Cloning of the cDNA encoding a novel rat mast-cell proteinase, rMCP-3, and its expression in comparison with other rat mast-cell proteinases

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A cDNA encoding a novel rat mast-cell proteinase (MCP) named rMCP-3 was successfully cloned and sequenced from the peritoneal cells of Lewis rats infected with the intestinal nematode *Nippostrongylus brasiliensis* by using the combination of reverse transcription–PCR and **r**apid-**a**mplification-of-**c**DNA-**e**nds ('RACE') methods. The cDNA was 979 bp long and included a 741 bp open reading frame. When the deduced amino acid sequence was compared with those of other known mast-cell proteinases, rMCP-3 was considered to be translated as a prepro-enzyme with a 19-amino-acid signal peptide, a two-amino-acid activation peptide and a 226-amino-acid mature enzyme. The amino acid identity in the mature enzyme was 52.9% and 55.1% with rMCP-1 and rMCP-2 respectively. The rMCP-3 mRNA was not detected in the peritoneal cells of mast-cell-deficient *Ws/Ws* rats, though it was strongly detected in those of littermate +/+ and Lewis rats, indicating the mast-cell origin of rMCP-3. In addition to being present in peritoneal mast cells, the rMCP-3 mRNA was strongly detected in the skin, tongue, and RBL2H3 rat basophilic leukaemia cells and weakly in the jejunum of *N. brasiliensis*-infected rats by RNA blot analysis using a rMCP-3 gene-specific probe. By reverse transcription–PCR, the rMCP-3 mRNA was also detected in the lung. While the expression of rMCP-1 and rMCP-2 are clearly restricted in connective-tissue mast cells and mucosal mast cells respectively, rMCP-3 was widely expressed in both types of mast cells with a predominance in connective-tissue mast cells.

INTRODUCTION

Mast cells are widely distributed in the whole body and play an important role in immune-mediated inflammatory reactions such as allergy and parasitic infections [1]. These reactions are, at least in part, triggered by neutral serine proteinases (mast-cell proteinases; MCPs) in their secretory granules with chondroitin sulphate or heparin proteoglycans [2–4]. Mast cells are usually classified into two subsets, mucosal (MMC) and connective-tissue mast cells (CTMC), in terms of their tissue distribution, histochemical properties and also cytokine-dependency [1]. Also, MCPs constitute a family of related enzymes, including proteinases of trypsin-like (tryptases) and chymotrypsin-like (chymases) substrate specificities. The expression of MCPs is markedly different between MMC and CTMC and also among species [2–4].

To date, only three major subclasses of MCPs have been identified and characterized in rats, though seven MCPs, five chymases (mMCP-1–mMCP-5) [5–8] and two tryptases (mMCP-6 and mMCP-7) [9,10] have been identified in mice. Rat mast-cell proteinase (rMCP)-1 is a chymase stored in the secretory granules of CTMC complexed with heparin proteoglycan [11,12]. After being released by mast-cell activation, rMCP-1 inactivates thrombin [13,14]. Another chymase, rMCP-2, is also stored in the secretory granules of MMC with chondroitin sulphate proteoglycan [15,16] and is used as a marker protein of immediate hypersensitivity reactions such as allergies and helminth infections [17,18]. The rat mast-cell tryptase (rMCT) is also stored in the secretory granules of the CTMC [19–21], the activity being regulated by plasma proteinase inhibitors [22,23]. However, no other additional rMCP is known, and the gene expression of these rMCPs and rMCT in various tissues and cells has never been explored in detail.

In this paper we report a full-length cDNA encoding a novel rMCP named rMCP-3, successfully cloned from peritoneal mast cells by using the combination of reverse transcription–PCR (RT-PCR) and **r**apid-**a**mplification-of-**c**DNA-**e**nds (RACE) methods. We also developed the rMCP-3 gene-specific probe and compared the expression of rMCP-3 with those of other rMCPs in various tissues and cells.

EXPERIMENTAL

DNA and RNA extraction

Genomic DNA was purified from liver of 8-week-old Lewis rats by SepaGene (Sanko Junyaku Co., Ltd., Tokyo, Japan) according to the manufacturer's protocol. Total RNAs were extracted by ISOGEN (Nippongene, Tokyo, Japan) from jejunum, lung, skin and tongue of 8–10-week-old Lewis rats before and 14 days after infection with *Nippostrongylus brasiliensis*. The peritoneal cells were collected by peritoneal lavage from 8–10-week-old Lewis rats, mast cell-deficient *Ws/Ws* rats [24,25] and their littermate +/+ rats before, and 14 days after, infection with the parasite. The RBL2H3 cell line, a subline of rat basophilic leukaemia cells having phenotypic characteristics of mast cells, was supplied from Japanese Cancer Research Resources Bank (JCRB) and the cells were cultured as described in [26]. Total RNAs of these cells were also extracted by ISOGEN. Polyadenylated RNA [poly(A)$^+$] selection was performed by using oligo(dT)–latex (Oligotex-dT30; Nippon Roche, Tokyo, Japan).

RT-PCR cloning and sequencing

A 1 μg portion of poly(A)$^+$ RNA extracted from peritoneal cells of Lewis rats infected with *N. brasiliensis* was reverse-transcribed by random hexamer [pd(N)$_6$] and SuperScript reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD, U.S.A.).

Abbreviations used: MCP, mast-cell proteinase; rMCP, rat MCP; mMCP, mouse MCP; CTMC, connective-tissue mast cells; MMC, mucosal mast cells; rMCT, rat mast-cell tryptase; RT-PCR, reverse transcription–PCR; RACE, **r**apid **a**mplification of **c**DNA **e**nds; poly(A)$^+$, polyadenylated.

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The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases under the accession number D38495.

The resulting cDNA was subjected to PCR for 30 cycles with the degenerated primers (P1 and P2; see below) designed within the well-conserved regions of known MCP (chymase) cDNA sequences [5–8,12,16,27,28]. The thermal cycle profile was 1 min at 94 °C, 2 min at 50 °C and 3 min at 72 °C. After electrophoresis, the PCR product of the expected size was cut from 2%-agarose gel, purified by GeneClean II (Bio 101 Inc., La Jolla, CA, U.S.A.) and cloned into a TA cloning vector pCR II (Invitrogen, San Diego, CA, U.S.A.). The nucleotide sequence was determined by the dideoxy chain-termination method using a Sequenase v2.0 kit (USB, Cleveland, OH, U.S.A.).

RACE

A 1 μg portion of poly(A)$^+$ RNA and 5 μg of total RNA extracted from rat peritoneal cells were reverse-transcribed with DT7 primer and P5 primer for 3′ and 5′ RACE respectively [29–32]. The DT7 primer contains oligo(dT)$_{17}$ at the 3′ end, followed by PT7 primer sequence, while the P5 primer is specific for rMCT. For 3′ RACE, the first-strand cDNA was amplified by PCR with the primers P3 and PT7, and the resulting first-round RACE products were amplified again with the primers P4 and PT7. For 5′ RACE we used the 5′-AmpliFINDER RACE Kit (Clontech, Palo Alto, CA, U.S.A.). Briefly, the single-stranded oligonucleotide anchor (5′ anchor) was directly ligated to the 3′ end of the first-strand cDNA with T4 RNA ligase, and the resulting anchor-ligated cDNA was amplified by PCR with the primer P6 and the anchor primer P7. These RACE products were also purified, cloned and sequenced as described above. Primers and anchor sequences used were as follows:

Primer P1 (sense)	5′-CCA(T)CAC(T)TCC(T)CGC(T)CCC(T)TACATGGC-3′
Primer P2 (antisense)	5′-GAGAC(T)TCG(T)GGTA(G)AAGAC(T)A(T)GCAGGGG-3′
Primer P3 (sense)	5′-CATGATATCATGTTACTGAA-3′
Primer P4 (sense)	5′-GGCAACCCCAAGAAGATGCA-3′
Primer P5 (antisense)	5′-AACTTCAGTAACATGATGTC-3′
Primer P6 (antisense)	5′-CTAGGAGGACGGTTATAGAC-3′
Primer P7	5′-CTGGTTCGGCCCACCTCTGAAGGTTCCAGAATCGATAG-3′
Primer PT7	5′-AATACGACTCACTATAG-3′
Primer DT7	5′-TAATACGACTCACTATAGGGATTTTTTTTTTTTTTTT-3′
5′-Anchor	3′-NH_3-GGAGACTTCCAAGGTCTTAGCTATCACTTAAGCAC-p-5′

The directions and locations of P1–P6 primers are shown in Figure 1 (below). After sequencing of the RT-PCR products using the degenerated primers P1 and P2, which were designed within the well-conserved regions of known MCP (chymase) cDNA sequences, the primers P3–P6 were designed in rMCP-3 specific inner regions.

Southern- and RNA-blot analyses

Three restriction enzymes, *Bam*HI, *Eco*RI, and *Xho*I were purchased from TOYOBO (Osaka, Japan). A 10 μg portion each of rat genomic DNA digested by one of these endonucleases was electrophoresed on 1%-agarose gel and transblotted on to Hybond-N nylon membrane (Amersham, Bucks., U.K.). Total RNAs (30 μg each) from the peritoneal cells, jejunum, skin, tongue and lung of uninfected and infected rats, and from RBL2H3 cells, were also electrophoresed on 1%-formaldehyde/agarose gel and transblotted on to nylon membrane. Hybridization was performed under high-stringency conditions using ^{32}P-labelled probes. The rMCP-3 gene-specific probe used for both Southern- and RNA-blot analyses was 3′-RACE products (corresponding to the nucleotide positions 601–979) having no internal restriction sites for the endonucleases used in the present study. The control rat β-actin probe was made by PCR as described [22]. These probes were labelled with [α-^{32}P]dCTP (Amersham) by a random-primed DNA labelling kit (Boehringer Mannheim). Alternatively, three oligonucleotide probes (corresponding to the nucleotide positions 325–362 for rMCP-3, 262–299 for rMCP-1 [12], and 316–353 for rMCP-2 [16]; see Figure 2 below) labelled with [γ-^{32}P]ATP (Amersham) by a DNA 5′-end labelling kit (Megalabel; Takara, Tokyo, Japan) were also prepared and used for comparative study of the expression of MCPs in various tissues and cells. The rMCP-1 and -2 oligonucleotide probes were designed in the positions of which sequences were already confirmed to be specific to each enzyme [12]. The rMCP-3 oligonucleotide probe was designed in the position corresponding to the rMCP-1 and -2 probes, where the nucleotide sequence is markedly different from those of rMCP-1 and -2 (see Figure 2 below). The membranes were exposed at −80 °C for 24 h in Southern blot, 72 h in RNA blot using 3′ RACE products as a probe, and 2 weeks in RNA blot using oligonucleotide probes.

RT-PCR analysis of rMCPs and rMCT in various tissues and cells

A 10 μg portion each of total RNAs from various tissues and cells was reverse-transcribed by oligo(dT)$_{12\text{-}18}$ primer and Super-Script reverse transcriptase (Life Technologies). The resulting cDNA was divided into five pieces and each piece of cDNA was subjected to PCR for 30 cycles with the primers described below. The thermal-cycle profile was 1 min at 94 °C, 2 min at 60 °C and 2 min at 72 °C. The sequences and combinations of the gene-specific primers designed for rMCP-1, -2, -3, rMCT and β-actin were as follows:

Primer rMCP-1 (sense)	5′-GCCTGTAAAAACTATTTT-3′
Primer rMCP-1 (antisense)	5′-CAGGCTGGTCAGATCCTGC-3′
Primer rMCP-2 (sense)	5′-GCCTGTGTGGACTACAGGTA-3′
Primer rMCP-2 (antisense)	5′-TCAGGCTTTTCAGCTACTTG-3′
Primer rMCP-3 (sense)	5′-TCCTGCAAACACTTCACCAG-3′
Primer rMCP-3 (antisense)	5′-CGAGATCCAGAGTAATTCT-3′
Primer rMCT (sense)	5′-CCACTGGTCTGCAAAGTGAA-3′
Primer rMCT (antisense)	5′-GGGACATAGTGGTGGATCCA-3′
Primer β-actin (sense)	5′-GACTACCTCATGAAGATCCT-3′
Primer β-actin (antisense)	5′-GTGGCCATCTCTTGCTCGAA-3′

The directions and locations of the primers for rMCP-1, -2 and -3 are indicated in Figure 2 (below). The nucleotide sequence data of rMCP-1, rMCP-2 and rMCT are taken from [12], [16] and [21], respectively.

RESULTS

Sequencing of rMCP-3 cDNA

The degenerated primers P1 and P2 were designed in the well-conserved regions of known MCP (chymase) cDNAs [5–8,12,16,27,28]. RT-PCR was performed to make rMCP-1 and -2 probes using total RNAs extracted from rat peritoneal cells of *N. brasiliensis*-infected Lewis rats and the primers P1 and P2. The expected size (approx. 600 bp) of RT-PCR product was obtained and subcloned. Surprisingly, sequence analysis revealed that six out of total 24 clones encode a novel rat MCP, named rMCP-3, while the rest encoded rMCP-2. Therefore we performed both 5′ and 3′ RACE using the primers P3–P6, which were designed for rMCP-3 on the basis of its partial cDNA sequence. Eventually a novel rat mast-cell chymase, rMCP-3, was discovered and its full-length cDNA sequence was determined. At least six individual clones each from the RT-PCR and RACE products were sequenced, and all sequencing was done once or more in both directions to obtain maximum accuracy for the sequence given. The total 979 bp of rMCP-3 nucleotide sequence and its deduced amino acid sequence are shown in Figure 1. An ATG codon was found 35 nucleotide residues downstream of the 5′ end. A stop codon (numbered 776) and a following 204 bp non-coding region, including a polyadenylation signal, AATAAA (numbered 955–960), were found at the 3′ end. Thus peptides with 247 amino acids were encoded by the single open reading frame. The first 19 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 (chymase). Consequently the initial methionine residue was numbered −21, and the following isoleucine residue, considered to be the N-terminus of the mature protein, was numbered 1.

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   1:                          agcctgcacagccctgagtagcccctcagagagg
  35: atgaatctccatgctctgtgtctgctgctccttctcctgggttccagcaccaaagctggg
(-21) M  N  L  H  A  L  C  L  L  L  L  L  L  G  S  S  T  K  A  G
  95: gagatcatcgggggcacggagtgcataccacactcccgcccctacatggcctatctagaa
 (-1) E  I  I  G  G  T  E  C  I  P  H  S  R  P  Y  M  A  Y  L  E
                                P1
 155: attgtcacttctgacaattacctgtcagcctgcagtggcttcctgatcagacgaaacttt
 (20) I  V  T  S  D  N  Y  L  S  A  C  S  G  F  L  I  R  R  N  F
 215: gtgctgactgcagctcactgtgcaggacggtctataaccgtcctcctaggagctcataac
 (40) V  L  T  A  A  H  C  A  G  R  S  I  T  V  L  L  G  A  H  N
                                                     P6
 275: aaaacatataaagaagacacatggcagaagctcgaggttgaaaagcaattcattcatcca
 (60) K  T  Y  K  E  D  T  W  Q  K  L  E  V  E  K  Q  F  I  H  P
 335: aactatgataagcgtttggttctccatgacatcatgttactgaagttgaaggagaaagcc
 (80) N  Y  D  K  R  L  V  L  H  D  I  M  L  L  K  L  K  E  K  A
                             P3                  P5
 395: aagctaaccctaggcgtgggaaccctcccactctcggccaacttcaacttcattccaccc
(100) K  L  T  L  G  V  G  T  L  P  L  S  A  N  F  N  F  I  P  P
 455: gggagaatgtgcagggcagttggctggggcagaacaaacgtgaatgaaccagcctctgac
(120) G  R  M  C  R  A  V  G  W  G  R  T  N  V  N  E  P  A  S  D
 515: acactgcaggaagtaaagatgagactccaggagccccaatcctgcaaacacttcaccagt
(140) T  L  Q  E  V  K  M  R  L  Q  E  P  Q  S  C  K  H  F  T  S
 575: tttcaacacaagtcccagttgtgtgtgggcaaccccaagaagatgcaaaatgtatacaag
(160) F  Q  H  K  S  Q  L  C  V  G  N  P  K  K  M  Q  N  V  Y  K
                                P4
 635: ggagactctggaggacctctcctgtgtgctgggatagcccaaggcattgcatcctacgta
(180) G  D  S  G  G  P  L  L  C  A  G  I  A  Q  G  I  A  S  Y  V
 695: catccgaatgcaaagccccctgctgtcttcaccagaatctcccattacagaccctggatc
(200) H  P  N  A  K  P  P  A  V  F  T  R  I  S  H  Y  R  P  W  I
                                                 P2
 755: aataagatcttgagggagaattaactctggatctcgggccagtgtgtgaggaaatctgga
(220) N  K  I  L  R  E  N  *
 815: gctggaatcgtgcaggttttctgtgacatgggatctggcctgtctgtagttcctgctgaa
 875: gcccttcctgatccctgagcttccagaaggttcttacaagtcacagaaagttcctaaaag
 935: tcaccatcttcataaacctcaataaagagccagatcctagactgc
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Figure 1 Nucleotide and deduced amino acid sequences of rMCP-3

Primers used for RT-PCR and RACE sequencing (P1–P6) are also indicated. Symbols used: underlining, signal peptide; broken line, activation peptide; asterisk, stop codon; shaded box, polyadenylation signal; closed circle, serine-proteinase catalytic triad residues; square, potential N-linked glycosylation sites. The amino acids were numbered with position one set to the start of the mature enzyme (in parentheses on the left-hand side).

Comparison of the deduced amino acid sequence with those of other rMCPs and mouse MCPs

When the deduced amino acid sequence of rMCP-3 was compared with those of rMCP-1 and -2 and mouse MCPs (mMCP), serine proteinase catalytic triad residues (His[45], Asp[89] and Ser[182]; shaded boxes) and six cysteine residues building the predicted intrachain disulphide bonds (closed circles) were well-conserved (Figure 2). The potential N-linked glycosylation site (open boxes) were seen in Asn[59], which was conserved only in mMCP-5. The sequence identities of rMCP-3 with rMCP-1 and -2 were 52.9 and 55.1 % in amino acids for mature enzymes respectively, whereas those against mMCP-1, -2, -4, and -5 were 53.5, 48.7, 58.4 and 95.1 % respectively. The rMCP-3 sequence therefore shows considerably more similarity to that of mMCP-5 than to those of rMCP-1 and -2, suggesting that the rMCP-3 might be the rat counterpart of mMCP-5.

Southern- and RNA-blot analysis of rMCP-3

In Southern-blot analysis using the 3′ RACE product as a probe under high-stringency conditions (Figure 3a), only a single band was detected regardless of the restriction enzymes used, suggesting that this probe is specific for the rMCP-3 gene. RNA blot analysis using this probe (Figure 3b) revealed that the rMCP-3 was expressed in the peritoneal cells from Lewis rats and +/+ rats, but not in those from mast-cell-deficient *Ws/Ws* rats, indicating the mast-cell origin of the rMCP-3. The mRNA was consistently detected in the skin and tongue before and after *N. brasiliensis* infection and in RBL2H3 rat basophilic leukaemia cells. Interestingly, in the jejunum, the rMCP-3 mRNA was weakly detected after, but not before, infection with *N. brasiliensis*. The mRNA signal was not detected in the lung even after the infection. Reproducibility of the results of rMCP-3 expression in various tissues was confirmed by two separate experiments using RNA preparations from different rats.

Comparison of the expression of rMCP-3 with those of rMCP-1 and -2 by RNA blot analysis using oligonucleotide probes and RT-PCR analysis

The distribution of rMCP-3 was compared with those of rMCP-1 and -2 by RNA-blot analysis using oligonucleotide probes (Figure 4a) and RT-PCR analysis (Figure 4b). The samples were obtained from various tissues of *N. brasiliensis*-infected rats and also from RBL2H3 cells. By RNA-blot analysis rMCP-1 mRNA was detected in the tongue and in peritoneal cells from +/+ rats. rMCP-2 mRNA was detected in the jejunum and RBL2H3 cells. rMCP-3 mRNA was detected only in peritoneal cells when an oligonucleotide probe was used. In more sensitive RT-PCR analysis, rMCP-1 and rMCT mRNAs were detected in the skin and lung in addition to the tongue and peritoneal cells, but not in the jejunum or in RBL2H3 cells. On the other hand, the

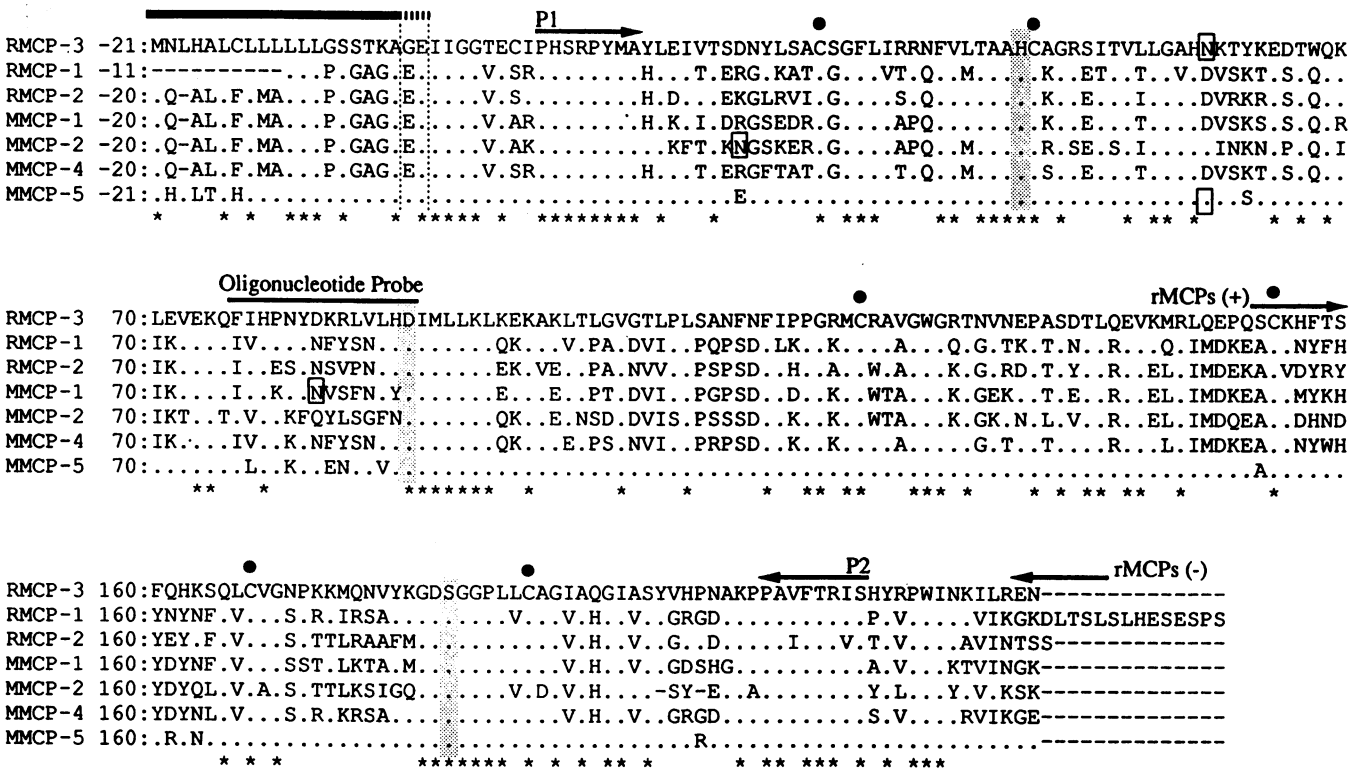

Figure 2 Comparison of the deduced amino acid sequence of rMCP-3 with those of other known rodent MCPs (chymases)

The deduced amino acid sequences of rMCP-1 and -2, and of mMCP-1, -2, -4, and -5, were taken from [12], [16], [5], [6], [7] and [8] respectively. Identical amino acid residues among the seven sequences are indicated by an asterisk. The other symbols used are as follows: black bar, signal peptide; broken bar, activation peptide; squares, potential N-linked glycosylation sites; closed circles, cysteine residues building the predicted intrachain disulphide bonds; shaded boxes, serine-proteinase catalytic triad residues. The locations and the directions of oligonucleotide probes and primers (degenerated primers P1 and P2 for the initial RT-PCR sequencing, and rMCPs primers used for RT-PCR analysis) are also indicated.

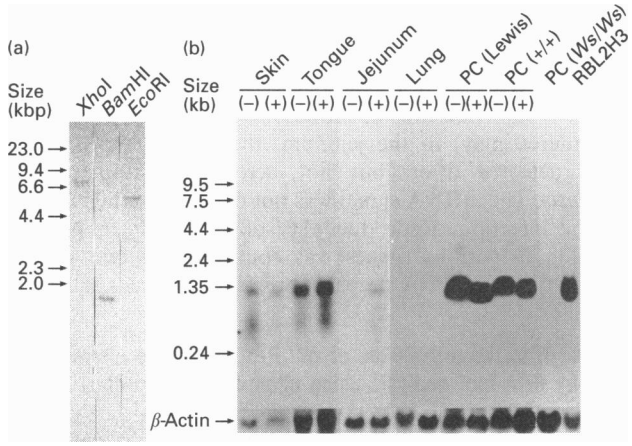

Figure 3 (a) Southern-blot analysis of rat genomic DNA digested with *XhoI*, *BamHI* and *EcoRI*, and (b) RNA-blot analysis of rMCP-3 before and after infection with *N. brasiliensis*

(**a**) The blot was performed under high-stringency conditions and was probed with 3′ RACE products corresponding to the nucleotide positions 601–979 having no internal restriction sites for the endonucleases used. Note that only a single band was detected regardless of the restriction enzymes used, indicating that this probe is specific for the rMCP-3 gene. (**b**) Portions (30 μg each) of total RNAs derived from various tissues and cells before (−) and 14 days after (+) infection were loaded. The blot was performed under the same conditions and the same probe as described for the Southern-blot analysis. The membrane was exposed at −80 °C for 72 h.

rMCP-2 mRNA was, in addition to the jejunum and RBL2H3 cells, detected also in the lung and peritoneal cells from +/+ rats, but not in the skin or tongue. Differently from the results for rMCP-1, rMCP-2 and rMCT, the rMCP-3 mRNA was detected in all the samples tested, except for the peritoneal cells from *Ws/Ws* rats, by RT-PCR analysis. The results of uninfected rats were the same as those from infected rats (results not shown). As summarized in Table 1, the expression patterns were basically consistent with previous reports that the rMCP-1 and rMCT are specific for CTMC, whereas the rMCP-2 is specific for MMC [12,16,21]. However, in the present results of RT-PCR analysis, not only rMCP-1 and rMCT, but also rMCP-2 and -3, mRNAs were detected in the lung and peritoneal cells, which are considered as the represesntatives of CTMC.

DISCUSSION

MCPs are considered as the useful phenotypic markers of mast-cell subsets because of their distinct distributions among tissue sites and among animal species [2,11,18]. While five chymases (mMCP-1–mMCP-5) [5–8] and two tryptases (mMCP-6 and -7) [9,10] were cloned and characterized, and their expression was confined to particular subtypes and/or maturation stages of mast cells in mice [33–35], only three major subclasses of MCPs have been identified and characterized in rats. The rMCP-1 [11,12] and rMCP-2 [15,16] are chymases specific for rat CTMC and MMC respectively, and the rat mast-cell tryptase (rMCT) is

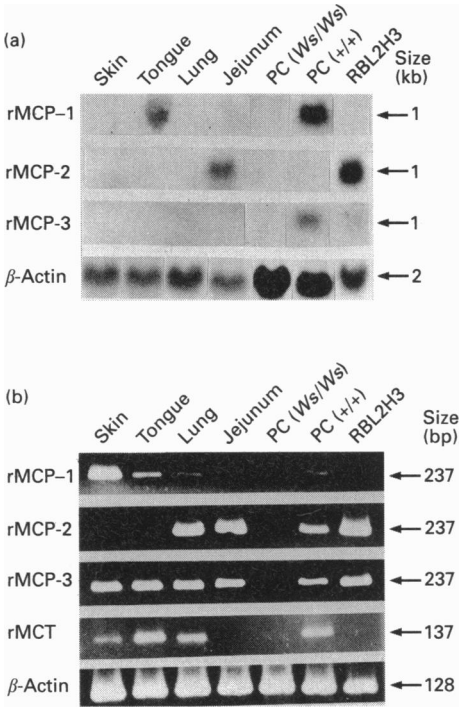

Figure 4 Comparison of the expression of rMCP-3 with those of rMCP-1 and -2 by RNA-blot analysis using oligonucleotide probes (a) and RT-PCR analysis (b)

(**a**) Portions (30 μg each) of total RNAs derived from various tissues and cells were loaded. The blot was performed under high-stringency conditions and was probed with an indicating oligonucleotide probe. The membranes were exposed for 2 weeks at −80 °C. (**b**) Portions (10 μg each) of total RNAs from various tissues and cells were reverse-transcribed by oligo$(dT)_{12-18}$ primer and SuperScript reverse transcriptase. The resulting cDNA was divided into five pieces and each piece of cDNA was subjected to PCR for 30 cycles with the indicating pair of primers. These results are summarized in Table 1.

specific for CTMC [19–21]. A novel mast-cell chymase, rMCP-3, reported here is the fourth rMCP having a unique tissue expression. The rMCP-3 cDNA was cloned from the peritoneal cells containing 5% or more mast cells. The mRNA was detected in the peritoneal cells of Lewis rats and +/+ rats, but not in mast-cell-deficient *Ws/Ws* rats. Even by histochemical analysis, mast cells were not observed in the peritoneal cells of *Ws/Ws* rats irrespective of whether there was *N. brasiliensis* infection or not (results not shown). In addition, the rMCP-3 mRNA was detected in RBL2H3 rat basophilic leukaemia cells having phenotypic characteristics of mucosal mast cells [26,36]. Therefore the rMCP-3 cDNA reported here definitely originated from mast cells and has unique tissue expression. The amino acid sequence deduced from the rMCP-3 cDNA was very similar to that of mMCP-5 (95.1%) but less so to those of rMCP-1 (52.9%), rMCP-2 (55.1%) or other mMCPs. In addition, similarly to mMCP-5 [7,8], the rMCP-3 had only one potential N-glycosylation site, asparagine-59, and an activation peptide composed of two amino acids, namely glycine and glutamic acid. Therefore, the rMCP-3 reported here is likely to be the rat counterpart of mMCP-5.

By RNA-blot analysis using 3′ RACE products as a gene-specific probe, regardless of the presence or absence of *N. brasiliensis* infection, the rMCP-3 mRNA was strongly detected in the skin and tongue in addition to the peritoneal cells, all of which are the representative source of CTMC. Moreover, the mRNA was also faintly detected in the jejunum of *N. brasiliensis*-infected rats containing numerous MMCs, but not in that of uninfected rats. Sensitive RT-PCR analysis revealed that, except for the peritoneal cells from *Ws/Ws* rats, the rMCP-3 mRNA was detected in all the samples tested, including the jejunum and lung. On the other hand, even using sensitive RT-PCR analysis, the mRNAs of rMCP-1 and rMCP-2 are restricted in CTMC and MMC respectively, except for the lung and peritoneal cells from +/+ rats. Thus, although rMCP-3 is predominantly expressed in CTMC, it seems to be present also in MMC. Related to this, RBL2H3 basophilic leukaemia cells, which closely resemble MMC [26,36], expressed rMCP-3 in association with rMCP-2, a representative marker of MMC [16,26], but not with rMCP-1 [12] or rMCT [21], markers of CTMC. Such expression patterns of rMCPs in RBL2H3 cells are the same as those of the jejunum, suggesting that RBL2H3 cells have phenotypic characteristics of MMC and provides the supportive evidence for the expression of rMCP-3 in MMC. In spite of the high sequence identity between rMCP-3 and mMCP-5, mMCP-5 mRNA was not detected in MMC, though its cDNA was cloned from peritoneal mast cells and Kirsten-sarcoma-virus-immortalized mast cells [8], and the gene was expressed in CTMC and bone-marrow-derived mast cells [8,37].

In rats, CTMC and MMC can be easily distinguished from each other by their granule proteinases, rMCP-1 and -2. Also, rMCT mRNA was detected only in the tissues containing CTMC [21]. Although the peritoneal mast cells have been considered representative of CTMC, they expressed rMCP-2, a marker for MMC [16]. The present results also show that, differently from the skin and tongue, the peritoneal mast cells expressed all rMCPs (rMCP-1 and -3 by RNA blot analysis and rMCP-1 to -3 and rMCT by RT-PCR analysis). Similarly to the peritoneal mast cells, all rMCPs were detected in the lung of *N. brasiliensis*-infected rats by sensitive RT-PCR analysis. While CTMC is a predominant mast-cell subtype in normal rat lung, MMC becomes predominant after infection with *N. brasiliensis* [38]. Thus it is likely that the peritoneal mast cells, as well as those of

Table 1 Expression of rMCPs and rMCT in various tissues and cells

Symbols used: ++, detected by both RT-PCR and RNA-blot analysis using an oligonucleotide probe; +, detected by RT-PCR but not by RNA-blot analysis; −, not detected by RT-PCR nor by RNA -blot analysis; (++), detected also by RNA-blot analysis using 3′ RACE products as a probe (379 bp) (see Figure 3b). Abbreviation used: PC, peritoneal cells.

	Skin	Tongue	Lung	Jejunum	PC (*Ws/Ws*)	PC (+/+)	RBL2H3
rMCP-1	+	++	+	−	−	++	−
rMCP-2	−	−	+	++	−	+	++
rMCP-3	+ (++)	+ (++)	+	+ (++)	−	++ (++)	+ (++)
rMCT	+	+	+	−	−	+	−

the lung, contain both CTMC and MMC, and their proportion is altered by infection. Alternatively, mast cells having the dual character of CTMC and MMC might be present in these tissues and their proportion might be altered by infection. Indeed, co-localization of rMCP-1 and -2 in the lung of *N. brasiliensis*-infected rats was reported by immunofluorescent histochemistry and by the measurement of these proteinases using an ELISA [39]. In the present study, in which an RNA-blot analysis using oligonucleotide probes was used, rMCP-1 was detected in tongue and peritoneal mast cells, rMCP-2 in the jejunum and RBL2H3 cells, and rMCP-3 in the peritoneal mast cells. The peritoneal cells indeed have chymase activity [19]. Therefore rMCP-3 might be a useful marker for mast cells present in the peritoneal cavity as well as in the lung. Further characterization of mast cells in these tissues is now underway in our laboratory.

In conclusion, we cloned and sequenced the cDNA encoding a novel rat MCP, named rMCP-3, from peritoneal mast cells. While the expression of rMCP-1 and rMCP-2 are clearly restricted in CTMC and MMC respectively, rMCP-3 was widely expressed in both types of mast cells with the predominance in CTMC. Therefore a study on the biological activity of the rMCP-3 protein compared with that of other rMCPs and rMCT would facilitate a further understanding of the differentiation and function of rat mast-cell subtypes.

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