A novel murine cathelin-like protein expressed in bone marrow

Anna E. Popsueva[a,*], Marina V. Zinovjeva[a], Jan W.M. Visser[b], J. Mark J.M. Zijlmans[c], Willem E. Fibbe[c], Alexander V. Belyavsky[a,b]

[a]*Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov str. 32, 117984 Moscow B-334, Russian Federation*
[b]*New York Blood Center, 310 East 67th Str., New York, NY, USA*
[c]*Department of Hematology, University Medical Center, Rijnsburgerweg 10, Leiden, The Netherlands*

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Abstract A novel cDNA encoding a putative secreted protein was isolated from murine bone marrow. The encoded protein named MCLP (murine cathelin-like protein) was found to be highly homologous to the pig cathelin, and to four neutrophil antimicrobial polypeptides: CAP 18, indolicidin, Bac 5 and FALL-39. Secondary structure prediction studies identified a highly cationic region in the C-terminal part of prepro-MCLP with a tendency to adopt an amphipathic α-helical conformation, as observed in many antimicrobial peptides. However, no antibacterial activity was observed with the synthetic peptide corresponding to this region of MCLP.

Key words: Antibacterial protein; Amphipathic α-helix; Hematopoietic stem/progenitor cells; cDNA; Cathelin

1. Introduction

A number of animal antimicrobial polypeptides are known at present [1]. Despite significant sequence variation, they share certain common features. In particular, these peptides are highly cationic and bind to bacterial lipopolysaccharide (LPS), the major component of the Gram-negative bacterial outer membrane. Antibiotic peptides act by perturbing the membranes of the target microorganisms, which leads to the formation of numerous channels in the bacterial membrane and eventually to cell death [2]. All animal antibacterial peptides, among them cecropins, magainins [1], defensins [1,3] and β-defensins [4], can form amphipathic α-helixes. Helix formation was shown to be important for the biological activity of antimicrobial peptides [5].

Antimicrobial peptides are synthesized as precursors, prepro-peptides, from which the mature peptides are released by proteolytic processing [6]. A major store of defense polypeptides is the granules of polymorphonuclear leukocytes, which play an important role in the inflammatory response to microorganisms.

A significant part of mammalian antibiotic peptides belong to the cathelin family [7]. The cathelin protein was first isolated as a new cysteine proteinase inhibitor from pig leukocytes [8]. Subsequently, several cDNAs encoding antibacterial polypeptides have been cloned from bone marrow cells. Sequences of signal regions and proregions of these antibiotic peptides turned out to be very similar to the cathelin sequence. The presence of a cathelin-like region was identified in several peptides: polypeptides Bac 5 and Bac 7 [9,10], prophenin [11] and PR-39 [12] belonging to the family of proline- and arginine-rich antibiotics, associated with the large granules of bovine neutrophils; in the cysteine-rich cyclic dodecapeptide [13] and protegrins [14]; in polypeptides CAP 18 [15] and p15 with two isoforms [16] from rabbit neutrophils; and in human polypeptide FALL-39 [17].

Here we report the cloning, sequencing and analysis of the cDNA encoding a novel protein which is highly similar to the members of the cathelin family.

2. Materials and methods

2.1. cDNA cloning

B252 clone was isolated previously [18] using subtractive cDNA cloning. Briefly, the oligo(dA)-tailed first cDNA strand from 10^4 rhodamine 123-dull cells [19,20] was hybridized with an excess of biotinylated poly(A)$^+$ RNA from peritoneal macrophages. Subtracted cDNA sequences were recovered by amplification with oligo(dT)-containing primer and cloned into lambda ZAP II vector. Filter replicas of subtracted library were sequentially hybridized with radiolabeled subtracted positive (Rho^{dull} cells minus macrophages) and negative (macrophages minus macrophages) probes, and differentially hybridizing clones were isolated.

MCLP cDNA clones were isolated from a murine adult bone marrow cDNA library (kindly given by Dr. M. Ershler) using a radiolabeled 400 bp B252 clone. About 1.5×10^6 recombinant clones were screened; 10 positive clones were detected, and 3 clones were purified to homogeneity. pBluescript plasmids were excised from lambda ZAP II vector using R408 helper phage. Isolated plasmids were sequenced by the termination method [21]. Primary and secondary sequence analysis was performed with program packages 'DNASIS' and 'DNA-SUN'; homology search was done using the blastn and blastp programs.

2.2. Northern blot analysis

Total RNA from various murine tissues was isolated using the guanidine thiocyanate method [22]. For Northern blotting, 20 μg RNA from each tissue was electrophoresed on 1.0% agarose/formaldehyde gel [23] and transferred by capillary blotting onto Hybond-N nylon membrane (Amersham) according to the manufacturer's instructions. The blot was probed with [^{32}P]dATP-labeled MCLP and washed in stringent conditions.

2.3. cDNA blot analysis

PCR-amplified total cDNA was prepared from sorted murine hematopoietic stem cell subpopulations: Rho^{bright}, Rho^{dull}, Rho$^-$/Ver^{dull}, Rho$^-$/Ver$^-$ [19,20], mono/granulocytes, macrophages, thymocytes and total bone marrow, by the modified procedure described earlier [24]. Amplified cDNA samples from each cell fraction were separated in 2% alkaline agarose gel and transferred to nylon membrane Hybond-N$^+$. Full size MCLP cDNA was used as a probe for hybridization.

3. Results and discussion

During searches for genes expressed preferentially in murine

*Corresponding author. Fax: (7) (95) 135-1405.
E-mail: popsueva@imb.imb.ac.ru.

The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under accession number X94353.

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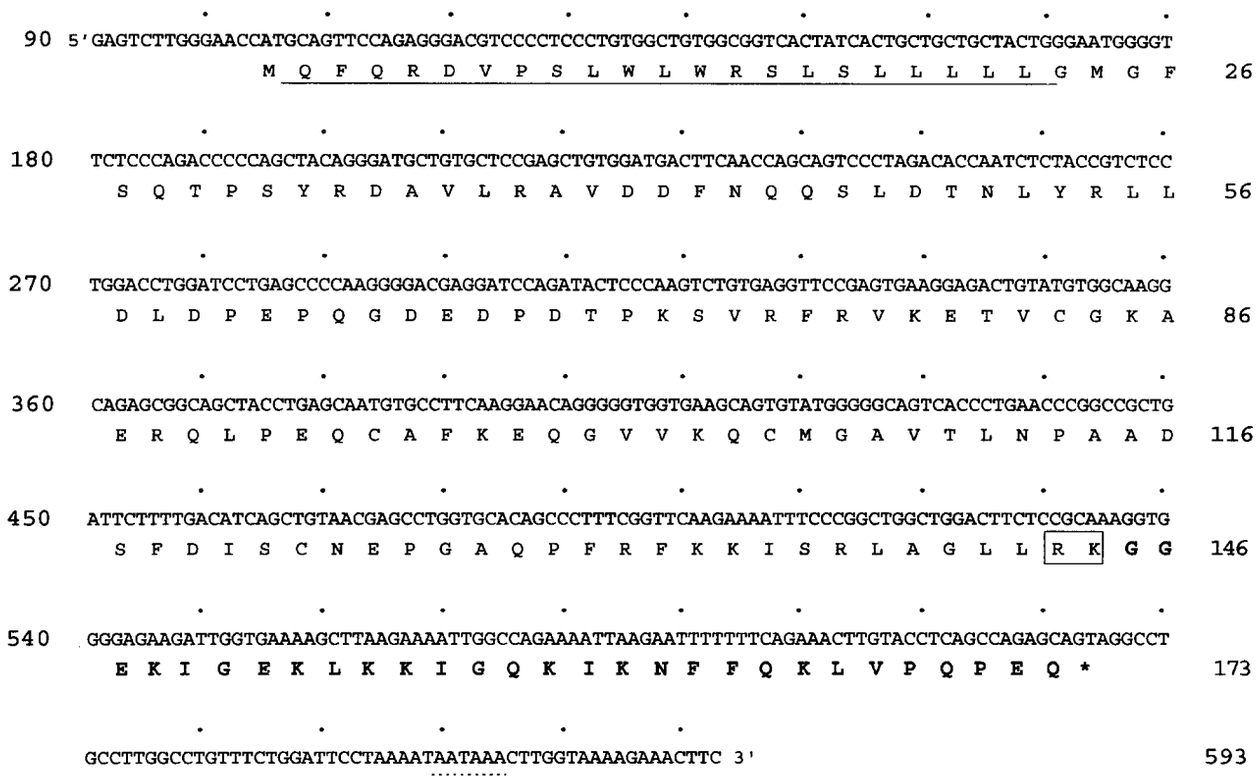

Fig. 1. The nucleotide and predicted amino acid sequence of MCLP. The polyadenylation signal is indicated by a dashed line and the stop codon is marked by an asterisk. The leader peptide is underlined, the putative mature processed peptide is in bold. Proteolytic cleavage sites are boxed.

hematopoietic stem/progenitor cells [18], a clone named B252 was isolated. Using B252 as a probe, a corresponding near full-length cDNA clone was isolated from the murine adult bone marrow cDNA library. On the basis of sequence similarity, the clone was renamed MCLP (murine cathelin-like protein). The 593 bp cDNA contains a 519 bp open reading frame (Fig. 1). A classical polyadenylation signal is found 23 bp upstream of the poly(A) tail. A putative initiation codon is surrounded by a satisfactory Kozak consensus sequence [25]. The initiator methionine is followed by a hydrophobic region

Fig. 2. Sequence similarities between MCLP and proteins of the cathelin family. The deduced amino acid sequence of MCLP is aligned with the precursor sequences of peptide antibiotics from rabbit (cathelin and CAP-18), cow (bactenecin, Bac5, indolicidin), human (FALL-39). Cysteines are boxed. The dibasic protease processing sites are underlined. Alignment was performed using the program DNA-SUN.

corresponding to a putative signal peptide. The predicted prepro-MCLP consists of 173 amino acids with a calculated mass of 22.7 kDa and pI 8.68.

The deduced sequence of the product of the MCLP gene was found to be highly homologous to the known members of the cathelin-like subfamily of antimicrobial peptides. The overall amino acid identity between prepro-MCLP and porcine cathelin, porcine PR-39, bovine Bac 5, bovine pro-indolicidin, human FALL-39 and rabbit CAP-18 is 63%, 59%, 57%, 54%, 51% and 51% respectively. Alignment of these proteins (Fig. 2) shows that their structural similarities are confined to the N-terminal regions, whereas the C-terminal regions are very different, with the exception of a short region of high similarity between MCLP and CAP-18. Similar to indolicidin [26], CAP-18, FALL-39, cathelin and bactenecins, the C-terminal moiety of the pro-region of MCLP has four cysteines which can form two intramolecular disulfide bridges (Fig. 2). The C-terminal region of MCLP has a dibasic proteolytic cleavage site RK (residues 143–144) that precedes a highly cationic sequence, corresponding to the residues 146–174 that can form an amphipathic helix (Fig. 4). Therefore, MCLP protein possesses all the characteristics of a typical antibacterial peptide precursor.

To verify whether mature MCLP has antibacterial activity, a peptide corresponding to residues 146–174 of the precursor protein was synthesized. The antibacterial activity of MCLP was tested with two strains of *E. coli* (NM 539, XL-1 blue) as described by Agerberth et al. [17]. No antibacterial activity was observed using peptide concentrations in the range of 0.1 to 100 μM. When tested for helix formation by CD spectroscopy, the synthetic peptide displayed in water and medium

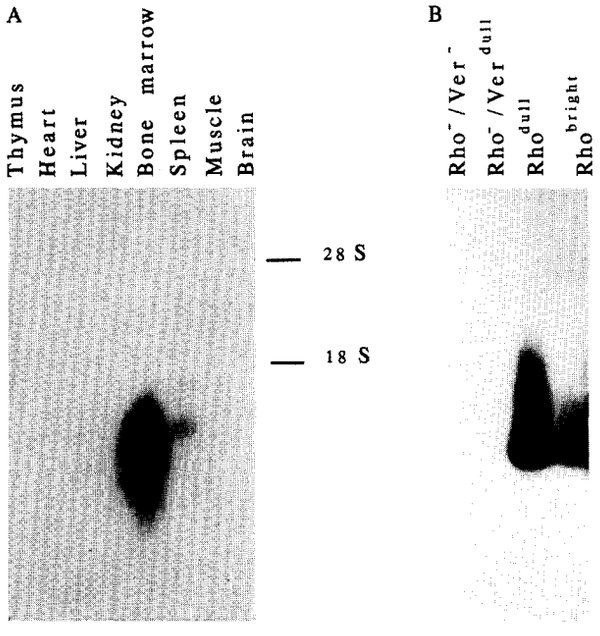

Fig. 3. (A) Northern blot analysis of MCLP gene expression in various tissues. Each lane contains 20 μg of total RNA from murine thymus, heart, liver, kidney, bone marrow, spleen, muscle and brain. (B) cDNA blot analysis of MCLP gene expression in different cell subsets of murine bone marrow stem/progenitor cell compartment. 1 μg of PCR-amplified cDNA from the corresponding subset was loaded onto each lane. Cell fractions are arranged according to their maturation stage, with the Rho^-/Ver^- fraction being the most primitive, and the Rho^{bright} fraction the most differentiated [19,20].

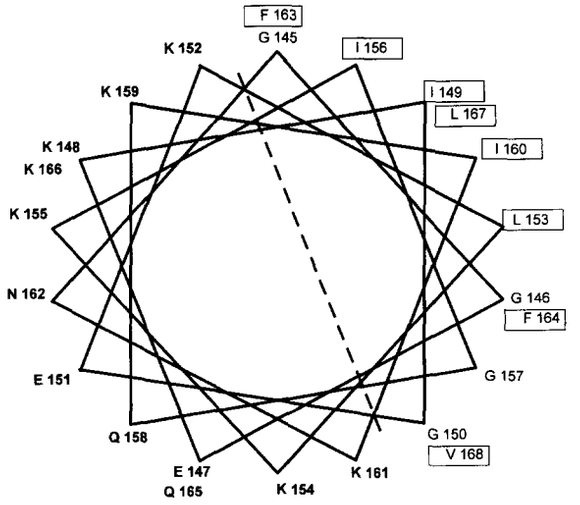

Fig. 4. Helical wheel representation of the region G^{145}-V^{168} of MCLP. The helix starts at the top of the figure. Strongly hydrophobic residues are boxed, and charged ones are in bold. The dashed line divides the helix into hydrophilic and hydrophobic parts.

E [27] a spectrum typical of an unordered conformation. However, under conditions favoring helix formation (30% trifluoroethanol), the helical content was about 50% (data not shown), which is similar to the peptides possessing antibacterial activity [15,17]. Therefore, the synthetic peptide does have a propensity for helix formation. The processing of the MCLP is not known yet, and it is possible that the mature endogenous peptide contains additional regions which may stabilize the helix conformation under physiological conditions to allow antibacterial activity. Alternatively, the conformation could be stabilized by accessory factors.

The presence of MCLP mRNA in various murine tissues was estimated by Northern blot analysis (Fig. 3A). A 0.9 kb mRNA was found in high amounts in bone marrow cells and to some extent in spleen cells. No hybridization signal was detected in brain, muscle, kidney, liver, heart, thymus. In addition, we hybridized the MCLP probe with the Southern blot of PCR-amplified cDNA from murine bone marrow fractions enriched with stem/progenitor cells fractionated on the basis of rhodamine 123 staining: Rho^{bright}, Rho^{dull}, Rho^-/Ver^{dull} and Rho^-/Ver^- [19,20]. No expression of MCLP mRNA was detected in the Rho^-/Ver^{dull} and Rho^-/Ver^- fractions (Fig. 3B), highly enriched in hematopoietic stem cells (30 cells of the most primitive Rho^-/Ver^- fraction are sufficient for reconstitution of lethally irradiated host). At the same time, significant hybridization signal was found in the Rho^{dull} and Rho^{bright} fractions (Fig. 3B) containing mostly committed progenitor cells, as well as in the cells of mono/granulocytic lineage isolated with antibody 15-1.1 (data not shown). No MCLP gene expression was detected in thymocytes and peritoneal macrophages (data not shown). According to these data, mRNA of the MCLP gene appears at the early to intermediate stages of hemopoiesis, presumably in progenitor cells committed to myeloid differentiation. In this respect, the expression of MCLP gene is similar to that of myeloperoxidase, cathepsin G and certain other markers of the myeloid lineage [28,29].

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