Cloning of the cDNAs for mast-cell chymases from the jejunum of Mongolian gerbils, *Meriones unguiculatus*, and their sequence similarities with chymases expressed in the connective-tissue mast cells of mice and rats

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By using the combination of reverse-transcription PCR and rapid amplification of cDNA ends methods, two distinct cDNAs encoding mast-cell proteases (chymases; MCPs), designated as gMCP-1 and -2, were successfully cloned and sequenced from the jejunum of Mongolian gerbil, *Meriones unguiculatus*, infected with *Nippostrongylus brasiliensis*. On the basis of a comparison of the deduced amino acid sequences with those of known rodent mast-cell chymases, gMCP-1 was found to be highly similar to mouse mast-cell protease (mMCP)-4 and rat mast-cell protease (rMCP)-1, while gMCP-2 was similar to mMCP-5 and rMCP-3. Although mMCP-4 and -5 and rMCP-1 and -3 were restrictedly or mainly expressed in connective-tissue mast cells and serosal

mast cells, the gMCP-1 and -2 genes were mainly transcribed in the jejunal mucosa and to a lesser extent in the skin and tongue. Moreover, kinetic study after infection revealed that the amounts of the gMCP-1 and -2 mRNAs in jejunum paralleled well the degree of intestinal mastocytosis. The expression of gMCP-1 and -2 in mucosal mast cells of gerbil jejunum was also confirmed by *in situ* hybridization. Since a tryptase, another type of MCP, was also expressed in mucosal mast cells of gerbils but not in those of mice and rats, the expression of MCPs in mucosal mast cells of gerbils is different from those of mice and rats. The Mongolian gerbil would be a useful model with which to investigate the physiopathological role of MCPs.

INTRODUCTION

Mucosal mast cells (MMCs) and connective-tissue mast cells (CTMCs) in mice and rats constitute distinct subpopulations. In these animals, MMCs contain chondroitin sulphate proteoglycan and a certain type of chymotrypsin-like protease (chymase) in their granules, while CTMCs have heparin and different kinds of chymases together with a trypsin-like protease (tryptase) [1–4]. However, we recently found that mast cells in the jejunum of the Mongolian gerbil, *Meriones unguiculatus*, were phenotypically, as well as biologically, different from those of mice and rats. Gerbils are unable to expel strongyloid parasites [5–7] in spite of prominent intestinal mastocytosis, though expulsion of these parasites was entirely dependent on MMCs in mice and rats [8–12]. Histochemical and cytochemical characteristics of gerbil MMCs were different from those of mice and rats. They contain heparin [13] and both chymase and tryptase activities [14] in their granules. In fact, we cloned the cDNA encoding the gerbil mast-cell tryptase (gMCT) from the jejunum and confirmed the expression in the jejunal mucosa by RNA-blot analysis and enzyme-histochemistry [15]. Typical MMCs in mice and rats lack heparin and tryptase, which are the markers for CTMCs in these animals [1–4]. Since MMCs and CTMCs of mice and rats express different kinds of chymases, characterization of cDNAs for chymases expressed in gerbil jejunum would give us much information on the nature of the MMCs of this animal. To this

end, we sequenced two distinct cDNAs for mast-cell chymases, designated gerbil mast-cell proteases (gMCP)-1 and -2, from the jejunum of gerbils by using a combination of reverse-transcription PCR (RT-PCR) and rapid amplification of cDNA ends (RACE) methods.

EXPERIMENTAL

DNA and RNA extraction

Genomic DNA was purified from the liver of an 8-week-old gerbil (an inbred strain: MGS/Sea, purchased from Seiwa Experimental Animals, Fukuoka, Japan) by SepaGene (Sanko Junyaku Co., Ltd., Tokyo, Japan) according to the manufacturer's protocol. Total RNAs were extracted using TRIZOL® (Life Technologies, Inc., Gaithersburg, MD, U.S.A.) from jejunum, skin and tongue of 8- to 10-week-old gerbils before and 7-, 10-, 14- and 21-days after infection with *Nippostrongylus brasiliensis*. Three gerbils were used at each designated time point, and the histological and RNA samples were prepared separately from each gerbil. For counting the number of mast cells, parts of samples were stained with Alcian Blue (pH 0.3) and Safranin O (pH 0.1) as described previously [13–15]. Poly(A)⁺ RNA selection was performed using oligo(dT) latex (Oligotex®-dT30; Nippon Roche, Tokyo, Japan).

Abbreviations used: MMC, mucosal mast cell; CTMC, connective-tissue mast cell; MCP, mast-cell protease; gMCP, gerbil MCP; mMCP, mouse MCP; rMCP, rat MCP; gMCT, gerbil mast-cell tryptase; rMCT, rat MCT; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; DIG, digoxigenin; RT-PCR, reverse-transcription PCR; RACE, rapid amplification of cDNA ends.

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The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession numbers, D45173 (gMCP-1) and D45174 (gMCP-2).

Primers and anchor sequences used were as follows: primer P1 (sense), 5'-CCA(T)CAC(T)TCC(T)CGC(T)CCC(T)TAC-ATGGC-3'; primer P2 (antisense), 5'-GAGAC(T)TCG(T)G-GTA(G)AAGAC(T)A(T)GCAGGGG-3'; primer P3 (sense), 5'-CATGACATCATGTTACTGAA-3'; primer P4 (sense), 5'-TCAGCCTACAAGGGAGACTC-3'; primer P5 (sense), 5'-ATCGCCTCCTACGTCCGTCGGAA-3'; primer P6 (antisense), 5'-CTTCAGTAACATGATGTCAT-3'; primer P7 (antisense), 5'-TCTTTCTTGCTCACATCATG-3'; primer P8 (antisense), 5'-CTAGGAGCACGGTTATAGAC-3'; primer P9, 5'-CTGGTTCGGCCACCTCTGAAGGTTCCAGAAT-CGATAG-3'; primer PT7, 5'-AATACGACTCACTATAG-3'; primer DT7, 5'-TAATACGACTCACTATAGGGATTTTTTTT-TTTTTTTTTT-3'; 5' Anchor, 3'-NH₃-GGAGACTCCAAG-GTCTTAGCTATCACTTAAGCAC-p5'. The directions and locations of the primers P1–P8 are shown in Figure 1. After

sequencing of the RT-PCR products using the degenerated primers P1 and P2, which were designed in the well-conserved regions of known MCP (chymase) cDNA sequences [16–26], the inner primers P3–P8 were designed in the regions specific for gMCP-1 and/or -2.

Southern- and RNA-blot analyses

Four restriction enzymes, *Bam*HI, *Eco*RI, *Hind*III and *Xho*I, were purchased from TOYOBO (Osaka, Japan). Samples (10 µg) of each gerbil genomic DNA digested by one of these endonucleases was electrophoresed on a 1% agarose gel and transblotted on to Hybond-N nylon membrane (Amersham International, Amersham, Bucks., U.K.). Total RNAs from the jejunum (20 µg), skin (100 µg) and tongue (100 µg) of uninfected and infected gerbils were also electrophoresed on a 1% formaldehyde/agarose gel and transblotted on to nylon membrane. Hybridization was performed under high-stringency conditions using ³²P-labelled probes as described elsewhere [27]. The probes used were 3'-RACE products (corresponding to the nucleotide positions 618–945 for gMCP-1 and 674–953 for gMCP-2 and having no internal restriction sites by the endonucleases used in this study) for both Southern- and RNA-blot analyses. The control human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe, which strongly cross-hybridizes to rodent G3PDH mRNA, was purchased from Clontech.

In situ hybridization

Small pieces of gerbil jejunum, resected at about 10 cm distal to the pylorus, were obtained from 14 days after *N. brasiliensis* infection as described previously [13–15]. The tissues were fixed with freshly prepared 4% (v/v) paraformaldehyde in 0.1 M sodium phosphate buffer overnight. Sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min, rinsed twice with 0.1 M phosphate buffer for 5 min, dehydrated with 70, 80, 90, and then 99.5% ethanol, and air-dried [33]. Digoxigenin (DIG)-labelled cRNA probes were prepared using the DIG RNA Labeling Kit (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacturer's instructions. Each clone of the 3'-RACE products subcloned into pCR II vector was linearized with *Bam*HI and transcribed with T7 RNA polymerase to generate 330- and 320-base antisense cRNA probes, specific for gMCP-1 and gMCP-2 respectively. Control sense cRNA probes were made by being linearized with *Xho*I and transcribed with SP6 RNA polymerase in the same manner. The hybridization solution was composed of 50% deionized formamide, 10% dextran sulphate, 1 × Denhardt's solution, 10 mM Tris/HCl (pH 7.6), 1 mM EDTA, 0.25% SDS, 0.6 M NaCl, 200 µg/ml of tRNA, and approx. 0.5 µg/ml of cRNA probe. Sections were hybridized with the solution at 50 °C for 16 h in a moisture chamber and then washed in 5 × standard saline citrate (SSC; SSC = 0.15 M NaCl/0.015 M sodium citrate) at 50 °C for 10 min and 2 × SSC containing 50% formamide at 50 °C for 30 min. After treatment with RNase A [30 µg/ml in 10 mM Tris/HCl (pH 7.6), 0.5 M NaCl and 1 mM EDTA] at 37 °C for 30 min, the sections were washed again in 2 × SSC at 50 °C for 20 min and twice with 0.2 × SSC at 50 °C for 20 min. RNA signals were visualized using the DIG Nucleic Acid Detection Kit (Boehringer) according to the manufacturer's instructions and the sections were counterstained with Safranin O (pH 0.1). To show the distribution of mast cells, an adjacent section was stained with Alcian Blue (pH 0.3) and Safranin O (pH 0.1), and compared with the section hybridized *in situ*.

RESULTS

Sequencing of gMCP-1 and -2 cDNAs

To search the MCP genes expressed in the jejunum of gerbils, we first performed RT-PCR with the degenerated primers P1 and P2, which were designed in the well-conserved region of the known MCP (chymase) cDNAs of other animals. The expected size (approx. 600 bp) of the RT-PCR product was obtained and subcloned into a pCR II vector. By screening and sequencing a total of 18 clones, two distinct types of gMCP cDNAs (12 of gMCP-1 and six of gMCP-2) were identified. Subsequently, to obtain the full-length gMCP cDNAs, both 5'- and 3'-RACE assays were performed using the inner primers P3–P8, which were designed for gMCP-1 and/or -2, based on each partial cDNA sequence. To obtain maximal accuracy, after RT-PCR and RACE treatment at least six individual clones obtained at each step were separately sequenced and their complete identities were confirmed. Total 945 and 953 bp of gMCP-1 and -2 nucleotide sequences and their deduced amino acid sequences are shown in Figures 1(A) and 1(B) respectively. ATG codons and stop codons were seen in the nucleotide positions 33 and 771 for gMCP-1 and 29 and 770 for gMCP-2, respectively. One and two polyadenylation signals were also found in gMCP-1 (numbered 925–930) and gMCP-2 (numbered 919–924 and 929–934) at the 3' ends. Thus, peptides with 246 (gMCP-1) and 247 (gMCP-2) amino acids were encoded by the single open reading frames. The first 19 (gMCP-1) and 20 (gMCP-2) amino acids and the following two amino acids probably represent the signal peptide and the activation peptide respectively, on the basis of a comparison with known MCPs (chymases) of other animals. Consequently the initial Met was numbered –20 (gMCP-1) and –21 (gMCP-2), and the following Ile residue, considered to be the N-terminal residue of the mature enzymes, was numbered 1.

Comparison of the deduced amino acid sequences with those of other species

When the deduced amino acid sequences of gMCP-1 and -2 were compared with those of other rodent MCPs (chymases) (Figure 2), serine protease catalytic triad residues (His⁴⁵, Asp⁸⁹ and Ser¹⁸²; shadowed boxes) and six cysteine residues building the predicted intrachain disulphide bonds (closed circles) were well-conserved. The N-linked glycosylation sites (open squares) were seen in several MCPs. The site at Asn⁵⁹ observed in gMCP-2 was conserved in mMCP-5 [20] and rMCP-3 [23], whereas no potential N-linked glycosylation site was observed in gMCP-1. The percentage amino acid and nucleotide identities of gMCP-1 and -2 with other rodent MCPs (chymases) are summarized in Table 1. The identities of gMCP-1 with mMCP-4 (85.4%) and rMCP-1 (82.3%), and those of gMCP-2 with mMCP-5 (87.6%) and rMCP-3 (87.2%) were far higher than those with other rodent chymases. In addition, similar to mMCP-4 [18] and rMCP-1 [22], the gMCP-1 had an activation peptide composed of two amino acids, Glu and Glu, whereas the gMCP-2 had an activation peptide, Gly and Glu, the same as mMCP-5 [20] and rMCP-3 [23]. Therefore, the gMCP-1 reported here is likely to be the gerbil counterpart of mMCP-4 and rMCP-1, while gMCP-2 is the counterpart of mMCP-5 and rMCP-3 (Table 2).

Southern-blot analysis

In Southern-blot analysis of gMCP-1 and -2 under high-stringency conditions (Figure 3), only a single band of DNA fragments was detected regardless of the restriction enzymes used. Since the

Figure 2 Comparison of the deduced amino acid sequences of gMCP-1 and -2 with those of other known rodent MCPs (chymases)

The deduced amino acid sequences of rMCP-1, -2 and -3, and mMCP-1, -2, -4 and -5 were taken from [22], [21], [23], [19], [17], [18], and [20] respectively. The identical amino acid residue among nine sequences is indicated by an asterisk. The other symbols used indicate as follows: signal peptide, black bar; activation peptide, dashed bar; potential N-linked glycosylation sites, squares; cysteine residues building the predicted intrachain disulphide bonds, closed circles; serine protease catalytic triad residues, hatched boxes.

Table 1 Amino acid and nucleotide identities of gMCP-1 and -2 with other rodent chymases

Results show the percentage identities of the deduced amino-acid sequences in the mature enzyme, while values in parentheses show those of the nucleotide sequences in open reading frames.

MCPs	gMCP-1	gMCP-2
gMCP-1	100.0 (100.0)	58.8 (65.8)
gMCP-2	58.8 (65.8)	100.0 (100.0)
mMCP-1	73.5 (81.3)	54.0 (63.2)
mMCP-2	63.3 (74.5)	50.4 (61.7)
mMCP-4	85.4 (85.1)	61.1 (67.0)
mMCP-5	56.6 (63.4)	87.6 (87.9)
rMCP-1	82.3 (84.5)	58.8 (66.8)
rMCP-2	70.8 (78.8)	54.4 (64.8)
rMCP-3	56.2 (63.4)	87.2 (86.9)

Table 2 Predicted counterparts among mouse, rat and gerbil MCPs

MCPs mainly expressed in MMCs are shown in bold type, while those mainly expressed in CTMCs are in plain text.

Protease phenotype	Mouse	Rat	Gerbil
Chymase	mMCP-1	rMCP-2	
	mMCP-2		
	mMCP-3		
	mMCP-4	rMCP-1	gMCP-1
Trypsin	mMCP-5	rMCP-3	gMCP-2
	mMCP-6	rMCT	
	mMCP-7		gMCT

Figure 3 Southern-blot analysis of gMCP-1 and -2

Samples (10 µg each) of gerbil genomic DNA were digested with *Xho*I, *Bam*HI, or *Eco*RI. The blot was performed under high-stringency conditions and was probed with 3'-RACE products (corresponding to the nucleotide positions 618–945 for gMCP-1 and 674–953 for gMCP-2 and having no internal restriction sites by the endonucleases used). Note that only a single band was detected regardless of the restriction enzymes used, indicating that these probes are specific for gMCP-1 and -2 genes. The membrane was exposed at -80 °C for 2 days.

probes used have no internal restriction sites and since the corresponding region is thought not to be divided by insertion of an intron [18,20], the probes used were specific for gMCP-1 and -2.

Figure 4 RNA-blot analysis of gMCP-1 and -2 before and after infection with *N. brasiliensis*

Total RNAs from gerbil small intestine (20 μ g; at the indicated time points), skin (100 μ g) and tongue (100 μ g) were electrophoresed on 1% formaldehyde agarose gel and transblotted on to nylon membrane. The blot was performed under the same conditions and with the same probe described in the Southern-blot analysis. The membrane was exposed at -80°C for 3 days (gMCP-1), 7 days (gMCP-2), and 24 h (G3PDH). Note that the RNA amounts of skin and tongue applied were 5-fold higher than those of jejunum. The number of mast cells per 10 villus crypt units (VCU) in the jejunum of gerbil were counted and indicated in the Figure.

RNA-blot analysis

The expression of gMCP-1 and -2 genes in different tissues before and after infection was examined by RNA-blot analysis. Both gMCP-1 and -2 genes were mainly transcribed in the jejunal mucosa and to a lesser extent in the skin or tongue (Figure 4: note that the RNA amounts of skin and tongue applied were 5-fold higher than those of jejunum). These results were basically consistent with our previous results showing that chymase activities were found in MMCs but not in CTMCs of gerbils by enzyme-histochemistry [14]. The lower amounts of mRNA in skin and tongue might be one of the reasons why chymase activities were not found in these tissues by enzyme-histochemistry. Using Alcian Blue and Safranin O staining, it was seen that the number of gerbil intestinal mast cells did not change significantly on day 7 after infection, but increased to reach a peak on day 14, and then decreased gradually for the next several weeks as described previously [15]. The amounts of gMCP-1 and -2 mRNAs in the skin and tongue remained unchanged at low levels during infection (results not shown), whereas those in the jejunum were drastically altered during infection. The amounts of gMCP-1 and -2 mRNAs paralleled well the number of mast

Figure 5 *In situ* hybridization of gMCP-1 (A) and -2 (B) in gerbil jejunum 14 days after infection with *N. brasiliensis*

The adjacent section was stained with Alcian Blue and Safranin O to show the distribution of gerbil MMC (C). Note that gMCP-1 and -2 genes were not expressed in all MMCs.

cells, gradually increasing for 2 weeks and subsequently decreasing by 3 weeks (Figure 4). Throughout the course of infection, enzyme-histochemically detectable chymase activity was limited to MMCs as described previously [14], indicating the MMC origin of gMCP-1 and -2. Reproducibility of the results of RNA-blot analysis was confirmed by two separate experiments using RNA preparations from different gerbils.

In situ hybridization

For further clarification of the expression of gMCP-1 and -2 genes in gerbil MMCs, *in situ* hybridization was performed. The results revealed that MMCs in gerbil jejunum were positive with gMCP-1 and -2 cRNA probes at the peak of mastocytosis on day 14 post-infection (Figure 5), although not all mast cells were positive with these probes in comparison with the adjacent section stained with Alcian Blue and Safranin O. With control gMCP-1 and -2 sense probes, mast cells or other cells were entirely negative (results not shown). Since chymase activities in

gerbil MMCs were already confirmed by enzyme-histochemical analysis in our previous study [14], these results indicate that functionally active gMCP-1 and -2 products were expressed in gerbil MMCs.

DISCUSSION

Two distinct cDNAs for chymases of Mongolian gerbils, designated gMCP-1 and -2, were cloned and sequenced by using the combination of RT-PCR and RACE methods. Based on the analyses of the deduced amino acid sequences, gMCP-1 was considered to be the gerbil counterpart for mMCP-4 and rMCP-1, while gMCP-2 was the counterpart for mMCP-5 and rMCP-3. In addition, similar to mMCP-4 [18] and rMCP-1 [22], gMCP-1 has an activation peptide, Glu and Glu, but no potential N-glycosylation site, whereas gMCP-2 has only one potential N-glycosylation site Asn⁵⁹ and an activation peptide composed of two amino acids, Gly and Glu, the same as mMCP-5 [20] and rMCP-3 [23].

The mRNAs for gMCP-1 and -2 were most abundantly detected in the jejunum of Mongolian gerbils and the amounts of these mRNAs paralleled well the degree of intestinal mastocytosis seen after infection with *N. brasiliensis*. Moreover, these enzymes were indeed expressed in MMCs of the jejunum by *in situ* hybridization. In mice and rats, however, the counterparts of gMCP-1 and -2 (mMCP-4 and -5 in mice and rMCP-1 and -3 in rats, respectively) are restrictedly or mainly expressed in CTMCs including serosal mast cells. In particular, mMCP-4 and rMCP-1 mRNAs were not detected in MMCs of each animal, even by sensitive RT-PCR analysis [18,19,22,23]. As shown in Table 2, MMCs of mice and rats express mMCP-1 [19] and rMCP-2 [21] respectively, and these enzymes increased in the serum during immediate hypersensitivity reactions such as allergies and helminth infections [34,35]. In the present study, the degenerated primers P1 and P2 used in the initial RT-PCR could actually amplify the mMCP-1 and rMCP-2 cDNAs from mouse and rat jejunum (results not shown). Still, the initial RT-PCR amplification using the total RNA prepared from the jejunum of *N. brasiliensis*-infected gerbils did not give cDNA corresponding to mMCP-1 or rMCP-2. These results suggest that gerbil MMCs do not express mMCP-1/rMCP-2-type chymases, which were characteristic in MMCs of mice and rats.

The gMCP-1 and -2 mRNAs were detected predominantly in the jejunum but also expressed constitutively in the skin and tongue. Similarly, a tryptase (gMCT) was also detected not only in the jejunum but also in other connective tissues with the predominance in the jejunum [15]. These findings seem to contradict our previous report that both chymase and tryptase activities were not detected in the gerbil CTMCs by enzyme-histochemical methods [14]. However, this discrepancy can be interpreted in several ways. The simplest explanation is that the enzyme activities in gerbil CTMCs were not sufficient enough to be detected by enzyme-histochemistry. In fact, the amounts of gMCPs and gMCT mRNAs in the skin and tongue were lower than those in the jejunum. Alternatively, even if gMCPs and gMCT were expressed in gerbil CTMCs, their activity might be inhibited by plasma proteinase inhibitors, such as trypstatin [27,29,30,36] or α 1-macroglobulin [37]. Another possibility is that the stability of chymase activity in MMCs and CTMCs of gerbils to various fixatives might be different between them. MMCs and CTMCs of gerbils contain heparin-like proteoglycan with different degrees of sulphation [13], and at least one rat chymase, rMCP-1, activity is known to be stabilized by binding with heparin [38,39]. Related to this, we could detect chymase

activity in the skin of gerbils by enzyme-histochemical staining after more gentle fixation (Y. Nawa, Y. Horii, M. Okada and N. Arizono, unpublished work).

Taking all these and previous findings together, gerbil MMCs are rather closer to CTMCs than to MMCs of mice and rats, though MMCs and CTMCs of gerbils were distinguishable from each other by histochemical and cytochemical methods [14]. According to the expression of proteases, human mast cells were divided into tryptase- and chymase-positive cells (MCTCs) located in connective tissues and tryptase-positive and chymase-negative cells (MCTs) seen in the mucosa [40,41]. Recently, however, both tryptase and chymase were detected immuno-histochemically in human intestinal mast cells [42]. In this regards, the MCP phenotype of gerbil MMCs is rather similar to that of human MMCs. We assume that such unique characteristics of gerbil MMCs might be due to different transcriptional regulation or fewer genes compared with those expressed in MMCs of mice and rats. In addition to the phenotypes of proteoglycans and MCPs, one of the most obvious characteristics of gerbil MMCs is that they never migrate into the intestinal epithelia through the basement membrane even after intestinal helminth infections [7,13]. Human [43,44] and rat MCPs [45] are thought to play an important role in activation of precursors for matrix metalloproteinases. In particular, rMCP-2 (but not rMCP-1) was shown to activate directly a procollagenase [45], suggesting the importance of mMCP-1/rMCP-2-type chymase for the migration of mast cells through extracellular matrices. Therefore, the absence of this type of chymase in gerbil MMCs would account for the lack of intraepithelial migration and the incapability to expel strongyloid parasites. Investigation of the fine specificity of isolated chymases of mice, rats and gerbils would facilitate further understanding of the physiology of mast cells in health and disease.

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