LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity[1]

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Received 20 June 2000; accepted 31 July 2000

Edited by Pierre Jolles

Abstract We report the isolation and characterization of a novel human peptide with antimicrobial activity, termed LEAP-1 (liver-expressed antimicrobial peptide). Using a mass spectrometric assay detecting cysteine-rich peptides, a 25-residue peptide containing four disulfide bonds was identified in human blood ultrafiltrate. LEAP-1 expression was predominantly detected in the liver, and, to a much lower extent, in the heart. In radial diffusion assays, Gram-positive *Bacillus megaterium*, *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus carnosus*, and Gram-negative *Neisseria cinerea* as well as the yeast *Saccharomyces cerevisiae* dose-dependently exhibited sensitivity upon treatment with synthetic LEAP-1. The discovery of LEAP-1 extends the known families of mammalian peptides with antimicrobial activity by its novel disulfide motif and distinct expression pattern.

Key words: Antimicrobial peptide; Liver; Hemofiltrate; Cysteine-rich peptide

1. Introduction

Antimicrobial peptides are an important component of innate immunity in many species, including plants, insects, fish, amphibians and mammals [1–3]. Although especially the latter have a highly efficient system of adaptive immunity, there is growing evidence that in animals antimicrobial peptides such as defensins [2,3], protegrins [4] and cathelicidins [5] represent a considerable part of the immune system in the defense of cellular pathogens. Antimicrobial peptides are predominantly expressed in epithelial cells lining the respiratory, alimentary and genito-urinary tract as well as in granules of circulating phagocytes, and they exert their activity mainly by disrupting the cell membranes of cellular pathogens [3,6–8]. This rather unspecific mode of action is suggested to be responsible for the broad-spectrum activity of many peptide antibiotics. Despite their diverging effects, most antimicrobial peptides share common structural elements in being amphipathic and carrying net positive charges at physiological pH due to basic amino acid residues [1,7].

As many antimicrobial peptides contain multiple disulfide bonds, we intended to isolate small cysteine-rich peptides from human blood ultrafiltrate, which was shown earlier to be a source of defensins [9]. Using a cysteine alkylation assay, we report the isolation and characterization of a novel extremely cysteine-rich 25-residue peptide that was termed LEAP-1 (liver-expressed antimicrobial peptide). By analysis of the primary structure and the corresponding cloned cDNA, we demonstrate that LEAP-1 is a novel peptide which resembles other peptide antibiotics in that it exhibits comparable structural elements and antimicrobial activity, but differs significantly in the predominant expression in liver.

2. Materials and methods

2.1. Isolation and structural characterization of LEAP-1

LEAP-1 was isolated during a comprehensive search for cysteine-containing peptides in fractions from human blood ultrafiltrate [10]. In brief, the cysteine content of a peptide is determined after reduction of disulfides and subsequent carboxamidomethylation of the resulting free thiol groups inducing a defined mass shift for each single cysteine residue. 10 μg of freeze-dried fractions were dissolved in 4-methylmorpholine (50 μl, 200 mM, pH 8.3) and dithiothreitol (5 μl, 400 mM) was added. After incubation for 60 min at 45°C, iodoacetamide (20 μl, 500 mM) was added, and after a further 30 min at ambient temperature, the samples were desalted by solid-phase extraction. Peptides were eluted with 100 μl 70% acetonitrile in 0.1% trifluoroacetic acid (TFA), and the fractions were subjected to MALDI-TOF mass spectrometry prior to and after carboxamidomethylation on a Voyager-DE STR (PerSeptive Biosystems). LEAP-1 was identified as a peptide with a molecular mass of 2789.8 Da and a content of eight cysteine residues calculated from the resulting mass shift of +464.5 Da in the alkylated fraction (Fig. 1). For purification of LEAP-1, a 10-mg aliquot of the corresponding peptide fraction was dissolved in 0.06% TFA and separated in three reverse-phase HPLC steps. Final purification was performed on a C18 ODS-AQ column (4.6×250 mm, 5 μm, 120 Å, UV detection at 214/280 nm, YMC Europe, Schermbeck, Germany). NH_2-terminal amino acid sequence analysis was performed by Edman degradation using a Procise 494 sequencer (PE Biosystems, Foster City, CA, USA).

2.2. Peptide synthesis

LEAP-1 was assembled on a preloaded Tentagel R Trt-Thr(tBu) resin (Rapp Polymere, Tübingen, Germany) using standard Fmoc chemistry and trityl-protected cysteines. The peptide was deprotected and cleaved from the resin with a mixture of TFA, ethanedithiol and water (94:3:3). After filtration and precipitation with *tert*-butylmethylether, the reduced peptide obtained was purified by reverse-phase HPLC on a Vydac C18 column (The Separations Group, Hesperia, CA, USA). Fractions containing the product were identified using electrospray mass spectrometry (ESIMS; M_r determined, 2797.5 Da; M_r calculated, 2797.3 Da) and analytical HPLC, and subjected

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[1] The nucleotide sequence data reported in this paper have been submitted to the GenBank/EBI Data Bank with accession number AJ277280. Scanning of this sequence against the data base resulted in the identification of related sequences with the accession numbers AD000684 and P81172.

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0014-5793/00/$20.00
PII: S0014-5793(00)01920-7

to oxidation in phosphate buffer at pH 7.4. The fully disulfide-bonded peptide was then purified by reverse-phase HPLC (Vydac C18). Purity was checked on a Nucleosil C18 PPN column (Macherey and Nagel, Düren, Germany) and by capillary electrophoresis on a Beckman P/ACE System 2000. Synthetic LEAP-1 coeluted and comigrated with native LEAP-1 during reverse-phase HPLC and capillary electrophoresis, respectively. The molecular mass of the product was determined by ESIMS (M_r experimental 2789.5 Da; M_r calculated 2789.3 Da).

2.3. Antimicrobial activity

2.3.1. Radial diffusion assay [11]. Bacterial cells in mid-logarithmic phase (500 μl) were added to 100 ml of previously boiled 10 mM sodium phosphate buffer (pH 7.2, 37°C) containing 0.03% (w/v) tryptic soy broth, 0.8% (w/v) NuSieve GTG agarose (FMC BioProducts, Rockland, ME, USA) and 0.02% (v/v) Tween 20. Bacteria growing at 37°C were plated with 0.2% type 1 agarose (w/v, low EEO, Sigma-Aldrich) and 0.6% NuSieve agarose. The mixture was pipetted into sterile Petri dishes and evenly spaced wells were obtained by punching out gel cylinders with a diameter of 3 mm. After incubation of the bacteria with synthetic LEAP-1 (1–11 μg) for 18 h, growth inhibition appeared as plain circular zones, and their diameters were determined [12]. Synthetic casein-(16–57), a 42-residue peptide fragment of human casein α-s1, was used as a negative control in order to exclude any effect due to contaminants derived from peptide synthesis procedures. Experiments were carried out in duplicate. Water was used as additional negative control.

2.3.2. Determination of colony forming units (CFU). *S. cerevisiae* was cultured overnight at 30°C in YPD medium (1% yeast extract, 2% peptone, w/v, pH 6.5). To obtain mid-logarithmic phase germs, cells were subcultured at 1:100 for 3 h and harvested by centrifugation at $600\times g$ and 4°C for 10 min. After washing of the pellets a dilution containing approximately 2×10^6 cells/ml in 10 mM sodium phosphate buffer was prepared. Dry LEAP-1 (1–12.5 μg) was then mixed with 100 μl of cell suspension and incubated for 2 h at 30°C. Subsequently, 10 μl of this mixture was withdrawn and the reaction was stopped by a 500-fold dilution with ice-cold sodium phosphate buffer, and aliquots were manually spread on YPD agar plates. After 16 h at 30°C, colonies were counted and inhibition percentiles were calculated. Three single experiments were carried out at least in triplicate. An analogous procedure was performed with *B. subtilis* using LB medium (pH 7.4).

2.4. Molecular biological standard methods and cDNA cloning

RNA extraction, cDNA first strand synthesis, polymerase chain reaction (PCR), reverse transcription/polymerase chain reaction (RT-PCR), and DNA fluorescence sequencing were performed as described earlier [13–15]. LEAP-1-specific cDNA fragments were amplified from human liver cDNA by means of standard RT-PCR methods using the primers SN-2S (CCGCTTGCCTCCTGCTCCTCC-TCCTCCTCG) and SN-6AS (CGATGACAGCAGCCGCAGCA-GAAAATGCAG) derived from a LEAP-1 gene-containing sequence (accession number AD000684, positions 31 400–34 200). Nested 3′- and 5′-RACE PCR for amplification of LEAP-1 cDNA ends was performed using Marathon-Ready human liver cDNA (Clontech, Palo Alto, CA, USA). The gene-specific primers SN-2S and SN-3S were used for 3′-RACE, and SN-5AS and SN-4AS for 5′-RACE according to the manufacturer's instructions. The fragments obtained were cloned in pGEM T vector (Promega, Mannheim, Germany) and subsequently sequenced. The specificity of the inserts for LEAP-1 was verified by sequence comparisons using the MacMolly program package (Softgene).

2.5. Real-time RT-PCR analysis

To quantitatively evaluate the expression of the LEAP-1 gene in various human tissues we performed real-time RT-PCR [16] by means of a Prism 7700 Sequence Detection System (PE Biosystems). Primers and probes were designed with the assistance of the Primer Express software (PE Biosystems). The probe constructed flanked intron 2 of the gene (FAM-CCAGCTGGATGCCCATGTTCCAGA-TAMRA) while the PCR primers were specific for exons 2 and 3. For relative quantification, the values calculated for LEAP-1 gene expression in each sample were divided by the corresponding values for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene. All quantitative real-time RT-PCR experiments were performed in duplicate using standard reagents and mixes from PE Biosystems and 96-well microplates containing cDNAs from 14 different human tissues, six standard curve samples (prepared from HUH-7 cells) and two non-template controls (purified water, ACS reagent, Sigma-Aldrich).

3. Results and discussion

The peptide isolation procedure utilizing a mass spectrometric assay for cysteine-rich peptides (Fig. 1) resulted in the isolation of a peptide which was shown to be homogeneous by HPLC and capillary electrophoretic analysis. Sequence analysis revealed the amino acid sequence DTHFPIX-IFXXGXXHRSKXGMXXKT (Fig. 2), where X represents residues which gave blank cycles during Edman sequencing. As a difference in molecular mass between the original sample and the reduced and subsequently carboxamidomethylated sample corresponded to eight cysteine residues, it was concluded that all Xs of the peptide sequence represent cysteine. The corresponding sequence of the peptide containing eight disulfide-bonded cysteine residues would have a molecular mass of 2789.3 Da. This value accords exactly with the molecular mass that was found experimentally for the isolated peptide which we termed LEAP-1. To our knowledge, a peptide containing four disulfide bonds within a stretch of 17 amino acid residues is unique in the human proteome. In the COOH-terminal moiety, LEAP-1 contains basic amino acid residues such as lysine and arginine. Therefore, LEAP-1 exhibits structural features which are known from other peptide antibiotics such as the defensins [3,8]. To verify the structure determined and to investigate antimicrobial effects of LEAP-1, a corresponding synthetic peptide was produced. Oxidation of the reduced synthetic precursor readily produced a major product identical with the native counterpart.

To determine the amino acid sequence of the precursor protein and to investigate the expression pattern of the corresponding gene, the cDNA specific for LEAP-1 was cloned and sequenced. For this purpose, sequence comparisons of the LEAP-1 amino acid sequence with entries of the EMBL data base were carried out. We could identify a putative gene for LEAP-1 being located within a sequenced contig of human chromosome 19 (accession number AD000684). From the given nucleotide sequence of the gene we then deduced the most probable exon regions by searching for typical exon/in-

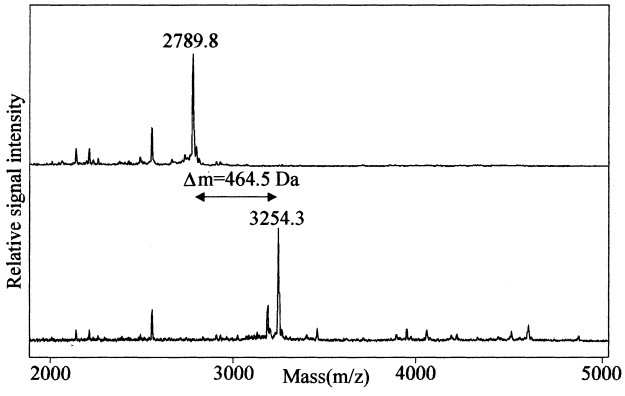

Fig. 1. Comparison of MALDI-TOF mass spectra of peptide fractions 7–30 before (upper trace) and after carboxamidomethylation (lower trace). The mass shift of 464.5 Da indicates the content of eight cysteine residues in LEAP-1 which was confirmed by Edman degradation with the purified peptide.

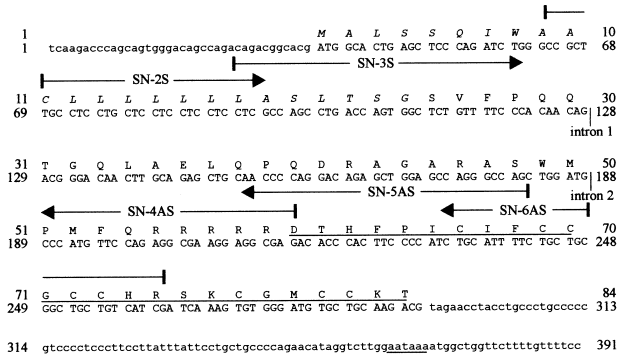

Fig. 2. LEAP-1 precursor cDNA sequence and deduced amino acid sequence. Coding regions are printed in capital letters. The positions of the primers used for preparative RT-PCR and the positions of the putative introns 1 and 2 within the corresponding gene are indicated. The sequence representing the isolated antimicrobially active LEAP-1 and the potential polyadenylation signal are underlined. The typical secretory signal sequence 1–24 is printed in italics.

tron boundary sequences and open reading frames. Following this strategy, six PCR primers located within the putative exons 1 and 3 of the LEAP-1 gene were derived. In PCR reactions with cDNA from human lung, kidney, liver and tonsils, we obtained homogeneous bands with each of the possible nine primer combinations. The most homogeneous and intensive band was obtained from human liver cDNA and the primer combination SN-2S/SN-6AS. The PCR product was cloned, sequenced and verified as LEAP-1-specific. To amplify the 3′- as well as the 5′-end of the LEAP-1 cDNA, we performed nested 3′- and 5′-RACE PCR. The RACE PCR products obtained exhibited comparable sizes and could both be verified as LEAP-1-specific. By identification of overlapping regions and the largest fragments obtained, a 391 bp LEAP-1-specific cDNA sequence exactly matching the three exon regions of the corresponding gene was finally determined. Approximately 40 bp upstream of the 5′-terminal nucleotide of the cDNA sequence, a potential TATA box motif (ATAAAA) was identified within the above-mentioned contig. Furthermore, a typical AATAAA polyadenylation signal is located approximately 28 bp upstream of the 3′-terminus of the cDNA. Both findings indicate the cloning of an almost full-length cDNA specific for LEAP-1.

The cDNA sequence exhibits an open reading frame encoding an 84-residue precursor protein which contains the isolated peptide at the COOH-terminus (Fig. 2). An NH_2-terminal signal sequence (residues 1–24) characteristic for secretory proteins was identified using prediction methods [17]. In addition, a basic penta-arginyl segment directly adjacent to the NH_2-terminus of the isolated peptide is contained within the precursor protein. It appears that the LEAP-1 precursor is a secretory protein that is proteolytically processed at the polybasic proteolysis site whereby the isolated peptide LEAP-1 is released. A further processing of LEAP-1 seems to be possible as an NH_2-terminally truncated form (sequence positions 65–84) was also identified in human blood ultrafiltrate by mass spectrometry. With its eight cysteine residues forming four disulfide bonds along a sequence of 17 amino acids, LEAP-1 is an exceptionally structured molecule. Interestingly, data base comparisons using the entire amino acid sequence derived from cDNA translation resulted in an entry (SwissProt, P81172) describing the partial amino acid sequence 50–84 of the LEAP-1 precursor. However, this entry does not provide information regarding the occurrence of LEAP-1 in plasma and the cysteine connectivity, gene expression and the antimicrobial activity. Furthermore, tBLASTn searches in the GenBank data base led to the identification of several mouse EST clone sequence entries (e.g. W41260, W36480, W34077) related to LEAP-1. The corresponding murine cDNA encodes a putative 84-residue protein which shows a sequence identity of approximately 51% with human LEAP-1, and exhibits an identical cysteine pattern at its COOH-terminus. Thus, it might represent the murine ortholog of LEAP-1.

Using the synthetic peptide, we examined the antimicrobial effects of LEAP-1 by performing an agar diffusion assay [11]. Here, mainly Gram-positive bacteria exhibited susceptibility to LEAP-1 treatment (Fig. 3). Nevertheless, LEAP-1 also inhibited the growth of Gram-negative *Neisseria cinerea* and the yeast *Saccharomyces cerevisiae*, thus resembling the antimicrobial spectrum of human β-defensin-1 [18]. In contrast, no inhibition of the growth of the yeast *Rhodotorula rubra* and Gram-negative germs *Escherichia coli* BL21 and *Pseudomonas fluorescens* was detected. In order to determine the in vitro IC_{50} values of LEAP-1 against two sensitive strains, we performed CFU assays. These experiments revealed half-maximal inhibitory activity at approximately 40 μg/ml (14.4 μM) against *B. subtilis* and 50 μg/ml (18 μM) against *S. cerevisiae*. These effective peptide concentrations are in the same order of magnitude as known for other antimicrobial peptides [19–22].

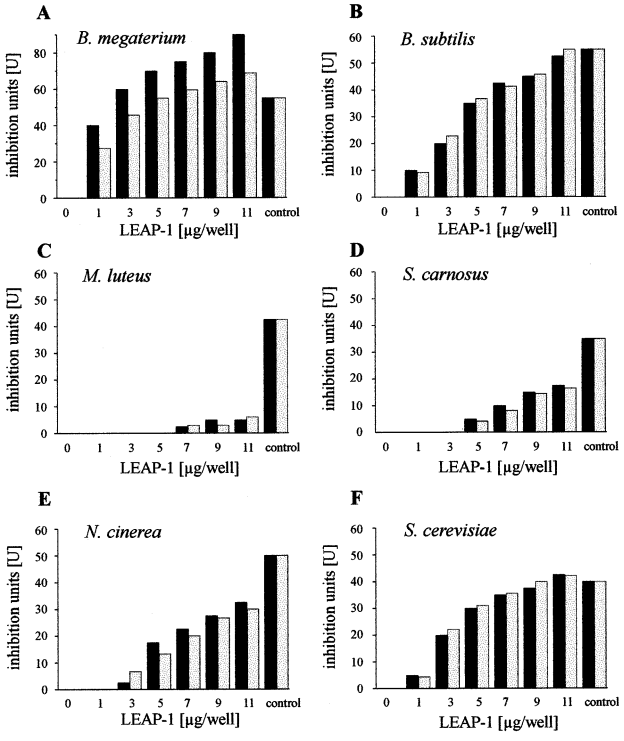

Fig. 3. Dose-dependent antibacterial activity of LEAP-1 using the radial diffusion assay. (A) *Bacillus megaterium* ATCC14581, (B) *Bacillus subtilis* ATCC6051, (C) *Micrococcus luteus* ATCC9341, (D) *Staphylococcus carnosus* DSM20501, (E) *Neisseria cinerea* ATCC14685, (F) *Saccharomyces cerevisiae* ATCC9763. 1 inhibition unit (U) corresponds to 0.1 mm radius of growth inhibitory area. The results of two independent experiments with casocidin-I at 11 μg as a positive control are shown. Casocidin-I has been shown to cause antimicrobial effects comparable to those of magainin II [18].

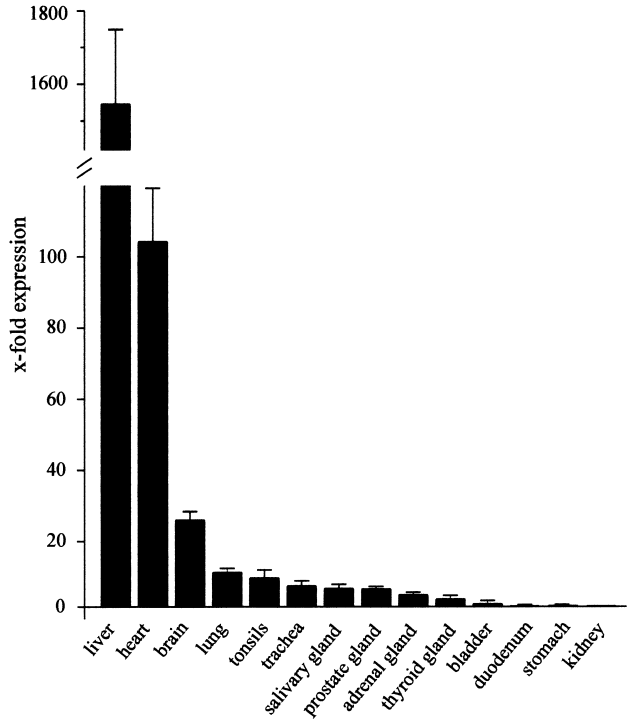

Fig. 4. Expression of the LEAP-1 gene by real-time quantitative RT-PCR. The indicated *x*-fold expression of LEAP-1 in different tissues was determined by standardization to the expression in the kidney, using a two-step calculation method. First, averaged LEAP-1 expression values of each tissue were divided by the values for the GAPDH housekeeping gene. Then, the normalized target gene values obtained were divided by the normalized calibrator value. Tissue from kidney with the lowest LEAP-1 expression level served as calibrator (i.e. kidney exhibits a 1-fold expression). For all tissues three experiments were carried out in duplicate.

Increased peptide concentrations due to local stimulation of expression at inflammatory sites or putative synergism between different antimicrobial components of suboptimal concentrations contribute to the fact that it is difficult to conclude in vivo effects from in vitro assays. Moreover, it might be possible for an antimicrobial peptide even in sublethal concentrations to be active against pathogens by receptor-mediated interactions or other mechanisms [20,23,24].

In order to analyze whether the expression pattern of LEAP-1 is similar to that of other human antibiotic peptides, we performed quantitative real-time RT-PCR assays (Fig. 4). The highest level of LEAP-1 gene expression was detected in the liver where it was found in a range comparable to that of the GAPDH housekeeping gene. Expression of LEAP-1 in heart and brain proved to be at least two times higher than in the other tissues tested. These organs have not been reported so far to harbor significant amounts of antimicrobial peptides. Lung, prostate gland, tonsils, salivary gland and trachea express LEAP-1 in a similar but low level. These results were confirmed using the superoxide dismutase gene as housekeeping gene for quantitative real-time RT-PCR and indicate a rather unusual expression pattern for an antimicrobially active peptide. In particular, the prevailing expression in liver gives rise to further investigations concerning the role of LEAP-1 in liver-specific functions. Thus, it would be important to evaluate whether LEAP-1 is involved in the activation of liver-derived acute-phase proteins. However, several findings suggest that antimicrobial peptides may have further functions other than the direct inhibition of microbes at mucosal barriers [23–25].

In conclusion, we identified a novel peptide that circulates in human blood plasma and exhibits a unique motif of cysteine connectivity. LEAP-1 extends the known families of endogenous peptides with growth inhibitory effects on microbial organisms. Future investigations concerning the role of LEAP-1 should address the extraordinary level of expression in the liver, and possible synergistic effects with other components of the immune system.

Acknowledgements: This work was supported in part by the Deutsche Forschungsgemeinschaft (grant to A.K., IIIGK-GRK99/2-98).

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