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Comparison of the amino acid and nucleotide sequences between human and two guinea pig major basic proteins

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By means of reverse-phase HPLC, 2 different proteins were obtained from apparently purified pig eosinophil major basic protein (MBP) and these proteins were named GMPB1 and GMBP2. It was revealed that these 2 components of MBP have similar molecular weights and pI values, although the amino acid compositions were slightly different. In the previous study, we cloned and sequenced GMPB1 cDNA. Here we obtained another clone by plaque hybridization using a screening probe synthesized by means of polymerase chain reaction. After sequencing, it became apparent that this clone corresponded to GMBP2. As in the case of GMBP1, the cDNA of GMBP2 encoded pre-proGMBP2 with 3 domains; signal peptide, acidic pro-portion, and mature GMBP2. By comparing the sequences of GMBP1 and GMBP2, it was revealed that the proteins were quite similar to each other. In addition, their sequences also resembled those of human MBP, especially in the basic domain of mature protein; but no such similarity existed in the pro-portion. Although the molecular weights determined by SDS-PAGE of guinea pig and human MBPs were 11 000 and 9300, respectively, the calculated molecular weights of these 3 MBPs were all 13.8 kDa. The calculated pI values of GMBP1, GMBP2 and human MBP were 11.7, 11.3 and 11.6, respectively. By means of Harr plot analysis, it was revealed that the amino acid sequences, not only in signal peptides but also in the basic domains of mature proteins, were well conserved between guinea pig and human MBPs.

Guinea pig eosinophil; Major basic protein; cDNA cloning; Nucleotide sequence; Amino acid sequence

1. INTRODUCTION

It is generally believed that the allergic diseases associated with eosinophilia are those in which immediate-type hypersensitivity reaction is predominant and that in all species the eosinophil is characterized by the presence of large intracytoplasmic granules. Ultrastructural studies indicate that these granules are bounded by a double-layered membrane and contain a rectangular crystalline-like core surrounded by a less electron-dense matrix [1]. Although the granule contains a number of biologically active agents, approximately half the granule consists of the arginine-rich major basic protein (MBP) [2]. In asthma, MBP is deposited in and around the bronchi [2]. In relation to this, it has been shown that MBP is not only capable of desquamating the bronchial epithelium [3], but also releasing histamine from mast cells [4]. The amino acid and cDNA sequences of human MBP obtained from HL-60 cells were reported by Baker et al. [5,6]. In experimental animals, guinea pig MBP was purified by Gleich et al. [7]. We have reported that guinea pig MBP was composed of 2 different proteins, named GMBP1 and GMBP2, which are similar in molecular weights and pI values [8]. After cDNA cloning of GMBP1, it was found that cDNA of GMBP1 encoded pre-proGMBP1 with 3 domains: signal peptide, acidic domain, and mature GMBP1 [8]. In the present study, cDNA cloning of GMBP2 was carried out. Comparing human MBP with 2 guinea pig MBPs, it was indicated that these 3 MBPs are similar not only in physical property but also in chemical structure.

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Abbreviations: MBP, major basic protein; SDS-PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis; IEF, isoelectric focusing; HPLC, high performance chromatography; TFA, trifluoroacetic acid; PCR, polymerase chain reaction

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number D00817

2. MATERIALS AND METHODS

2.1. *Purification of guinea pig eosinophil MBP*

The procedures employed to collect the purified guinea pig MBP are the same as those reported previously [8]. Briefly, male Hartley guinea pigs (300–350 g) were intraperitoneally injected once a week with 1 mg/animal of polymixin B (Sigma) and 1 mg/animal of ascaris suum extract. Six weeks later, the peritoneal exudated cells were collected by perfusing the abdominal cavity of anesthetized animals with 50 ml of physiological buffered solution (PBS, in mM: NaCl, 154; KCl, 2.7; $CaCl_2$, 0.9; HEPES, 5 (pH 7.4)). By means of Percoll densi-

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ty gradient centrifugation, eosinophils were purified to more than 95% purity. The eosinophils were disrupted in a solution consisting of 0.25 M sucrose, 5 mM HEPES (pH 7.4) and 1 mg/ml heparin and the granules were collected by centrifugation at 4°C. The granules were dissolved with 0.1 N HCl and the solution was applied to a Sephadex G-50 column and eluted with acetate buffer (pH 4.3) [7]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) were carried out by means of the Phast system (Pharmacia). MBP fractions were further purified by means of reverse phase high performance liquid chromatography (HPLC; Waters). An MBP fraction dissolved in 0.1% trifluoroacetic acid (TFA) was injected onto a Vydac C-4 column and eluted with a solution consisting of solvent A (0.1% TFA) and solvent B (CH_3CN/0.1% TFA, 9:1) [8].

2.2. *Determination of amino acid composition and partial sequence*

Edman degradations of MBP and its fragments obtained from digestion with lysyl-endopeptidase were carried out using a gas phase sequencer (Applied Biosystems Inc., Forster City). Phenylthiohydantoin derivatives of amino acids from each cycle were analyzed by reverse phase HPLC.

2.3. *Construction of cDNA library of guinea pig eosinophils*

As reported previously, purified guinea pig eosinophils were disrupted and the lysate layered onto a cesium trifluoroacetic acid solution. This was centrifuged at 85 000 × *g* for 25 h [8]. The total RNA was collected and poly(A)$^+$ RNA was selected using Oligotex-d(30 (Takara). The λgt10 cDNA library was constructed using the cDNA synthesis system (Amersham) and the cDNA cloning system-λgt10 (Amersham) from 1 μg poly(A)$^+$ RNA, and more than 3×10^6 independent clones were obtained.

2.4. *Polymerase chain reaction (PCR) and synthesis of screening probes*

After the method previously described [8], PCR primers were synthesized, taking the partial amino acid sequence of GMBP2 as a model. PCR was performed using the primers and cDNA of guinea pig eosinophils to obtain a screening probe for GMBP2 using a Gene Amp DNA Reagent Kit (Perkin Elmer Cetus) and a DNA Thermal Cycler (Perkin Elmer Cetus). The PCR was run for 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 30 s) and extension (72°C, 30 s). The amplified 185 bp fragment was ligated into plasmid pUC118. The 185 bp fragment was labeled by PCR using 10 mM [α-^{32}P]dCTP in the reaction mixture. In this process, the 185 bp fragment-inserted pUC118 was used as a template and Takara M13 primers were employed (M13 primer M4: d(GTTTTCCCAGTCACGAC), and M13 primer RV: d(CAGGAAACAGCTATGAC)). After PCR, the reaction mixture was passed through a NICK column (Pharmacia) to separate the amplified DNA from unincorporated [α-^{32}P]dCTP.

2.5. *Screening of the cDNA library and determination of the cDNA sequence*

The cDNA library was screened with the ^{32}P-labeled 185 bp fragment, following the method of Benton and Davis [9]. The hybridization solution was 6 × SSC (1 × SSC = 0.15 M sodium chloride, 0.015 M citrate), 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin, 0.1% SDS and 100 μg/ml denaturated salmon sperm DNA. The filters were hybridized with a PCR-amplified labeled probe overnight at 65°C, and were washed in 2 × SSC and 0.1% SDS at 60°C. Subsequently, the filters were exposed to Kodak XAR5 film. A positive clone was selected from the library and, following the method of Sanger et al. [10], a cDNA insert of about 1 kbp was subcloned into pUC118 in order to determine the sequence.

2.6. *Data analysis for the amino acid and nucleotide sequences*

Based on the obtained cDNA sequence and corresponding amino acid sequence, calculation of homologies between the MBPs and Harr plot analyses were carried out by means of a personal computer (NEC, PC-9801RA5) with GENETYX software (Software development Co. Ltd.) at Okayama University Gene Research Center.

3. RESULTS

The partial amino acid sequence of mature GMBP2 compared with that of human mature BMP is indicated in Fig. 1a. According to this partial sequence, PCR primers, which correspond to the underlined portion of GMBP2 in Fig. 1a, were synthesized as indicated in Fig. 1b.

Using the oligonucleotide primers and cDNA obtained from guinea pig eosinophils, PCR was carried out to obtain the hybridization probe for the screening of the λgt10 cDNA library. The PCR-amplified 185 bp DNA sequence is indicated in Fig. 2. The nucleotide sequence coincided well with that determined from the amino acid sequence, and this oligonucleotide was used for the screening of the cDNA library of eosinophils. After plaque hybridization using a ^{32}P-labeled probe, a positive clone was obtained from 2.8×10^6 clones.

Fig. 3 indicates the determined sequence of the cDNA of GMBP2. As shown in this figure, GMBP2 cDNA was 899 bp in length excluding the poly(A)$^+$ tail flanking 17 bp 3′ to a typical poly(A)$^+$ addition sequence (AATAA). On the basis of the cDNA sequences seen in those of human MBP and GMBP1, it was revealed that GMBP2 cDNA encoded 3 domains: 15 amino acids of

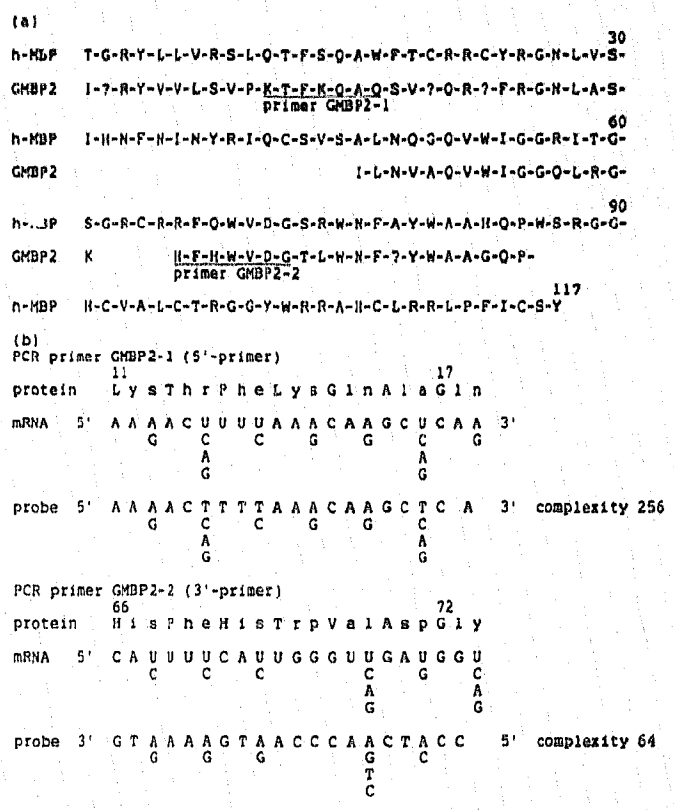

Fig. 1. Partial amino acid sequence of GMBP2 and nucleotide sequence of the synthesized PCR primers. (a) Partial amino acid sequence of GMBP2 in comparison with human MBP [4]. Nucleotide sequences of the synthesized PCR primers in the underlined positions in Fig. 1a.

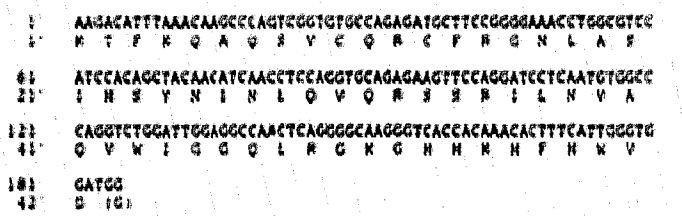

Fig. 2. Nucleotide sequence of PCR-amplified oligonucleotide corresponding to the partial sequence of GMBP2.

the signal peptide, 100 amino acids of the pro-portion, and 119 amino acids of the mature GMBP2. The calculated molecular weight of mature GMBP2 was 13.8 kDa. Mature GMBP2 contains 17 basic amino acids and one acidic amino acid, and the calculated pI value was 11.3. The limited pro-portion of GMBP2 contains 31 acidic amino acids and 8 basic amino acids, and the calculated pI value was 3.8. However, the total pI value of proGMBP2 was 5.6.

The amino acid sequence of pre-proGMBP2 is shown in Fig. 4 in comparison with those of human pre-proMBP and pre-pro-GMBP1. The homology in pre-pro-protein sequences between human MBP and GMBP2 was 48.7%, although in mature MBPs the

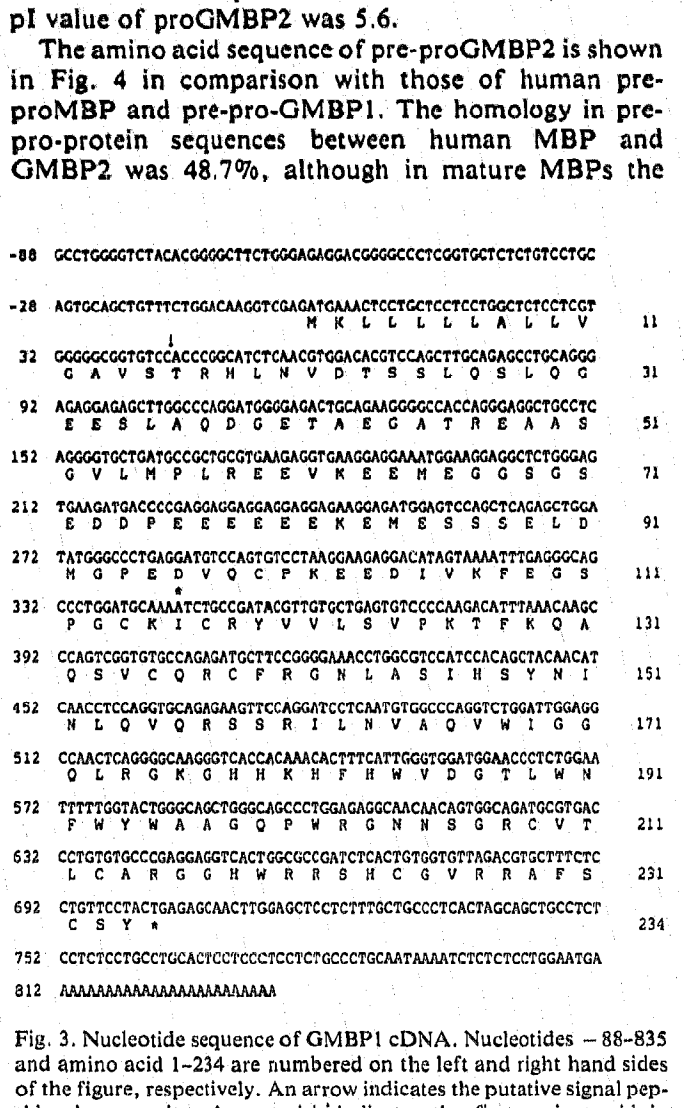

Fig. 3. Nucleotide sequence of GMBP1 cDNA. Nucleotides −88–835 and amino acid 1–234 are numbered on the left and right hand sides of the figure, respectively. An arrow indicates the putative signal peptide cleavage site. An asterisk indicates the first amino acid in eosinophil granule MBP.

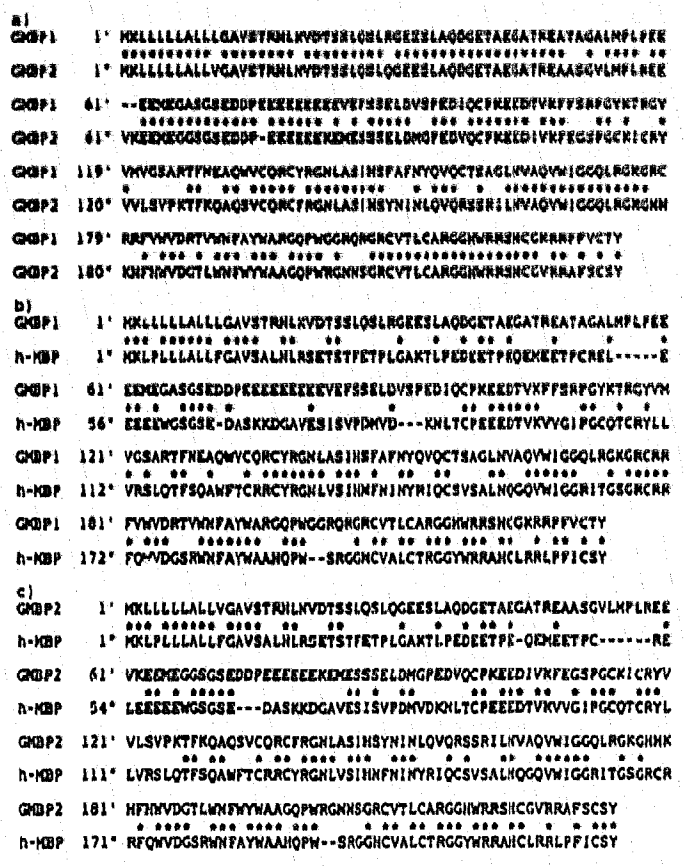

Fig. 4. Comparison of the amino acid sequences of pre-proMBP in guinea pig (GMBP1 and GMBP2) and human (h-MBP) [4]. (a) GMBP1 and GMBP2, (b) GMBP1 and h-MBP, and (c) GMBP2 and h-MBP. Identical residues are indicated by an asterisk. Gaps have been introduced to achieve maximum sequence homology.

homology was 55.4%. The homologies in pre-pro-proteins and mature proteins between GMBP1 and GMBP2 were 74.2% and 67.5%, respectively. In contrast, the nucleotide sequences of the coding regions of GMBP1 and GMBP2 were homologous by 81.1%.

Fig. 5 represents Harr plot analyses on the primary structures between the pre-pro-proteins of GMBP1, GMBP2 and human MBP. As indicated in Fig. 5a, GMBP1 and GMBP2 were homologous in both pro-portion and mature regions. On the other hand, when human MBP was compared with GMBP1 and GMBP2, the basic regions in the mature proteins were similar and it was considered that the basic region is well conserved, although the primary sequences in the acidic regions of proportions were not very homologous despite their similar pI values (Fig. 5b,c). In each case, the signal peptides closely resembled each other.

4. DISCUSSION

It is well known that MBP is a major constituent of eosinophil granules. Since isolated guinea pig MBP ex-

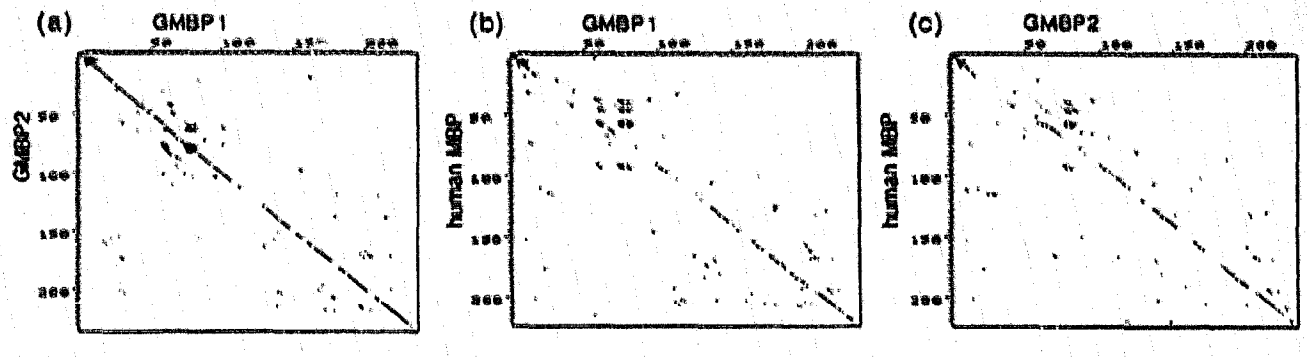

Fig. 5. Harr plot analyses on the primary structures between GMBP1, GMBP2 and human MBP. (a) GMBP1 and GMBP2, (b) GMBP1 and human MBP, and (c) GMBP2 and human MBP. Harr plot was carried out by GENETYX software with a personal computer. The program was conditioned as follows: minimum length = 5 and matching = 3/5 or more. Each dot represents a common amino acid residue between indicated proteins.

hibits a single band on SDS-PAGE and IEF (migrated to the cathode position) [7], it had been thought that guinea pig MBP was a single protein. However, when guinea pig MBP was applied to reverse phase HPLC, 2 peaks were recognized [8]. These two proteins exhibited almost identical molecular weights and pI values on SDS-PAGE and IEF (migrated to cathode position), respectively. Therefore, it is indicated that guinea pig MBP consists of 2 components, namely GMBP1 and GMBP2 [8]. Even in cDNA sequences determined in previous [8] and present studies, the calculated molecular weights of GMBP1 and GMPB2 were almost identical (13.8 kDa). This value is exactly the same as that of human MBP [5]. The calculated pI values of GMBP1 and GMBP2 were also quite similar (11.6 and 11.3, respectively). Since the pH range of the IEF is usually 10 at the highest, it was supposed that both proteins migrated to the cathode position. The molecular weights of guinea pig MBP and human MBP determined on SDS-PAGE were 11 kDa and 9.3 kDa [11]. The difference in the molecular weights between the calculated values and those determined by SDS-PAGE may be ascribed to the strong basicity of MBPs, which may hinder the migration of proteins to the position corresponding to the molecular weights. The similarity in pI values and molecular weights may well be the reason for the difficulty in separating these 2 proteins by SDS-PAGE or IEF.

It has been supposed that the cytotoxic activity of MBP may be due to its highly basic charge [3,7]. Therefore, the acidic domain of pro-protein may be of use to neutralize the basicity of mature GMBP2, as in the cases of human MBP and GMBP1 [5,8]. It was assumed that GMBP2 would be firstly translated as a pre-pro-protein, which would be converted to proGMBP2 and mature GMBP2 in order as in the case of GMBP1 [8].

The amino acid sequence near the cleavage site between pro-portions and mature GMBP2 was slightly different from that seen in GMBP1 (G-S-P-G-C-K*I-C-R-Y-V-V and S-R-P-G-Y-K*T-R-G-Y-V-M, respectively). The common amino acid sequence between these sites is x-x-P-G-x-K*-x-x-x-Y-V-x. In the case of human MBP, the cleavage site processing from proMBP to mature MBP is G-I-P-G-C-Q*T-C-R-Y-L-L [5]. This sequence more closely resembles the sequence in GMBP2 than that seen in GMBP1. The common amino acid sequence at this cleavage site between human MBP and GMBP2 is G-x-P-G-C-x*x-C-R-Y-x-x. Although it is not known what kind of enzyme(s) participates in the processing of proMBP to mature MBP, the above sequence may be a plausible candidate for the recognition site of the endoprotease. In contrast, the amino acid sequences of putative signal peptides and the cleavage sites of these 3 proteins are quite similar to each other (GMBP1: M-K-L-L-L-L-L-A-L-L-L-G-A-V-S*T-R-H-L, GMBP2: M-K-L-L-L-L-L-A-L-L-V-G-A-V-S*T-R-H-L, and human MBP: M-K-L-P-L-L-L-A-L-L-F-G-A-V-S*A-L-H-L [5,12]). The lengths of signal peptides of these 3 proteins are all 15 amino acids. The common sequence in these 3 proteins is M-K-L-x-L-L-L-A-L-L-x-G-A-V-S*x-x-H-L. It was assumed that a quite similar mechanism would participate in the cleavage process in these proteins. As indicated in the Harr plot analysis, the signal peptides of these 3 peptides closely resembled each other. Homologous sequences were observed in the basic region of mature MBPs. Although the pI values of the pro-portions of 3 MBPs are all 3.8, the homology was rather low between human MBP and guinea pig MBPs.

From the present study, it was indicated that GMBP1 and GMBP2 may be 2 subclasses of guinea pig MBP. Although only one MBP is reported in human eosinophils, it is possible to assume that there exists a subclass of MBP in other species, including humans.

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