

# cDNA cloning and nucleotide sequence of lipocortin-like 33 kDa protein in guinea pig neutrophils

Eisuke F. Sato, Yoshikazu Tanaka\* and Kozo Utsumi

*Department of Medical Biology, Kochi Medical School, Nankoku-shi, Kochi 781-51 and \*Institute for Fundamental Research Center, Suntory, Ltd, Shimamoto-cho, Mishima-gun, Osaka 618, Japan*

Received 30 November 1988

cDNA clones of guinea pig neutrophil 33 kDa protein, a lipocortin like-protein, were isolated from two  $\lambda$ gt10 libraries and one primer-extended  $\lambda$ gt10 library of guinea pig neutrophils using synthetic oligonucleotide probes or cDNA fragment probe. The cDNA consists of 1389 nucleotides, and contains 1038 nucleotides encoding 346 amino acids of 33 kDa protein and a poly(A) tail. The deduced amino acid sequence shows high homology with those of lipocortin 1 from human U937 cells (89% homology) and rat lung (86%).

Protein, 33 kDa; Lipocortin; cDNA; DNA sequence

## 1. INTRODUCTION

The 33 kDa protein (p33) of guinea pig neutrophil is a calcium-dependent phospholipid-binding protein belonging to the family of lipocortin-like proteins [1,2]. This protein is neutrophil-specific and is translocated from the cytoplasm to the plasma membrane following treatment with various stimuli [3,4]. The protein inhibits phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity, binds muscle actin under certain conditions [2], and is phosphorylated by protein kinase C (PKC) in the presence of phosphatase inhibitors [5]. Moreover, it was found to have a high degree of homology with human lipocortin 1 from amino acid composition and partial sequence data [2]. We report here a complete amino acid sequence of P33 deduced from the nucleotide sequence of the cDNA.

*Correspondence address:* E.F. Sato, Department of Medical Biology, Kochi Medical School, Nankoku-shi, Kochi 781-51, Japan

*Abbreviations:* cDNA, complementary DNA; p33, 33 kDa protein from guinea pig neutrophils; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PKC, Ca<sup>2+</sup>- and phospholipid-dependent protein kinase; [ $\alpha$ -<sup>32</sup>P]dCTP, deoxycytidine 5'-[ $\alpha$ -<sup>32</sup>P]triphosphate; [ $\gamma$ -<sup>32</sup>P]-ATP, adenosine 5'-[ $\gamma$ -<sup>32</sup>P]triphosphate; SSC, sodium chloride and sodium citrate buffer

## 2. MATERIALS AND METHODS

### 2.1. Materials

Conventional enzymes were obtained from Toyobo (Osaka) and Takara Shuzo (Kyoto). The cDNA synthesis system, cDNA cloning system -  $\lambda$ gt10, [ $\alpha$ -<sup>32</sup>P]dCTP and [ $\gamma$ -<sup>32</sup>P]ATP were products of Amersham Japan (Tokyo). The DNA sequence kit was purchased from Toyobo. Nick-translation kit was from BRL (MD, USA).

### 2.2. Oligonucleotide probe

p33 was purified from guinea pig neutrophils and several peptide fragments were sequenced with an automatic amino acid sequencer as described [2]. Amino acid sequence information from one of the tryptic peptides (T20) and two of the cyanogen bromide peptides (CNBr2, CNBr15) corresponded to that of human lipocortin 1 [6]. Based on the determined nucleotide sequence of human lipocortin 1, three oligonucleotide probes corresponding to the sequence of amino acids of T20, CNBr2 and CNBr15 were chemically synthesized for use as screening probes (fig.1).

### 2.3. Construction of cDNA library from guinea pig neutrophil poly(A)-rich RNA

Total RNA from guinea pig neutrophils was extracted with guanidine thiocyanate [7]. Poly(A) RNA was fractionated by oligo(dT)-cellulose column chromatography [8]. Two cDNAs were prepared by the method of Gubler and Hoffman [9]. The essential difference between these two cDNAs is the primer used to direct the first strand synthesis: primers used were oligo(dT) (T) or random hexanucleotides (R). The cDNAs were cloned into the *Eco*RI site of  $\lambda$ gt10. The constructed cDNA libraries consisted of  $8 \times 10^5$  (R) and  $4 \times 10^5$  (T) clones, respectively.

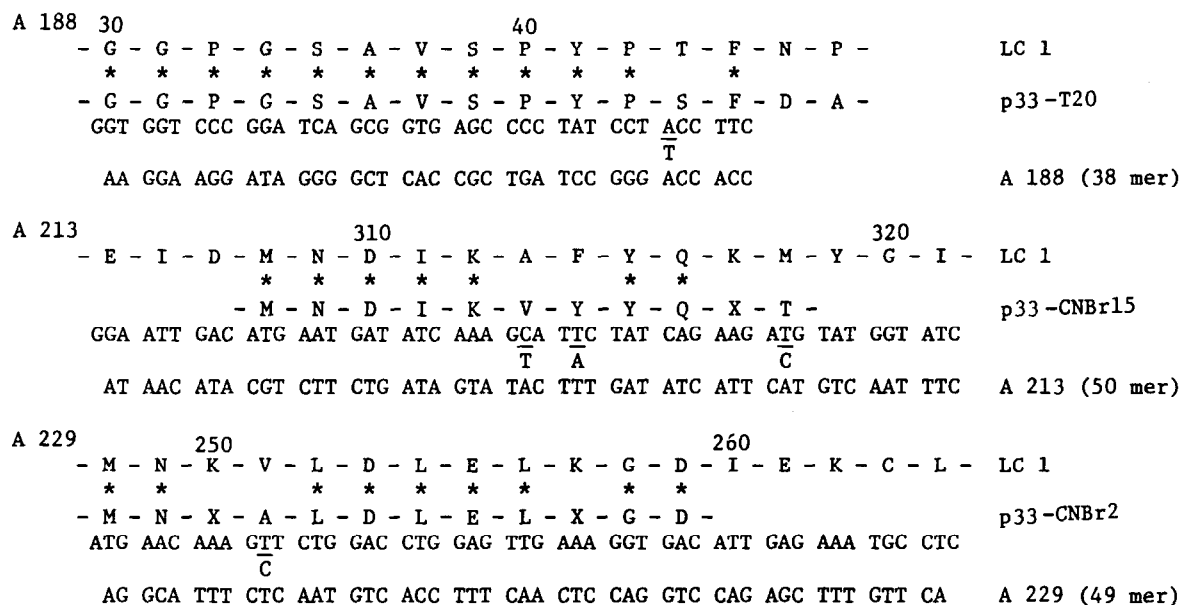


Fig.1. Synthetic oligonucleotides employed for screening cDNA clones for guinea pig p33. Amino acid sequence information from one of the tryptic peptides (T20) and two of the cyanogen bromide peptides (CNBr2, CNBr15) [2] corresponds to human lipocortin 1 [6]. The underlined base was exchanged with that of p33 residues. 38-mer (A188), 50-mer (A213) and 49-mer (A229) oligonucleotides are complementary to all possible coding sequences corresponding to the three hexapeptides T20, CNBr15 and CNBr2.

#### 2.4. Screening of guinea pig neutrophil p33 cDNA

The two cDNA libraries (T and R) were screened essentially as in [10] with  $^{32}\text{P}$ -labeled oligonucleotide probe A213. Isolated positive recombinant phages were characterized further by hybridization with all three oligonucleotide probes (A188, A213, A229). Hybridization was carried out at 55°C for 16 h in a solution containing 5 × SSC (0.15 M NaCl, 0.015 M sodium citrate buffer, pH 7.0), 5 × Denhardt's solution, 100 µg/ml heat-denatured salmon sperm DNA, 0.1% SDS and the  $^{32}\text{P}$ -labeled oligonucleotide probe. After hybridization, filters were washed by immersion (4 times) in a sufficient volume of

2 × SSC containing 0.1% SDS at 55°C for 20 min. The filters were then dried and autoradiographed with an intensify screen.

#### 2.5. DNA sequencing

DNA sequence analysis was carried out by subcloning a suitable restriction endonuclease fragment into M13mp18 RF DNA. The DNA sequence of the cDNA was determined by the method of Sanger et al. [11].

### 3. RESULTS AND DISCUSSION

#### 3.1. cDNA sequencing with oligonucleotide probes

Using a  $^{32}\text{P}$ -labeled oligonucleotide probe (A213),  $8 \times 10^5$  (R) and  $4 \times 10^5$  (T) independent recombinant phages from the two libraries were screened and many positive plaques were obtained. These phages were used as mini-libraries and screened by two probes (A213, A229). One positive clone from the R library was obtained which obviously hybridized to both probes. The  $\lambda\text{gt}10$  phage DNA from this clone, designated  $\lambda\text{gpL7}$ , was subjected to Southern blot analysis. The  $\lambda\text{gpL7}$  insert was hybridized to probes A213 and A229 but not to A188 (not shown). To confirm the presence of the p33 sequence in  $\lambda\text{gpL7}$ , the DNA sequence of the

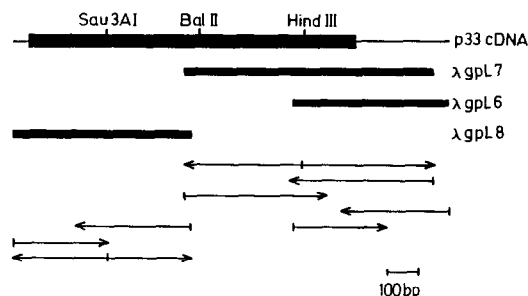


Fig.2. Guinea pig p33 cDNA sequencing strategy and major restriction enzyme sites used in preparing subclones. The thick line indicates the coding region, the thin line denoting the non-coding region. Horizontal arrows indicate regions and directions of sequencing.

$\lambda$ gpL7 insert was determined [11]. It was found that this clone contained a similar nucleotide sequence coding for the amino acid sequence at the carboxyl-terminal region of p33. To obtain the 3'-sequence of p33 cDNA, the T library was screened by the cDNA ( $\lambda$ gpL7) probe, which was  $^{32}$ P-labeled by nick-translation, and one positive clone was obtained. The cDNA of the  $\lambda$ gt10 DNA from this positive clone, designated  $\lambda$ gpL6, encoded the carboxyl-terminal region of p33 and the poly(A) tail. The oligonucleotide probes of A268 (5'AGAG-CCTTCTGGAAATC3': 17-mer) and A269 (5'AG-TCAGCCAAGTCATCA3': 17-mer), which was the (-) strand of the 5'-end of  $\lambda$ gpL7 (fig.3), were then synthesized to obtain the remaining 5'-sequence of the complete cDNA sequence by primer extension. Using this oligonucleotide as a primer, a primer-extended cDNA library was constructed from guinea pig neutrophil poly(A)-rich

RNA in  $\lambda$ gt10. This library was screened by three oligonucleotide probes (A188, A268, A269). One clone of the  $1 \times 10^5$  phages screened, designated  $\lambda$ gpL8, covered the 5'-sequence of p33. The total nucleotide sequence was determined [11]. The restriction endonuclease cleavage map and strategy of nucleotide sequence determination are summarized in fig.2.

### 3.2. Nucleotide sequence of the cDNA

The cDNA of p33 consists of 1389 nucleotides and a poly(A) tail (fig.3). The coding region for p33 includes 1038 nucleotides and corresponds to 346 amino acids. The 3'-noncoding region contains the polyadenylation signal, AATAAA, which is found 15 nucleotides upstream of the poly(A) homopolymer stretch. Comparing the amino acid sequences of T13 and CNBr15 with those obtained from the cDNA for p33, only two residues differ

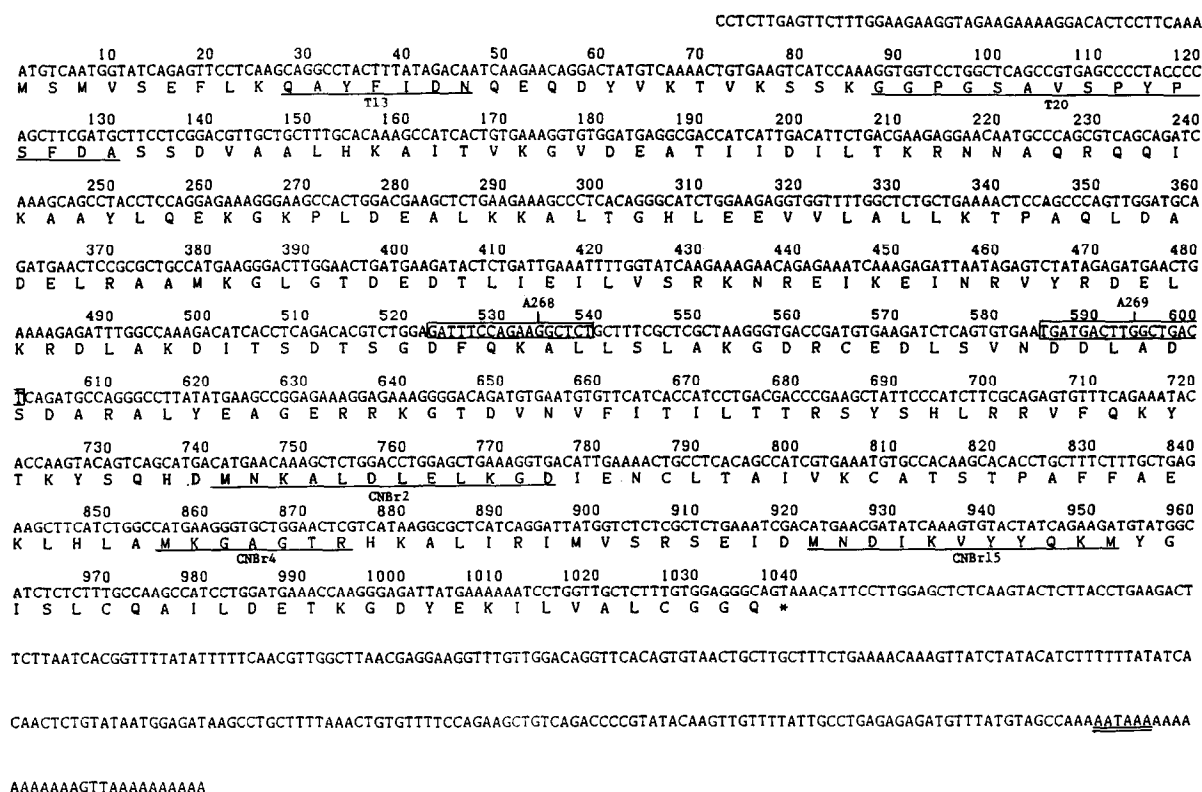


Fig.3. Nucleotide sequence of the p33 cDNA and the deduced amino acid sequence of guinea pig p33. The open reading frame is translocated to amino acids. Nucleotides are numbered in the 5'- to 3'-direction. The poly(A) additional signal is double-underlined. Amino acid sequences determined by direct peptide sequencing as described in [2] are underlined (T13, CNBr2, CNBr4, CNBr15). Two boxes surround the primer extension probe site (A268, A269).

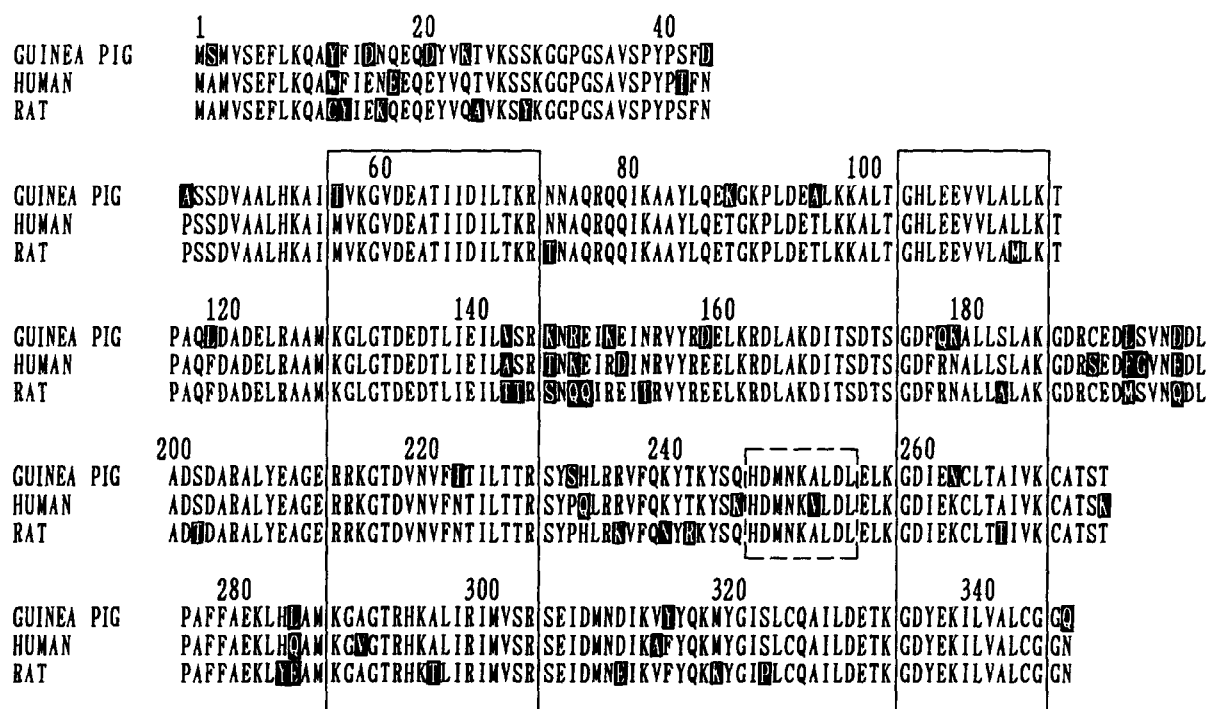


Fig.4. Sequence alignments for guinea pig p33, rat lipocortin 1, and human lipocortin 1. Amino acid residues of the proteins which are not identical among those of three proteins, are shaded. Boxed regions correspond to the two consensus sequences of guinea pig p33, human lipocortin 1 [6] and rat lipocortin 1 [12]. The (---) box indicates the PLA<sub>2</sub> inhibitory site. GUINEA PIG, guinea pig p33; HUMAN, human lipocortin 1; RAT, rat lipocortin 1.

(fig.3: T13, residues 7; CNBr15, residues 11), assuming that the partially purified p33 may be a mixture of a few closely related polypeptides.

### 3.3. Amino acid sequence of the protein and its characteristics

The complete primary structure of the protein is quite similar to those of lipocortin 1 from human U937 cell [6] and rat lung [12]: the percent homologies were approx. 89 and 86%, respectively. From the amino acid sequence, the molecular mass of p33 was calculated to be 38 541 Da, which is higher than the approximate value of 33 kDa estimated by SDS-polyacrylamide gel electrophoresis. Analysis of the p33 amino acid sequence reveals the 4-fold repetition of a p33-amino-acid-long unit with a singular N-terminal extension (fig.4). Geisow and Walker [13] have reported that lipocortin consists of two domains, each containing two of the homologous sequences. Fig.4 shows that the first box comprises 17-residue consensus sequences and the second, 12-residue consensus se-

quences. This structure is characteristic of members of the recently discovered family of Ca<sup>2+</sup>-dependent phospholipid-binding proteins termed annexins [13]. Mele et al. [14] have reported recently that synthetic oligopeptides corresponding to a region of high amino acid sequence similarity between uteroglobin and lipocortin 1 have potent PLA<sub>2</sub> inhibitory activity in vitro and striking anti-inflammatory effects in vivo. Like lipocortin 1, the third repetition of p33 (residues 246-254) shows the highest similarity with those of

246	H D M N K A L D L	p33
39	M Q M K K V L D S	Uteroglobin
246	H D M N K V L D L	LC 1
	M Q M N K V L D S	LC 1 like synthetic peptides

Fig.5. Amino acid sequence similarity to PLA<sub>2</sub> inhibitory region. p33, guinea pig p33; LC1, human lipocortin 1; LC1 like synthetic peptides, synthetic oligopeptides corresponding to human lipocortin 1.

synthetic oligopeptides (figs 4,5). Since the inhibitory activity of p33 is present in vitro [2], the region of p33 may be responsible for the inhibitory activity of PLA<sub>2</sub>.

As reported in [3], p33 occurred only in neutrophils and the amount of the protein is extremely high [1,3]. These facts suggest that p33 may have not only PLA<sub>2</sub> inhibitory activity but also some other biological functions. To clarify the biological roles of p33, further investigation is required. Therefore, we are now attempting the genomic DNA cloning of this protein.

*Acknowledgements:* The authors wish to express their gratitude to Dr Toshihiko Ashikari, Institute for Fundamental Research Center, Suntory, Ltd for helpful discussions on cDNA cloning. We thank Dr Toshinobu Suzuki and Mr Masahiko Takahashi for preparing the manuscript.

## REFERENCES

- [1] Utsumi, K., Sato, E., Okimasu, E., Miyahara, M. and Takahashi, R. (1986) FEBS Lett. 201, 277-281.
- [2] Sato, E.F., Miyahara, M. and Utsumi, K. (1988) FEBS Lett. 227, 131-135.
- [3] Sato, E.F., Morimoto, Y.M., Matsuno, T., Miyahara, M. and Utsumi, K. (1987) FEBS Lett. 214, 181-186.
- [4] Sato, E.F., Okimasu, E., Takahashi, R., Miyahara, M., Matsuno, T. and Utsumi, K. (1988) Cell Struct. Funct. 13, 89-96.
- [5] Nobori, K., Okimasu, E., Sato, E.F. and Utsumi, K. (1987) Cell Struct. Funct. 12, 375-385.
- [6] Wallner, B.P., Mattaliano, R.J., Hession, C., Chow, E.P., Browning, J.L., Ramachandran, K.L. and Pepinsky, R.B. (1986) Nature 320, 77-81.
- [7] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294-5299.
- [8] Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408-1412.
- [9] Gubler, U. and Hoffman, B.J. (1983) Gene 25, 263-269.
- [10] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- [11] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
- [12] Tamaki, M., Nakamura, E., Nishikubo, C., Sakata, T., Shin, M. and Teraoka, H. (1987) Nucleic Acids Res. 15, 7637.
- [13] Geisow, M.J. and Walker, J.H. (1986) Trends Biochem. Sci. 11, 420-423.
- [14] Miele, L., Cordella-Miele, E., Facchiano, A. and Mukherjee, A.B. (1988) Nature 335, 726-730.