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Characterization of cDNA clones encoding guinea pig neutrophil cationic peptides

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cDNA clones encoding antimicrobial guinea pig neutrophil cationic peptides GNCP-1 and GNCP-2 were isolated from a bone marrow cell cDNA library. Analysis of these clones indicated that both GNCPs were produced as precursor proteins comprising 93 amino acid residues, which were composed of signal sequences (N-terminal 19 residues), pro-peptide sequences (43 residues) and mature GNCP sequences (31 residues). The deduced amino acid sequences showed that there were only two amino acid differences between GNCP-1 and GNCP-2, one in the pro-peptide region and one in the mature peptide region. Interestingly, Northern blot analysis and transcription run-off assay revealed that the expression of GNCP mRNA and the transcription of GNCP gene was observed in bone marrow cells but not in mature neutrophils. These observations suggest that mature neutrophils, despite their abundant content of GNCPs, lose the capacity to synthesize GNCPs.

Neutrophil cationic peptide; cDNA cloning; Amino acid sequence; Gene expression; Transcription run-off; Guinea pig

1. INTRODUCTION

Neutrophils play an important role in protecting hosts against microbial infections. Both oxidative and non-oxidative mechanisms are used by neutrophils to restrict infections [1–3]. Molecules such as H_2O_2, O_2^- and HOCl are examples of microbicidal agents that are derived from oxidative metabolism [1,3]. On the other hand, neutrophil granules contain antimicrobial proteins and peptides that contribute to the oxygen-independent host defense mechanism [2,3]. The most abundant of these antimicrobial components are low molecular-weight cationic peptides with potent microbicidal activity against bacteria, fungi and viruses [2–8]. In addition, the cationic peptides have been shown to display cytotoxic activity [9] and histamine-releasing activity [10].

Six rabbit, one guinea pig and four human cationic peptides have been purified and characterized [11–14]. Recently, we have purified two guinea pig neutrophil cationic peptides, GNCP-1 and GNCP-2, and found that GNCP-1 and GNCP-2 are single-chain peptides comprising 31 amino acid residues, which differ only by the substitution of an isoleucine (GNCP-1) for a leucine (GNCP-2) at position 21 [10], and that GNCP-1 is the same peptide as that previously purified [12]. However, the precursors of GNCPs are not clear at this stage. In this study, therefore, we have isolated cDNA clones for GNCPs, and analyzed the nucleotide sequences to deduce the amino acid sequences. Furthermore, the expression of the GNCP gene was studied in bone marrow cells and mature neutrophils using GNCP cDNA as a probe.

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Abbreviations: GNCP, guinea pig neutrophil cationic peptide; bp, base pairs

2. MATERIALS AND METHODS

2.1. *Preparation of cells*

Bone marrow cells were obtained from guinea pig femoral bones. Guinea pig peritoneal exudate neutrophils and peripheral blood neutrophils were prepared as previously described [15]; the purity of the cells was >95%.

2.2. *cDNA cloning and sequencing*

Total cellular RNA was isolated from bone marrow cells by the guanidinium thiocyanate extraction method [16], and poly(A)$^+$ RNA was selected by oligo-dT cellulose column chromatography. Double-stranded cDNAs were synthesized by a cDNA synthesis kit, and were ligated with *Eco*RI adaptors, according to the manufacturer's instructions (Pharmacia LKB Biotechnology). *Eco*RI-terminated cDNAs were inserted into phage vector λgt10 and packaged in vitro (Promega), and then the phages were grown on *Escherichia coli* C600Hfl. About 20000 recombinant phages were screened with a synthetic oligonucleotide probe (5′-GTTCTGGAAGATGCAGGTGCCGAGGCGGCGGTATGGGAAGCGGCAGGT-3′, 48-mer) corresponding to the amino acid sequences Thr-9 to Asn-24 of mature GNCP-1; this sequence was 90% identical to that of the GNCP cDNA clones isolated. The probe was labeled with [^{32}P]dCTP (3000 Ci/mmol, Amersham) using random hexanucleotide primers (Promega) [17]. The nucleotide sequences of the cDNA inserts were determined from both directions using a sequencing kit (United States Biochemical) by the dideoxy chain-termination method [18] after subcloning the cDNA inserts into a plasmid vector pBluescript SK(−) (Stratagene).

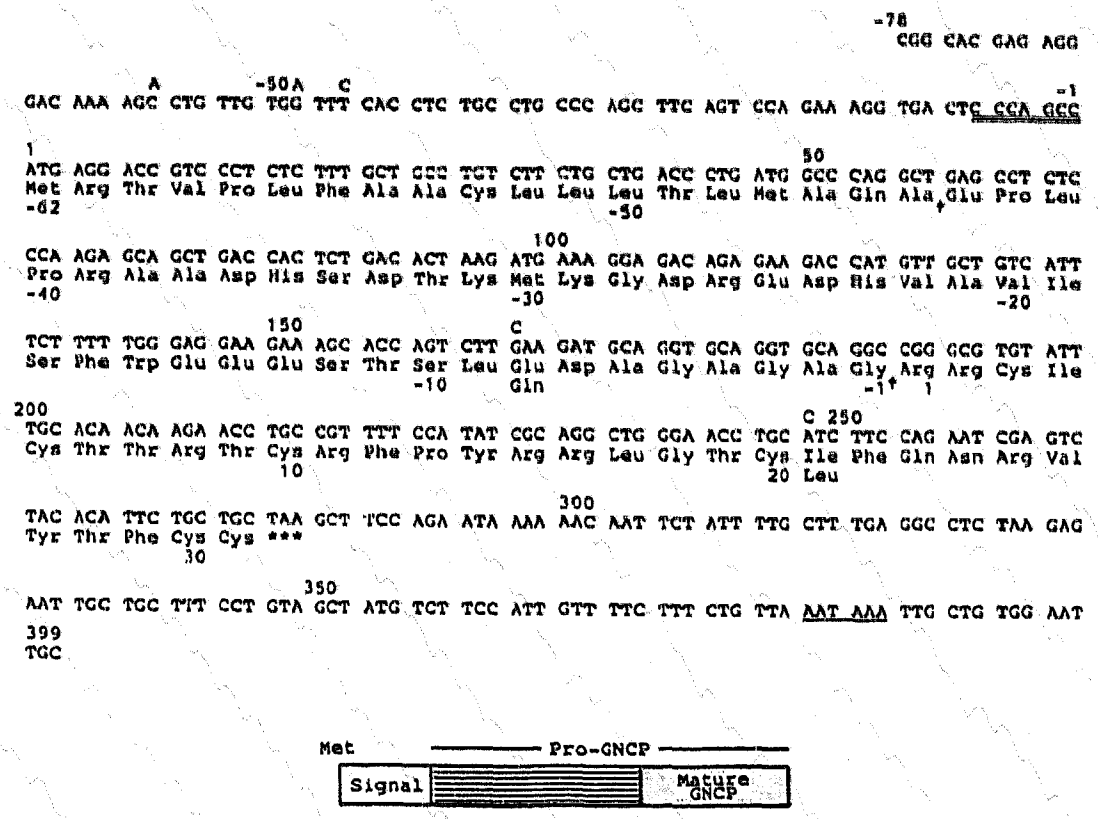

Fig. 1. Nucleotide sequences of GNCP cDNA clones and the deduced amino acid sequences of prepro-GNCPs. The nucleotide sequence shown is that of the four overlapping GNCP-1 clones, and is numbered starting at the initiation codon ATG. Negative numbers show the upstream region. The deduced amino acid sequence for the precursor of GNCP-1, shown under the nucleotide sequence, is numbered beginning at the N-terminal residue of mature GNCP-1. Negative numbers indicate pre- and pro-sequences. The GNCP-2 cDNA sequence is identical to that of GNCP-1 except where indicated over the given sequence. The amino acid differences between GNCP-1 and GNCP-2 are also indicated below the amino acid sequence. The consensus sequence for translation initiation [22] is double-underlined. The arrows indicate the most probable cleavage sites in post-translational processing [23–25]. The triple stars indicate the termination codon. The polyadenylation signal [36] is underlined. The structure of the predicted protein product is shown beneath the sequence data.

2.3. *Evaluation of GNCP mRNA transcript*

Expression of GNCP mRNA was evaluated by Northern blot analysis. Total cellular RNA was isolated from bone marrow cells and neutrophils as described above, and electrophoresed on a formaldehyde-containing agarose gel. Then, RNA was blotted onto a nylon membrane (Schleicher and Schuell), and hybridized with GNCP-1 cDNA probe or γ-actin cDNA probe (pHFγA-1 [19]; graciously provided by P. Gunning and L. Kedes, Stanford University) labeled with [^{32}P]dCTP by the random hexanucleotide primer method [17].

2.4. *Evaluation of GNCP gene transcription*

The transcription of the GNCP gene was evaluated by a transcription 'run-off' assay, as described previously [20,21]. Nuclei were isolated from bone marrow cells and neutrophils, and incubated with [^{32}P]UTP (800 Ci/mmol, Amersham) to label nuclear RNA. Transcription of the GNCP gene and, as a control, the γ-actin gene, was determined by hybridization (40 h, 42°C) of the ^{32}P-labeled nuclear RNA (10^7 cpm/ml) to the filter-bound (nylon membrane, Schleicher and Schuell; dot-blot apparatus, Bio-Rad laboratories), unlabeled cDNA targets (GNCP-1 cDNA and γ-actin cDNA) in the presence of 50% formamide and 10% dextran sulfate.

When the GNCP-2 cDNA was used for Northern blot analysis and transcription run-off assay as a labeled cDNA probe and an unlabeled cDNA target, respectively, the same results as those with the GNCP-1 cDNA were obtained (not shown).

3. RESULTS AND DISCUSSION

3.1. *Isolation of cDNA clones for GNCPs and its sequence analysis*

To isolate GNCP cDNAs, a cDNA library was prepared from bone marrow cells, and about 2×10^4 independent plaques were screened with a synthetic oligonucleotide probe based on the amino acid sequences of GNCP-1. Fifteen clones were selected randomly from 40 recombinant clones hybridized with the probe, and their cDNA inserts were analyzed by agarose gel electrophoresis. The sizes of the cDNA inserts were 450–500 bp. Among these inserts, 6 inserts were sequenced, and it was found that 4 inserts encoded GNCP-1 and 2 inserts encoded GNCP-2. An open reading frame of 282 nucleotides was in the various clones preceded by a 5′-untranslated region of 55–78 nucleotides and followed by a 3′-untranslated region of 104–117 nucleotides (Fig. 1). The amino acid sequences deduced from the cDNA sequences contained sequences identical with the previously published mature GNCP-1

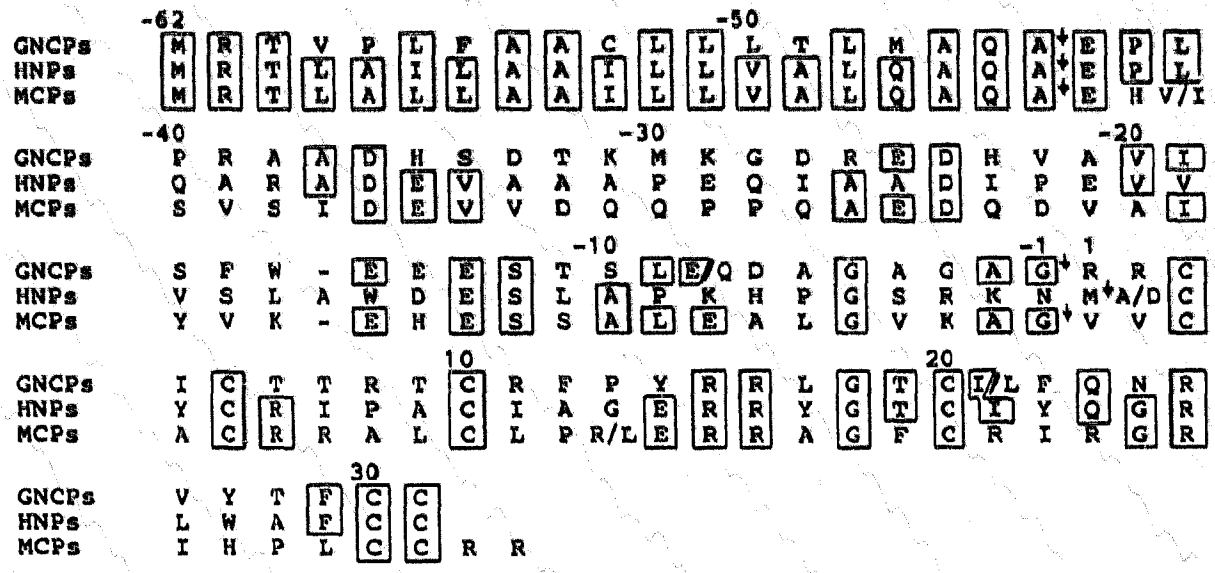

Fig. 2. Comparison of amino acid sequences of guinea pig neutrophil cationic peptides (GNCPs), human neutrophil cationic peptides (HNPs) and rabbit macrophage cationic peptides (MCPs). The deduced amino acid sequences begin at the initiation methionine, and are numbered beginning at the N-terminal residues of mature GNCPs and MCPs. Negative numbers indicate pre- and pro-sequences. Arrows indicate the most probable cleavage sites in post-translational processing; the N-terminal signal sequences (19 amino acid residues) are followed by the pro-peptide regions and the C-terminal mature peptide regions. Amino acid residues which differ among GNCP-1 and GNCP-2, HNP-1 and HNP-3 [27], or MCP-1 and MCP-2 [28], are indicated by slashes. Gaps, shown by dashes, are introduced for maximal alignment. Amino acid residues common to at least two sequences are boxed.

and GNCP-2 sequences [10]. Furthermore, the sequence analysis indicated the possibility that GNCPs are made as 93 amino acid precursor proteins with a molecular mass of 10.5 kDa. The initial methionine codon was preceded by a consensus sequence for translation initiation [22]. The initial methionine was followed by a hydrophobic sequence, which appeared to be a signal sequence. The most probable site for

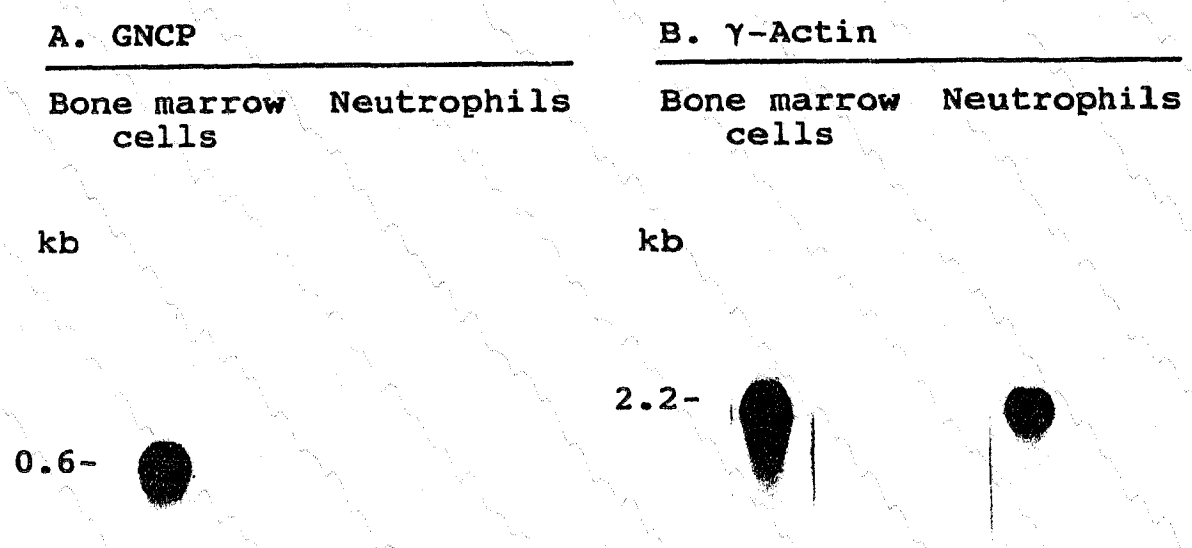

Fig. 3. Northern blot analysis of GNCP mRNA. Total cellular RNA from bone marrow cells (left lanes) and peritoneal exudate neutrophils (right lanes) was electrophoresed on an agarose gel, and blotted onto a nylon membrane. The blot was hybridized to ^{32}P-labeled GNCP or γ-actin cDNA probe. (A) GNCP mRNA; (B) γ-actin mRNA.

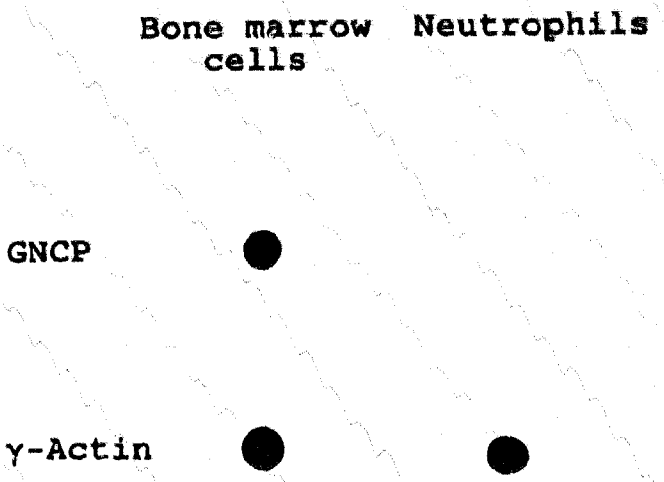

Fig. 4. Analysis of GNCP gene transcription. Nuclei were isolated from bone marrow cells (left lane) and peritoneal exudate neutrophils (right lane), and labeled with [^{32}P]UTP. The labeled nuclear RNA was hybridized to filter-bound GNCP (top) and γ-actin (bottom) cDNA targets.

signal peptide cleavage seemed to occur between alanine-44 and glutamic acid-43, although it was also possible that the cleavage occurs after alanine-46 or proline-42 [23–25]. Following signal peptide removal, the 74 amino acid pro-GNCPs must be further cleaved to generate 3.8-kDa mature GNCPs. It is interesting to note that the pro-peptide region contains many acidic amino acid residues, and may serve to mask the toxic effect of the mature peptides and protect the cells from damage during transport to the granules, as suggested for the eosinophil granule major basic protein [26].

The number and organization of GNCP genes have not been determined yet. However, the sequence data showed GNCP-1 and GNCP-2 cDNAs differed by only 5 nucleotide substitutions, out of 459 positions compared; 3 substitutions in the 5′ noncoding region, one substitution leading to a coding difference in the pro-peptide region (glutamic acid vs glutamine) and one substitution leading to a coding difference in the mature peptide region (isoleucine vs leucine). Because of this high degree of similarity, it is likely that the two sequences diverged recently or represent two alleles of the same gene.

cDNAs encoding human neutrophil cationic peptides (HNPs) and rabbit macrophage cationic peptides (MCPs) have been cloned [27,28]. Fig. 2 shows the deduced amino acid sequences of GNCPs, HNPs and MCPs. The overall homologies of amino acid sequences were 37–45% among GNCPs, HNPs and MCPs. Interestingly, the putative signal sequences were highly homologous (58–95%) among these peptides. In contrast, the homologies of pro-peptide and mature peptide sequences were 23–30% and 32–45%, respectively. It is interesting that the antimicrobial activity is conserved among these species, despite the low homology of the mature peptide sequences. The conserved amino acid residues such as cysteine and arginine may be important for the microbicidal activity.

3.2. *Expression of GNCP gene in bone marrow cells and neutrophils*

The expression of the GNCP gene was studied using bone marrow cells and mature neutrophils. Northern blot analysis revealed that bone marrow cells expressed a 0.6-kb GNCP mRNA (Fig. 3). Assuming a poly(A) tail of 100–200 nucleotides [29], the GNCP gene must encode an mRNA of 400–500 nucleotides. The lengths of GNCP cDNA clones isolated were 454–468 bp. Therefore, the GNCP cDNA clones obtained seem to represent complete or nearly complete transcripts of GNCP mRNA.

Interestingly, GNCP mRNA was not detected in mature neutrophils, although γ-actin mRNA, a control, was detected in the same cells. Transcription run-off assay indicated that the GNCP gene was transcribed in bone marrow cells but not in mature neutrophils, whereas the γ-actin gene was transcribed in both types of cells (Fig. 4). In peripheral blood neutrophils, neither expression of GNCP mRNA nor transcription of the GNCP gene were found, as described with peritoneal exudate neutrophils (not shown).

It is reported that neutrophils, terminally differentiated short-lived cells, have relatively low biosynthetic capacity, and the synthesis of their granule proteins such as lactoferrin and elastase is limited to a period of maturation that takes place in the bone marrow [30–35]. The observations in this study seem to suggest that GNCP, one of the major antimicrobial components of the neutrophil granules, is also synthesized only by maturing neutrophils in the bone marrow, as for lactoferrin and elastase. The mechanisms regulating GNCP gene expression during neutrophil maturation remain to be elucidated.

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