HPLC-grade water and acetonitrile and analytical grade acetic and formic acids were purchased from Fisher (Pittsburgh, PA) . Trifluoroacetic acid and phenylisothiocyanate were purchased from Pierce (Rockford, IL). Acrylamide and molecular weight standards were from Bethesda Research Laboratories (Bethesda, MD).

Native and performic acid-oxidized samples were hydrolyzed in vacuo with 6 N HCl at 110°C for 40 h. Amino acid analysis was performed after phenylthiocarbamylation [15] by the Picotag technique using a Millipore–Waters 510 binary solvent delivery system and a Nova-Pak C-18 column (Millipore–Waters, Milford, MA). Amino acid sequences were determined by gas-phase Edman degradation with a Porton Model 2090 instrument, using purified 500 pmol samples of protegrins that had been reduced and alkylated with iodoacetic acid (Sigma) in 6.0 M guanidine HCl, 0.5 M Tris-HCl, 2 mM EDTA (pH 8.1) and desalted by RP-HPLC on a Vydac C18 column. The mean initial yield was 55.2% (range 47 5–63.8%) and the mean repetitive yield was 86.4% (range 77 6–92.2%)

Fast atom bombardment (FAB)-mass spectrometric analyses were performed by Kristine Swiderek and Terry D Lee at the Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, CA. The sample (approximately 200 pmol) was taken up in 1–2 μl of 5% acetic acid and added to 1 μl of a mixture of dithiothreitol: dithioerythritol (5·1) on a 1.5×6.0 mm stainless-steel sample cage, ionized with a 6 KeV Xe atom beam, and analyzed in a double focussing JEOL HX100HF magnetic sector mass spectrometer.

Chymotrypsin digests were prepared by incubating 3 nmol of each carboxymethylated protegrin with 0.05 μg of TLCK-treated chymotrypsin (Worthington, Freehold, NJ) overnight at 37°C in 0.1 M ammonium bicarbonate, pH 8.5. The reaction was quenched by adding 1 vol. of glacial acetic acid to 4 vols. of incubation mixture. After lyophilization and resuspension in 0.1% trifluoroacetic acid/water, the peptide fragments were separated by RP-HPLC on a 0.46×25 cm Vydac C18 column, and subjected to amino acid analysis.

2.4. *Other methods*

Tricine SDS-PAGE [16] and acid-urea (AU)-PAGE [17] were performed in minigel formats, using a model SE 250 vertical gel unit (Hoefer, San Francisco, CA) Gels were stained with a solution that contained 1 g of Coomassie brilliant blue R-250 (Sigma), 270 ml methanol, 630 ml water and 150 ml formaldehyde and destained in methanol/water/formaldehyde (1:3.0.04) and then silver-stained [18] . The protein concentrations of column fractions and other mixtures of proteins mixtures was measured by the bicinchoninic acid (BCA) technique (Pierce Chemicals, Rockford, IL). The concentration of NP-1 was determined by quantitative amino acid analysis and the concentration of purified protegrins and synthetic tachyplesin 1 (Bachem, Philadelphia PA) was assessed by A_{280} measurements, using a millimolar extinction coefficient of 1,280 for protegrins (which contain 1 tyrosine/molecule) and 8,250 for tachyplesin 1, which contains 2 tyrosines and 1 tryptophan/molecule [19,20].

3. RESULTS

We applied an ultrafiltration step early in our purification scheme to focus this analysis on molecules of approximately 5 kDa and below. The top portion of Fig. 1 illustrates the elution of the ultrafiltered leukocyte extract from a BioGel P-10 column, and Fig. 2 shows the antimicrobial activity of these P10 fractions against *E. coli, L. monocytogenes* and *C. albicans*.

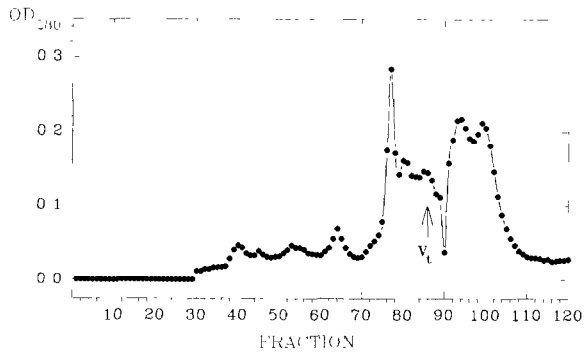

Fig. 1 Biogel P-10 chromatogram. A concentrated ultrafiltrate of porcine leukocyte peptides was prepared as described in section 2, eluted from the Biogel P-10 column with 5% acetic acid, and collected in 6 6 ml fractions. V_t signifies the total column volume.

Fractions 76–78, which showed strong activity against all of these organisms, contained a mixture of three peptides, the migration of which on AU-PAGE resembled that of the highly cationic rabbit defensins, NP-1. NP-2 and NP 3a,b (Fig. 3). The individual porcine peptides were designated PG-1, PG-2 and PG-3, in the order of their migration towards the cathode on AU-PAGE, with PG-1 being the fastest. Unlike the rabbit defensins, which constituted >15% of the total protein in rabbit granulocytes and were clearly evident in crude extracts (lane 10), the protegrins were only faintly visible in crude extracts (lane 1) , and became prominent only after the ulrafiltration step (lane 2). Additional antimicrobial peptides, devoid of cysteine and therefore unrelated to protegrins (data not shown), were eluted from the column in earlier fractions and will be described in a future report.

The three protegrins were readily separated by RP-

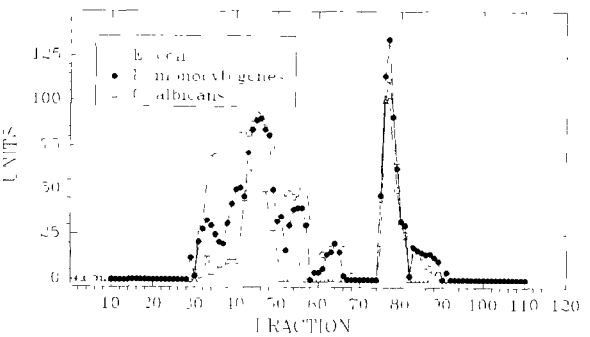

Fig. 2. Antibacterial activity. Aliquots (66 μl) of the 6.6 ml P-10 fractions shown in the previous figure were lyophilized, resuspended in 6.6 μl of 0.01% acetic acid to effect a 10-fold concentration, and tested against *E coli* ML-35p, *L. monocytogenes* strain EGD and *C albicans* in radial diffusion assays. The peptides of interest for this report were most abundant in Fractions 76–78.

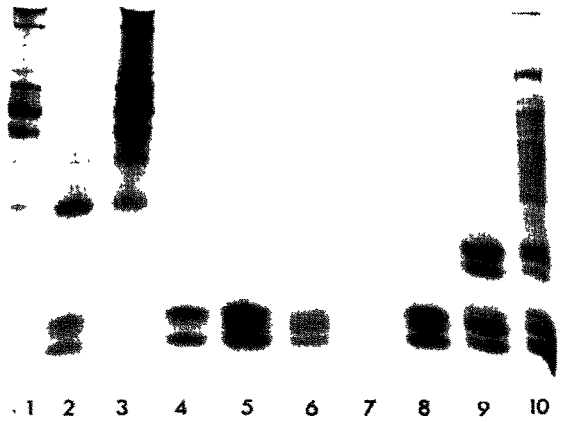

Fig. 3. AU-PAGE of the P-10 fractions. Lane 1, crude porcine leukocyte extract (6.0 μg protein); lane 2, YM-5 filtrate (7 μg protein), lane 3, YM-5 retentate (18.8 μg protein); lane 4, Fraction 76 (20 μl, 3.5 μg protein); lane 5, Fraction 77 (20 μl, 6.0 μg protein): lane 6, Fraction 78 (20 μl, 2.6 μg protein); lane 7, Fraction 79 (20 μl, 1.2 μg protein) lane 8, mixture of equal volumes of Fractions 76–78 (4.2 μg protein). Lane 9, a mixture of rabbit defensins NP-1, 2, 3a, 3b, 4 and 5 (4.2 μg protein), The most cationic (NP-1) and least cationic (NP-5) rabbit defensins are labeled. Lane 10, crude extract of rabbit neutrophils. The gel was stained with Coomassie blue.

HPLC. As shown in Fig. 4, PG-1 emerged first from a preparative Vydac C-18 column, followed by PG-3 and then by PG-2, FAB-MS measurements yielded the following values for monoisotopic mass: PG-1, 2,154.5; PG-2, 1,955.6; and PG-3 2,055.5, indicating that protegrins were considerably smaller than defensins (M_r 3,500–4,500).

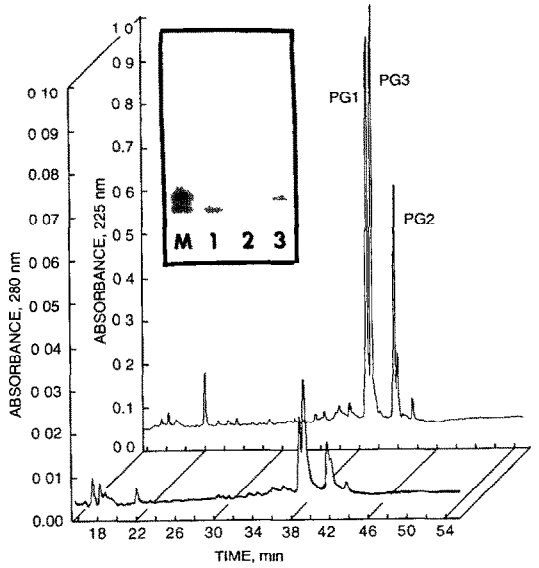

Fig. 4. HPLC purification of the protegrins. Fractions 76–78 from the P10 column were pooled and chromatographed on a 1 × 25 cm Vydac 218TP1010 column with a gradient (Buffer A, 0.1% TFA; Buffer B, 0.1% TFA in acetonitrile) that increased in acetonitrile concentration by 1%·min^{-1}. The inset shows an AU-PAGE gel, stained with Coomassie blue, that contains the starting mixture (M), composed of pooled Fractions 76–78, and the individual protegrin (PG) species, which are labeled 1, 2 and 3 on the inset

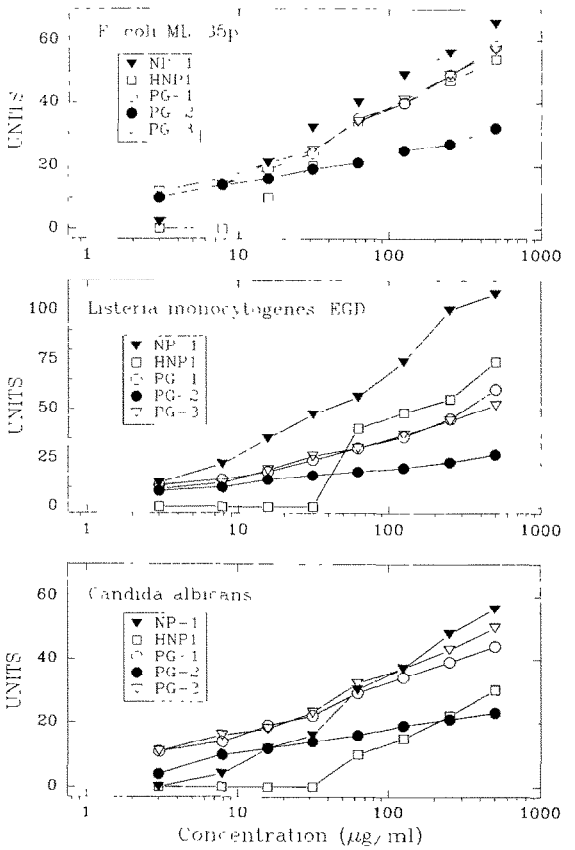

Fig. 5 Antimicrobial activity of purified porcine protegrins. Purified porcine protegrins PG-1, -2 and -3, rabbit defensin NP-1 and human defensin HNP-1 were prepared in 0 01% acetic acid at a concentration of 500 μg/ml, and seven additional 2-fold serial dilutions were prepared in 0.01% acetic acid. 5 μl of each concentration was tested for microbicidal activity against *E. coli* ML-35p, *L. monocytogenes* EGD and *C albicans* by radial diffusion in thin agarose gels. Each set of 10 units corresponds to a 1 mm diameter clear zone, devoid of viable bacteria, around the 3 mm sample well

We used a radial diffusion assay in agarose gels [14] to test the purified protegrins against *L. monocytogenes*, *E. coli*, and *C. albicans* (Fig. 5). Under the selected study conditions, PG-1 and PG-3 killed the Gram-negative bacterium, *E. coli* ML-35p less effectively than NP-1 but more effectively than HNP-1. PG-1 and PG-3 also killed the Gram-positive organism, *L. moncytogenes* strain EGD, with considerable efficacy, approximately half that of the human and rabbit defensins on a weight basis. Both peptides, especially PG-3, also showed excellent activity against *C. albicans*. Although PG-2 was also active against each of these organisms, its potency was substantially less than that of PG-1 and PG-3, especially at concentrations exceeding 25 μg/ml.

Amino acid analysis was performed on each peptide three times: in its native state, after its oxidation by performic acid, and after its reduction and alkylation by iodoactetic acid. As the results from these analyses were concordant, Table I shows only the values we obtained

Table I

Amino acid analysis of S-carboxymethylated protegrins

Amino acid	PG-1		PG-2		PG-3	
	mol %	n_{18} (n_{seq})	mol %	n_{16} (n_{seq})	mol %	n_{18} (n_{seq})
Cys[a]	21.42	3.9 (4)	25.4	4.1 (4)	21.7	3.9 (4)
Asx	1.01	0.2 (0)	0.15	0.0 (0)	0.63	0.1 (0)
Glx	1.26	0.2 (0)	0.27	0.0 (0)	0.70	0.1 (0)
Ser	0.50	0.1 (0)	0.00	0.0 (0)	0.00	0.0 (0)
Gly	16.24	2.9 (3)	11.70	1.9 (2)	21.6	3.9 (4)
His	0.00	0.0 (0)	0.00	0.0 (0)	0.00	0.0 (0)
Arg	33.0	5.9 (6)	29.2	4.6 (5)	27.2	4.9 (5)
Thr	0.00	0 0 (0)	0.00	0.0 (0)	0.00	0.0 (0)
Ala	0.29	0.1 (0)	0.00	0.0 (0)	0.00	0.0 (0)
Pro	0.13	0.0 (0)	0.10	0.0 (0)	0 00	0.0 (0)
Tyr	6.18	1.1 (1)	6.20	1.0 (1)	5.90	1.1 (1)
Val	9.74	1.8 (2)	8.31	1.3 (1)	10.38	1.9 (2)
Met	0.00	0.0 (0)	0.00	0.0 (0)	0.00	0 0 (0)
Ile	0.20	0.0 (0)	4 94	0.8 (1)	0.74	0.1 (0)
Leu	4 63	0.8 (1)	6 43	1.0 (1)	5.87	1.1 (1)
Phe	5.25	1.1 (1)	7.13	1.1 (1)	5 16	0.9 (1)
Trp	n.d.		n.d.		n.d.	
Lys	0.16	0.0 (0)	0.16	0.0 (0)	0.10	0.0 (0)
Total	100.0	18.1 (18)	100 0	15.8 (16)	100.0	18 0 (18)

[a]Cysteine was determined as S-carboxymethylcysteine; n.d. signifies not determined. n_{18}, n_{16}, amino acid residues calculated from mol % determination, assuming 18 or 16 residues/molecule.

with the carboxymethylated peptides. The peptides were unusually rich in cysteine (21–25 mol%), arginine (27–33 mol%) and glycine (11.2–21.6 mol%), and contained tyrosine, accounting for their absorbance at 280 nm, but no lysine, which is prominent in tachyplesins. The remaining residues were hydrophobic, and consisted of valine, phenylalanine, leucine, and isoleucine (PG-2 only).

The amino acid sequences of the protegrins were determined and are shown in Fig. 6. Chymotryptic fragments were prepared and purified from each of the PG-peptides. Amino acid analysis of chymotyrptic peptides representing residues 1–5 and 1–7 were consistent with the $Arg^4 \rightarrow Gly^4$ substitution found in PG-3, and fragments containing Cys^{13} to the carboxy-terminal residue confirmed the presence of an isoleucine in PG-2 and its truncation (data not shown). PG-1 and PG-3 contained 18 amino acids, and were identical except for residue 4, which was an argininc in PG-1 and a glycine in PG-3. The resulting charge difference explained the slower migration of PG-3, relative to PG-1, in AU-PAGE gels. PG-2 was identical to PG-1 except that it contained isoleucine at residue 14, instead of valine, and its carboxyl terminus had been truncated after Val^{16}. PG-2 was smaller than PG-3, but had the same net positive charge, accounting for its slightly faster migration on AU-PAGE.

Although the primary amino acid sequences of the protegrins showed minimal homology to tachyplesins or β-defensins, their first 3 cysteine residues were spaced identically to those of classical defensins, which are exemplified by HNP-1 and NP-3a in Fig. 6. In addition, they showed remarkable homology to the 'corticostatic' rabbit defensin, NP-3a, in that eight of the ten PG-3 residues from Gly^4 to Cys^{13} (GLCYCRRRFC) were identical to residues 1–10 of the rabbit defensin NP-3a (GICACRRRFC), and another (Leu^5 of PG-3) showed a highly conservative substitution (Ile^2). Additionally, a tyrosine residue that corresponds to Tyr^7 of PG-3 occurs in many defensins, including human HNP-1, -2 and -3 , rat NP-1, -2 and -4 and mouse 'cryptdins' (intestinal defensins) [4,5,7].

The monoisotopic molecular masses calculated from these sequence data are: PG-1 2,155.04, PG-2, 1,955.94 and PG-3, 2,055.96. The comparable values obtained by FAB-MS were: PG-1, 2,154.5; PG-2, 1,955.6; PG-3, 2,055.5. Because FAB-MS measurements are accurate to within $\approx$ 0.5 mass unit (0.025%) for protegrin-sized peptides, the experimental and calculated values are in excellent agreement. Nevertheless, these values do not exclude the possibility that the carboxy-terminal residues of protegrins are amidated, since post-translational carboxy-terminal amidation of protegrins would reduce their respective masses by 1 unit, and these smaller masses would also be consistent with the FAB-MS data. More definitive information about carboxy-terminal amidation of native protegrins requires additional studies, such as solid-phase synthesis of amidated and non-amidated protegrins and comparison with their native forms.

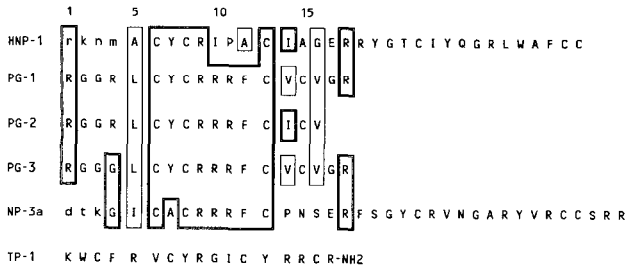

Fig. 6 Amino acid sequences of protegrins. The three protegrins have been aligned with human defensin HNP-1 and rabbit defensin NP-3a. Identical residues are boxed with double lines, conservative substitutions are boxed within a single line. Defensin residues shown in lower case are present in prodefensins, but are absent from the fully processed, mature peptides. TP-1 signifies tachyplesin I.

4. DISCUSSION

Four principal families of cysteine-rich antimicrobial peptides have been recognized in animals during the past few years: tachyplesins [13,21], defensins [4,5], β-defensins [6,12] and insect defensins [22,23]. Plants also produce small cysteine-rich antimicrobial peptides, which differ from those thus far detected in animal cells [24,25].

Tachyplesins are found within the small cytoplasmic granules of horseshoe crab hemocytes, cells that resemble mammalian granulocytes in morphology, and are so abundant that approximately 10 mg of tachyplesin was recovered from the hemolymph of a single horseshoe crab [26,27] and 70 mg of tachyplesin I was obtained from 100 g (wet weight) of hemocytes [28]. Tachyplesins contain 17 or 18 amino acid residues, including four cysteines that form two intramolecular disulfide bonds, and have an amidated carboxy-terminal residue [28]. Although several cysteine-free antimicrobial peptides, including insect cecropins [29–31] and bovine leukocyte indolicidin [32] also have an amidated carboxy-terminal residue, this feature is not found in defensins [4,5], β-defensins [6,12] and insect defensins [22,23]. Whether the carboxy-terminal protegrin residue is amidated or not remains to be determined.

Tachyplesins inhibited growth of various Gram-positive and Gram-negative bacteria and of *C. albicans* at concentrations between 1.6 and 6.3 μg/ml [13] and inactivated vesicular stomatitis virus, influenza A virus and HIV in vitro [33,34] . They were also noted to bind to lipopolysaccharide (LPS) and to inhibit LPS-mediated activation of hemocyte factor C [13,20]. Extremely stable, tachyplesins retained activity after boiling for 30 min or exposure to 0.1% trifluoroacetic acid [20]. By two-dimensional NMR I spectroscopy [21], tachyplesin I had an antiparallel β-sheet configuration that was connected by a midsection β-turn involving residues 8–11 and stabilized by the disulfide bridges linking its $Cys^3 \rightarrow Cys^{16}$ and $Cys^7 \rightarrow Cys^{12}$.

The porcine protegrins described in this report resemble tachyplesins in several aspects, including: (i) their broad spectrum of antimicrobial activity, which encompasses Gram-positive and Gram-negative bacteria and the fungus, *C. albicans*; (ii) their molecular mass of approximately 2 kDa; (iii) the presence of four cysteine residues and two intramolecular cystine disulfide bonds; and (iv) their potent activity against certain enveloped viruses (Kokryakov et al., unpublished). However, the very different placement and spacing of the cysteine residues of protegrins and tachyplesins (Fig. 6) indicates that they belong to distinct peptide families.

Is there a relationship between protegrins and defensins? At first sight, this seems unlikely, since protegrins have only 16–18 residues with four cysteines while mature defensins, which have 29–35 residues with 6 cysteines, are almost twice as large. Nevertheless, this possibility cannot be casually dismissed. The six cysteine residues of defensins are invariant, and constitute a critical component of the 'defensin motif' [4,5]. Fig. 6 shows that the first three cysteine residues of the protegrins are spaced identically with the first three cysteines of classical defensins. While this may be due to chance alone, it is more difficult to overlook the remarkable similarities between the (G^4LCYCRRRFC^{13}) decapeptide region of PG-3 and corresponding residues (G^1ICACRRRFC^{10}) of rabbit defensin NP-3a. Eight of these residues are identical, while another (Leu^5) is represented by a highly conservative substitution (Ile^2). Future studies at the mRNA and gene level should clarify whether this resemblance is coincidental, or signifies relatedness between the protegrin and defensin gene families.

NP-3a, the primary sequence and antimicrobial properties of which were first reported in 1985 [35], was later found to have additional properties, including 'corticostasis', namely the ability to competitively antagonize ACTH-mediated steroid synthesis by adrenocytes [36–38]. This property is believed to result from the ability of NP-3a to bind reversibly and with high affinity to the ACTH receptor of rat adrenocytes [37,38], which itself may derive from the mimicry of the RRR motif of NP-3a to the residues of ACTH responsible for binding to its receptor [38]. Although the in vivo significance of corticostasis remains conjectural, the effects of protegrins on adrenal steroid production in vitro and in vivo warrants further study.

Why have so many cysteine-rich antimicrobial peptides emerged in recent studies? The wide distribution of such peptides among animals, including arthropods, birds and mammals, suggests that they are not newcomers to the scene. In contrast, the elaborate lymphocyte-regulated immune system found in higher vertebrates is relatively new, at least on the time-scale of evolution. Since bacteria and fungi preceded them, it is reasonable to assume that the earliest animals derived survival benefit from chemical defense systems that supplemented

the protective effects of their exoskeletal armor. Endogenous antimicrobial peptides, cysteine-rich and otherwise, may have constituted such a phylogenetically primitive host defense system. The purification of a cecropin-like molecule from the intestinal tissues of pigs [39], as well as the occurrence of defensin and tachyplesin-like molecules in phylogenetically ancient taxa, such as dragonflies [40] and horseshoe crabs [13], lends support to this notion. Delineation of such endogenous antimicrobial peptides should not only illuminate the molecular mechanisms of innate immunity, it may also provide time-tested molecular templates that can be used to develop novel therapeutic agents.

Acknowledgements. This work was supported, in part, by research Grants AI 22839 and AI 32930 from the National Institutes of Health. Protein sequencing was performed at the UCLA Protein Sequencing Facility, which is supported by a BRS Shared Instrumentation Award 1 S10RR05554 01 from the National Institutes of Health. We thank Audree Fowler (UCLA) for expertly performing the amino acid micro-sequencing, Ken Miyasaki (UCLA) for helping us with the figures, Dr. S.V. Slepenkov (St. Petersburg University) for assisting with the leukocyte harvest and Leonie Tan (UCLA) for skilled technical assistance. We thank Dr. Sci. Olga Mirgorodskaya, Head of the Laboratory of Biochemical Researches, Institute for Analytical Instrumentation of the Russian Academy of Sciences, St. Petersburg, Russian Federation, for her support.

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