Cloning and expression of human neutrophil lipocalin cDNA derived from bone marrow and ovarian cancer cells

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Abstract Human neutrophil lipocalin (HNL) cDNA was amplified by PCR technology in combination with deoxyinosine containing oligonucleotides for cloning, sequencing and production of the recombinant protein in *E. coli*. The primers were targeted to the corresponding DNA backtranslate of the mature protein resulting in a PCR amplified 534 bp cDNA from different reverse transcripts of ovarian cancer cell line and bone marrow cell mRNAs. Sequence analysis revealed that two different cDNAs from ovarian cancer and bone marrow cells could be obtained. Cloning and expression of HNL cDNAs were performed in *E. coli* strain HMS 174 [DE3] using the pET system yielding in two recombinant proteins with a molecular weight of 21 kDa which is consistent with an 178 amino acid residues containing sequence of the mature HNL protein. N-terminal amino acid sequence analysis of the expression products showed an identical polypeptide sequence missing the *E. coli* processed starting methionine.

Key words: Lipocalin; Ovarian cancer; Inosine; PCR; Gelatinase

1. Introduction

HNL was first identified by Triebel et al. [1] as a 25 kDa gelatinase-associated glycosylated component of the 125 kDa gelatinase from human PMN leukocytes. Furthermore, the protein was localized in monomeric and dimeric forms in the specific granules of human neutrophils [2]. HNL belongs to the lipocalin family. It has strong homologies with the rat α_2 microglobulin-related protein [3] and the mouse oncogene protein 24p3 [4]. Most lipocalins are carriers of small hydrophobic substances through the hydrophilic medium of the body [5]. HNL has been shown to bind chemoattractants of the type of *N*-formyl peptides [6]. But its physiological role is still not clear. Although the amino acid sequence similarity among the members of the lipocalin family is low, there are three conserved regions containing the invariant residue tryptophan in one motif [7]. Cristallographic studies of several lipocalins revealed that they exhibit also a high similarity in the folding pattern with an eight-stranded antiparallel β-barrel fold into an orthogonal calyx [8]. Chan et al. [9] have shown that lipocalin genes are part of conserved syntenies at HSA9q.

Here we report on both the PCR amplification using oligonucleotides containing deoxyinosine and on the sequencing of the cDNA of the HNL from the reverse transcripts of ovarian cancer cell line and bone marrow cell mRNA. The obtained two different cDNAs could be cloned in *E. coli* and the rHNL could be expressed as a 21 kDa protein.

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; OC, ovarian cancer; PMN, polymorphonuclear; TIMP-1, tissue inhibitor of metalloproteinases.

2. Materials and methods

Human bone marrow cells were a gift from Dr. F. Lindemann (Krankenanstalten Bethel, Bielefeld, Germany), human ovarian cancer cell lines OC 1, OC 2, OC 6 and OVCAR 3 were from Dr. O. Wilhelm (Frauenklinik der TU München, Klinikum r. d. Isar, Germany), *E. coli* strain HMS 174 [DE3] and plasmid vector pET 11a were from Novagen (Madison, USA), restriction enzymes were from Biolabs (Schwalbach, Germany).

2.1. RNA preparation and reverse transcription

Total RNA from 1×10^7 bone marrow cells and the ovarian cancer cell lines was prepared by the guanidinium isothiocyanate extraction procedure followed by ultracentrifugation through a cesium chloride gradient according to Chirgwin et al. [10]. First strand cDNA synthesis from 5 μg of total RNA was performed with Superscript (Lifetechnologies, Inc.) by the manufacturer's protocol.

2.2. Oligonucleotide synthesis

Oligonucleotides were synthesized by Genosys Biotechnologies, Inc. according to the N-terminal and C-terminal amino acid sequences of the mature HNL protein. Deoxyinosine was introduced at the ambiguous codon positions in cases where more than two alternate deoxynucleotides would have been possible leading to following HNL-primers: 5′-CAGGACTCIACITCIGACCTIATCCCIGCCCC-3′ and 5′-CCGTCIATGCAT TGATCIATIGGIACIGGGAA-3′.

2.3. PCR amplification and cloning of HNL

50 ng of first strand cDNA were used as template to amplify the HNL gene by PCR technology. Each reaction consisted of 100 pmol of primers, 0,2 mM dNTP, 1,5 mM $MgCl_2$ and 1 U of Taq DNA polymerase (Lifetechnologies, Inc.) in 50 μl of reaction buffer recommended by the manufacturer. Two combined amplification cycles with 40 s at 95°C, 45 s 40°C, 90 s 72°C (5 cycles), 40 s 95°C, 60 s 50°C, 40 s+1 s/cycle 72°C (33 cycles) were carried out on a MiniCycler (MJ Research, Inc.).

2.4. Cloning and sequencing of the PCR amplification products

PCR fragments were electrophoresed in agarose gels and purified by phenol extractions and ethanol precipitation before ligating the PCR product into the TA cloning vector pCR II (Invitrogen Corp., San Diego, USA) using the manifacturer's protocol. This system takes advantage of the nontemplate dependent activity of thermostable polymerases used in PCR that add single deoxyadenosines to the 3′-end of all duplex molecules provided by PCR. Sequencing of the HNL cDNA in the plasmid pCR II was performed according to the dideoxy termination method [11].

2.5. Southern blot analysis

HNL cDNA containing plasmids pCR II and pET 11a were separatly digested with *Eco*RI, *Nde*I and *Bam*HI followed by agarose gelelectrophoresis, capillary transfer of restriction fragments on nylon membrane

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and hybridization using standard methods [12]. 'HNL PAT': 5'-CAAGAG CTACAATGTCACCTCCGTCCTG-3' was labelled with digoxygenine (MWG-Biotech, Germany) and used as a probe in Southern blot hybridization.

2.6. Bacterial expression

For HNL expression the cDNA was selectively amplified with two oligonucleotide primers: 'HNLF' 5'-ATTCATATGCAGGACTCGACGTCGGAC-3' and 'HNLR' 5'-CAGGGATCC CTAGCCGTCGATGCATTGATCGA-3' using PCR II-HNL cDNA as template. 'HNLF' incorporates sequences for a unique *Nde*I site and an initiating methionine. 'HNLR' introduces sequences for a stop codon and a unique *Bam*HI site. Ligation into the plasmid pET 11a and expression in *E. coli* strain HMS 174 [DE3] followed as previously described [13].

2.7. SDS-PAGE and Western blot analysis

SDS-PAGE was performed by the method of Laemmli [14]. Western blots were performed as published by Bergmann et al. [15]. After blotting the proteins were further subjected to automated amino acid sequence determination in a Knauer sequencer by the method described in [16].

3. Results and discussion

Total HNL RNA was isolated from different ovarian cancer cell lines and bone marrow cells and used as templates for reverse transcription. Bone marrow cells were expected to be a good source for HNL mRNA as the protein was found to be expressed in human leukocytes [2]. Ovarian cancer cell cDNA seemed also to be a suitable source for HNL cDNA because the supernantant of these cells contain this protein (not published) as revealed by specific ELISA procedures [17].

Since only protein and no DNA information of HNL was available when we started these studies, the degeneracy of codons for amino acids had to be considered in the design of primers for the PCR technique. The use of selective oligonucleotides had to represent the potential nucleic acid sequences for each alternate codon. Oligonucleotide design which take account of this problems requires neutral bases like deoxyinosine [18]. Therefore HNL primers were designed by the DNA backtranslate (Genetic Computer Group), containing five deoxyinosines each, according to the mature HNL amino acid sequence [19]. Fig. 1A shows the products of the PCR amplification reactions obtained with these oligonucleotides at the optimized low annealing temperature of 50°C. Variation of the annealing temperature below 45°C or above 55°C resulted in unspecific amplification products or in no amplification reaction (not shown). The amplification product obtained by the use of cDNA from OC 1, OC 2, OC 6 or bone marrow cells had a lenght of 534 bp which was in accordance with the expected size of the DNA encoding the whole mature part of HNL. Possible prepro sequences of the HNL at the 5'- and 3'-coding region could not be revealed by this approach but were not essential for cloning and expression of rHNL in *E. coli* [13].

Sequence determinations of the 534 bp OC 1, OC 6 and bone marrow cDNAs were carried out in the pCR II vector. Southern blot analyses were performed to obtain positive pCR II-HNL cDNA clones (Fig. 1B). This pCR II vector system takes advantage of the 3'-deoxyadenylate extension which the Taq DNA polymerases added to the termini of amplified cDNA [20] and allows direct cloning of the PCR product in appropriate constructed vectors (TA cloning). HNL oligonucleotide design therefore needed no additional unique restriction sites which were candidates for unspecific template hybridization yielding in various PCR amplification products.

The determined sequence data are shown in Fig. 2. The isolated 534 bp cDNA corresponded to the amino acid sequence of the mature HNL protein [2]. The HNL cDNA sequence using OC 6 transcripts as template is compared with the related lipocalin cDNA sequences of mouse (24p3) [4] and rat ($\alpha_2$microglobulin related protein) [3] indicating strong conserved nucleotide homologies with an overall conformity of

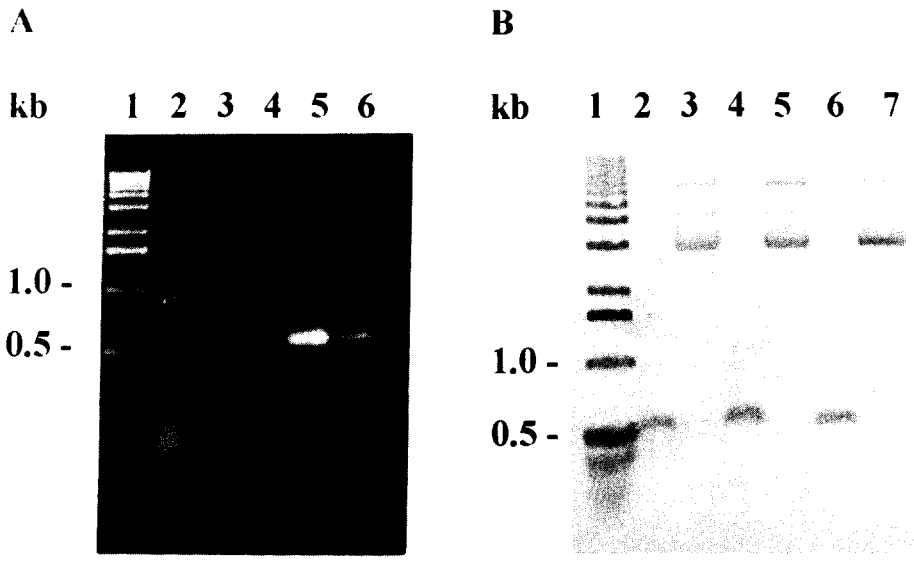

Fig. 1. (A) A 1% agarose gel electrophoresis of PCR products obtained by PCR reaction (see section 2) with inosine containing HNL primers and different templates. Lane 1, pBR 322 fragments (DNA Längenstandard X, Boehringer Mannheim, Germany) as a molecular weight standard. Template cDNA from; lane 2, OVCAR 3; lane 3, OC 1; lane 4, OC 2; lane 5, OC 6 and lane 6, bone marrow cells. The DNA samples were visualized by ethidium bromide fluorescence. (B) Southern blot analysis of *Eco*RI digested pCR II containing HNL cDNA. Lane 1, molecular weight standard. HNL cDNA from; lane 2, OC 1; lane 4, OC 6; lane 6, bone marrow cells. Lane 3, 5, 7 plasmids before digestion as control. Sequence analysis of the 534 bp cDNA revealed identity with the DNA backtranslate (Genetics Computer Group) of the mature HNL protein.

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HNL
rat   ctcttcctcc tccggcacac atcggaccta gtagctgctg aaaccatggg 50
24p3                          agaccta gtagctgtgg aaaccatggc 27

HNL
rat   cctgggtgtc ctgtgtctgg cccttgtcct gcttggggtc ctgcagaggc 100
24p3  cctgagtgtc atgtgtctgg gccttgccct gcttggggtc ctgcagagcc 77

HNL        cagga ctcGacgtcg gacctgatcc cggccccacc tctgagcaag 45
rat   aggcccagga ctcaactcag aacttgatcc ctgccccacc tctgatcagt 150
24p3  aggcccagga ctcaactcag aacttgatcc ctgccccatc tctgctcact 127

HNL   gtccctctgc agcagaactt ccaggacaac caattccagg ggaagtggta 95
rat   gtgcccctgc agccaggctt ctggaccgaa cggttccagg gcaggtggtt 200
24p3  gtcccctgc agccagactt ccggagcgat cagttccggg gcaggtggta 177

HNL   tgtggtaggc ctggcaggga atgcaattct cagag.aaga caaagacccg 144
rat   cgttgtcggc ctggcagcga atgcggtcca gaaagaaaga caaag..ccg 248
24p3  cgttgtgggc ctggcaggca atgcggtcca gaaaaaaaca gaagg..cag 225

HNL   c.aaaagatg tatgccacca tctatgagct gaaagaagac aagagctaca 193
rat   ctttaccatg tacagcacca tctatgagct acaggaagac aatagctaca 298
24p3  ctttacgatg tacagcacca tctatgagct acaagagaac aatagctaca 275

HNL   atgtcacctc cgtcctgttt aggaaaaaga a......gtg tgactactgg 237
rat   acgtcacttc catcctcgtc ag......gg gccagggctg tcgctactgg 342
24p3  atgtcacctc catcctggtc agggaccagg accagggctg tcgctactgg 325

HNL   atcaggactt ttgttccagg ttgccagccc ggcgagttca cgctgggcaa 287
rat   atcagaacat tcgttccaag ctccaggcct ggccagttca ccctgggaa 392
24p3  atcagaacat ttgttccaag ctccagggct ggccagttca ctctgggaaa 375

HNL   cattaagagt taccctggat taacgagTta cctcgtccga gtggtgagca 337
rat   tattcacagc taccctcaga tacagagcta cgatgtgcaa gtggccgaca 442
24p3  tatgcacagg tatcctcagg tacagagcta caatgtgcaa gtggccacca 425

HNL   ccaactacaa ccagcatgct atggtgttct tgaagaaagt ttctcaaaac 387
rat   ctgactacga ccagtttgcc atggtatttt tccagaagac ctctgaaaac 492
24p3  cggactacaa ccagttcgcc atggtatttt tccgaaagac ttctgaaaac 475

HNL   agggagtact tcaagatcac Gctctacggg agaaccaagg agctgacttc 437
rat   aaacagtact tcaaagtcac cctgtacgga agaaccaagg ggctgtccga 542
24p3  aagcaatact tcaaaattac cctgtatgga agaaccaagg agctgtcccc 525

HNL   ggaactaaag gagaacttca tccgcttctc caaatctctg ggcctccctg 487
rat   tgaactgaag gagcgattcg tcagctttgc caagtctctg ggcctcaagg 592
24p3  tgaactgaag gaacgtttca cccgctttgc caagtctctg ggcctcaagg 575

HNL   aaaaccacat cgtcttcccC gtcccgatcg atcaatggat cgacggc    534
rat   ataacaacat cgttttctct gttcccaccg accaatgcat tgacaactga 642
24p3  acgacaacat catcttctct gtccccaccg accaatgcat tgacaactga 625

rat   acagacggtg agcgtggctg actgggatgt gcagtggcct gatggttcag 692
24p3  atgggtggtg agtgtggctg actgggatgc gcagagaccc aatggttcag 675

rat   gtcccacctg tctgtctgcc gctccatctt tcctgttgcc agagaatcac 742
24p3  gcgctgcctg tctgtctgcc actccatctt tcctgttgcc agagagccac 725

rat   ctggctgccc caccagccat gattccatca agcatctgat ccc...tctt 789
24p3  ctggctgccc caccagccac cataccaagg agcatctgga gcctcttctt 775

rat   atttgatcag ctctccccat ccacctgtgt taacgctgcc ccaccaacgg 839
24p3  atttggccag cactccccat ccacctgtct taa.....ca ccaccaa.tg 819

rat   gctcccccct tctgctgaat aaacacatgt ccccaaa              876
24p3  gcgtcccctt tctgctgaat aaatacatgc cccc                  853
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Fig. 2. cDNA sequence of the mature HNL protein compared with the cDNA sequences of mouse oncogene protein *24p3* and *rat* α_2 microglobulin-related protein. Two clones of HNL cDNA derived from OC 6 (shown) and bone marrow cells show differences in four base pairs (big letters, see text). Boldface letters indicate base pairs conserved among the three lipocalins. Underlined letters indicate different base pairs compared with the cDNA sequence described by Bundegaard et al. [21].

71% (HNL, mouse), 72% (HNL, rat) and 86% (rat, mouse). Alignment of HNL-OC 6 cDNA with HNL-bone marrow cDNA shows base pair exchanges in four cases ($G^{9}\rightarrow C$; $T^{315}\rightarrow C$; $G^{408}\rightarrow C$; $C^{507}\rightarrow T$) which, however, do not lead to an amino acid exchange. Bundgaard et al. [21] described a cDNA of HNL from a chronic myelogenous leukemia cDNA library which differs at 10 highly degenerated codon positions (see Fig. 2) resulting also in the same derived amino acid sequence of rHNL protein. Further investigations are needed to show whether mutations of the cDNAs or/and the use of syn-

thesized primers for PCR [21] are responsible for the sequence differences found.

Computer HNL cDNA alignment of the three lipocalin motifs [25] with other lipocalin members (Fig. 3), like α_1 microglobulin [22], retinol binding protein [23] and α_1 acid glycoprotein [24] showed high homologies of 50–68%. In contrast to this there were only 30–40% homologies on the level of the amino acids. This underlined the conservation of these lipocalin motifs at the cDNA level and facilitates the screening for new members of the lipocalin family by PCR techniques using degenerated oligonucleotides designed according to the conserved sequence motifs.

In order to produce high amounts of the HNL protein for the purpose of functional and structural studies we expressed the protein in *E. coli*. For this purpose the pET 11 vector system [26] which allows the expression of the mature amino acid sequence starting with an initiating methionine was chosen. HNL cDNA containing a unique *Nde*I, a unique *Bam*HI site and a stop codon was selectively amplified with 'HNLR' and 'HNLF' oligonucleotides using pCR II-HNL plasmid as template. After restriction digestion the amplification product was ligated into the *Nde*I and *Bam*HI sites of the T7 expression vector 11a as previously described for TIMP-1 [13]. The plasmid pET 11a-HNL was transformed into *E. coli* strain HMS 174 [DE3]. In this host vector system the expression of rHNL was very efficient and rHNL was stored intracellularly in inclusion bodies in a denatured, insoluble form (not shown). Fifteen minutes after induction of the gene expression SDS-PAGE

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1. motif

HNL  61   aacttccaggacaaccaattccagggaagtggtatgtggtaggcctggca
          N  F  Q  D  N  Q  F  Q  G  K  W  Y  V  V  G  L  A
rat  61   ggcttctggaccgaacggttccagggcaggtggttcgttgtcggcctggca
          G  F  W  T  E  R  F  Q  G  R  W  F  V  V  G  L  A
almg 43   aacttcaatatctctcggatctatgggaagtggtacaacctggccatcggt
          N  F  N  I  S  R  I  Y  G  K  W  Y  N  L  A  I  G
RBP  40   aacttcgacaaggctcgcttctctgggacctggtacgccatggccaagaag
          N  F  D  K  A  R  F  S  G  T  W  Y  A  M  A  K  K
alac 43   aacgccaccctggaccagatcactggcaagtggttttatatcgcatcggcc
          N  A  T  L  D  Q  I  T  G  K  W  F  Y  I  A  S  A

2. motif

HNL  322  gtccgagtggtgagcaccaactacaaccagcatgctatggtgttc
          V  R  V  V  S  T  N  Y  N  Q  H  A  M  V  F
rat  322  gtgcaagtggccgacactgactacgaccagtttgccatggtatttt
          V  Q  V  A  D  T  D  Y  D  Q  F  A  M  V  F
almg 301  tcctatgtggtccacaccaactatgatgagtatgccatttttcctg
          S  Y  V  V  H  T  N  Y  D  E  Y  A  I  F  L
RBP  310  cactggatcgtcgacacagactacgacacgtatgccgtacagtac
          H  W  I  V  D  T  D  Y  D  T  Y  A  V  Q  Y
alac 292  ttcgctcacttgctgatcctcagggacaccaagacctacatgctt
          F  A  H  L  L  I  L  R  D  T  K  T  Y  M  L

3. motif

HNL  409  ctctacgggagaaccaaggagctgacttcggaactaaaggagaacttc
          L  Y  G  R  T  K  E  L  T  S  E  L  K  E  N  F
rat  409  ctgtacggaagaaccaagggctgtccgatgaactgaaggagcgattc
          L  Y  G  R  T  K  G  L  S  D  E  L  K  E  R  F
almg 391  ctctacgggcgggcgccgcagctgagggaaactctcctgcaggacttc
          L  Y  G  R  A  P  Q  L  R  E  T  L  L  Q  D  F
RBP  406  gtgttttcccgggaccccaacggcctgcccccagaagcgcagaagatt
          V  F  S  R  D  P  N  G  L  P  P  E  A  Q  K  I
alac 376  gtctatgctgacaagccagagacgaccaaggagcaactgggagagttc
          V  Y  A  D  K  P  E  T  T  K  E  Q  L  G  E  F
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Fig. 3. Comparison of three conserved sequence motifs of some lipocalin family members [25]. DNA sequence numbers are related to the amino acid sequence of the mature proteins. Sequences were compared by the wordsearch program (Genetics Computer Group). Boldface letters indicate the only invariant residue throughout the lipocalin superfamily tryptophan [7]. Footnotes: rat, rat α_2 microglobulin-related protein; almg, human α_1 microglobulin [22]; RBP, human retinol binding protein [23]; alac, human α_1 acid glycoprotein [24].

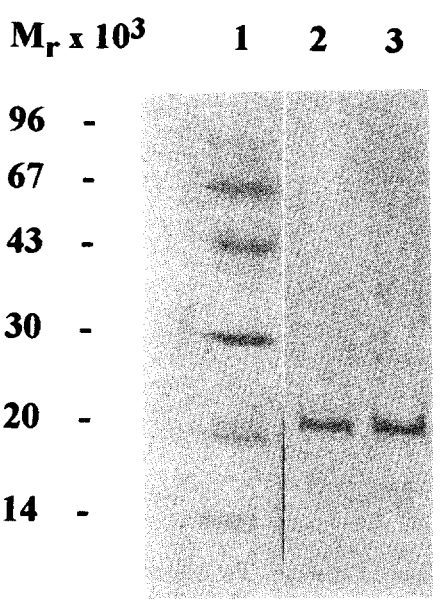

Fig. 4. Western blot analysis of *E. coli* cell pellet 4h after induction of rHNL gene expression with specific anti-HNL IgG [17] after SDS gelelectrophoresis: lane 1, molecular weight markers (Pharmacia, Freiburg, Germany); lane 2, rHNL (OC 6); lane 3, rHNL (bone marrow).

analysis indicated a production of an additional protein band of 21 kDa, the molecular weight of which was in accord with the calculated molecular mass of the HNL protein. Four hours after induction, the amount of the expression product was increased to about 15% of total bacterial protein. Western blot analysis of the *E. coli* cell pellet with rabbit polyclonal anti-HNL antibodies [17] showed cross reactivity with the recombinant 21 kDa protein indicating the production of rHNL (Fig. 4). The N-terminus was determined to be QDSTSDLIPAPP which was identical to the one of the mature HNL. The starting methionine was processed by *E. coli* [27].

In this paper we demonstrated for the first time that ovarian cancer cells produce HNL mRNA. As neutrophils during leukodiapedesis, cancer cells have to penetrate through the basement membrane when they become invasive. Further investigations have to show whether the lipocalin contributes to this process.

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