Identification and Molecular Cloning of a Novel Mouse Mucosal Mast Cell Serine Protease*

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A novel 28,000 M, serine protease, designated mouse mast cell protease-2 (MMCP-2), that is stored in the secretory granules of Kirsten sarcoma virus-immortalized mouse mast cells (KiSV-MC) has been identified and its NH₂-terminal amino acid sequence has been determined. Analysis of a 963-base pair cDNA that encodes MMCP-2 revealed that this serine protease is a basically charged protein, possessing the histidine-aspartic acid-serine charge relay system that is characteristic of other serine proteases. DNA blot analysis using the full-length MMCP-2 cDNA indicated the existence of a family of highly related serine protease genes in the mouse genome. When the same DNA blot was probed with the 149-base pair KpnI-3' fragment of the cDNA, the probe hybridized to a single DNA fragment, thereby demonstrating that this 3' fragment could be used as a gene-specific probe. The presence of high levels of the MMCP-2 mRNA transcript in the intestines of nematode-infested mice, and its absence in mouse bone marrow-derived mast cells and peritoneal cavity-derived connective tissue mast cells, suggest that this member of the mouse mast cell protease family is preferentially expressed late in the differentiation of mucosal mast cells.

In both rats and mice, at least two subclasses of mast cells have been identified based on differences in tissue distribution, histochemical staining properties, and T cell-factor dependence (reviewed in Ref. 1). The connective tissue mast cell (CTMC) is the predominant type present in the skin and peritoneal cavity, whereas the T cell-dependent mucosal mast cell is the major type present in the intestinal mucosa of nematode-infected animals. In the rat, both types of mast cells contain large amounts of basically charged serine proteases (2–7), which are stored in active form bound to acidi-}

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EXPERIMENTAL PROCEDURES

Isolation and Culture of Cells—CTMC were obtained from RA1L/r mice (The Jackson Laboratory, Bar Harbor, ME) by peritoneal lavage.
tonal lavage and were purified to >97% as previously described (16-18). The CTMC were washed once in Hanks’ balanced salt solution and processed as described below to isolate RNA. The mouse WEHI-3 myelomonocytic tumor cell line (line TIB-68; American Type Culture Collection, Rockville, MD) and mouse 3T3 fibroblasts (line CCL-92; American Type Culture Collection) were cultured in enriched medium (RPMI-1640 containing 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 5 mM HEPES (GBCO)). BMCC were obtained by culturing BALB/c mouse bone marrow cells for 3-6 weeks in 50% enriched medium, 50% WEHI-3 conditioned medium (19). Kirsten sarcoma virus-immortalized mouse mast cell lines KiSV-MC1, KiSV-MC5 and KiSV-MC6 (15) were cultured in 24-well plates at 37 °C. The [3H]DFP-labeled proteins were recombined with the proteins in enriched medium that lacked HEPES. Kirsten sarcoma virus-immortalized 3T3 fibroblasts were cultured as described (15).

Infection of Mice with N. brasiliensis and Schistosoma mansoni Larvae—Mucosal mast cell production was elicited in 4-week-old female CFW mice (Charles River, Kalamazoo, MI) (20, 21) by inoculating animals subcutaneously with approximately 500 infective larvae of N. brasiliensis in 0.1 ml of distilled water using a 1-ml syringe fitted with a 22-gauge needle. Four mice were inoculated, two each on subsequent days. Four other mice were sham inoculated in an identical manner. On day 10 after inoculation, after overnight fasting, the mice were killed by cervical dislocation, the peritoneal cavities were opened, and the 10 cm of small intestine were removed from each animal. For RNA extraction, the portions of intestine were each flushed with 4 ml of ice cold phosphate buffered saline, placed in 2 ml of ice-cold extraction buffer (see below), and quickly minced into 1-mm pieces. Mincing was completed within 2 min after the animals had been killed. The tissues were sonicated with a Branson sonifier (power setting 7, 50% duty cycle, 10 pulses; Branson Sonic Power Co., Danbury, CT) and debris was removed by centrifugation at 83 × g for 5 min at 4 °C. Total RNA was purified from each supernatant as described below. The RNA samples from the infected and uninfected mice (4 samples of each) were coded and then analyzed as described below.

Separation of Mouse Mast Cell Proteins by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis, and NH2-terminal Amino Acid Sequence Analysis of a 28,000 M, Mouse Mast Cell Protein—To radiolabel the serum proteases (22), a whole cell sonicate of 5 × 10⁸ KiSV-MC5 was prepared in 0.5 ml of Hanks’ balanced salt solution. Ten percent of the sonicate was added to 50 pl of 0.15 M NaCl, 0.02 mM Tris-HCl, pH 7.4, containing 5 μCi of [3H]diisopropyl fluorophosphate (DFP) (4 Ci/nmol; Amersham Corp., Arlington Heights, IL). The remaining 95% of the sonicate was incubated with 10 μM of 103-Cys-containing protein (Difco). Sixty-two hours later, the mice were killed, and their peritoneal cavities were lavaged with 3 ml of RPMI-1640 containing 10 units/ml of heparin (Sigma). The 2.4 × 10⁸ isolated cells were pooled, washed once with RPMI-1640, and their total RNA was extracted as described below. As assessed by their Wright’s/Giemsa staining and after exclusion with trypan blue, 95% of the cells were between 24% eosinophils, 38% lymphocytes, 37% monocytes/macrophages, 1% neutrophils, and 0.5% connective tissue-type mast cells.

DNA and RNA Blot Analysis-Samples (-10 μg/lane) of mouse genomic liver DNA were separately digested for 4 h at 37 °C under conditions of low stringency in 0.15 M NaCl, 15 mM sodium citrate, 0.1% SDS, 1 mM EDTA, and 10 mg/ml of salmon sperm DNA carrier and fractionated by agarose gel electrophoresis. DNA blots were analyzed sequentially by probe, hybridization, and autoradiography with nick-translated rat cDNA that encodes RMCPC-2 (9) in hybridization buffer (50% formamide, 0.75 M NaCl, 75 mM sodium citrate, 2 X Denhardt’s buffer, 0.1% SDS, 1 mM EDTA, 100 μg/ml salmon sperm DNA carrier, and 10 mg/ml of salmon sperm DNA carrier) at 32 °C for 3 h. The nitrocellulose filters were washed at 37 °C under conditions of low stringency in 0.15 M NaCl, 15 mM sodium citrate, 0.1% SDS, 1 mM EDTA, and 10 mg/ml of salmon sperm DNA carrier, pH 7.0. Two individual KiSV-MC-derived clones, designated cDNA-2 and cDNA-4, were isolated. The KiSV-MC DNA library was rescreened with cDNA-2 to isolate cDNA-14. The three cDNAs (designated cDNA-2, cDNA-4, and cDNA-14) were then digested with HindIII and cloned into the BamHI site of pSV-β-gal (Amersham Corp.) and sequenced by the deoxyxil chain termination method of Sanger et al. (33). Both strands of cDNA-2 were sequenced.

DNA and RNA Blot Analysis-Samples (-10 μg/lane) of mouse genomic liver DNA were separately digested for 4 h at 37 °C with BamHI, RflI, HindIII, or SphI. The resulting DNA fragments were resolved on 1% agarose gels and transferred to Zeta-blot (Cuno Inc., Meriden, CT) (34). The DNA blots were analyzed separately using either the full-length cDNA-2 or the 149-base pair (bp) KpnI fragment of cDNA-2. The blots were washed under conditions of high stringency (55 °C; 30 mM NaCl, 3 mM sodium citrate, 0.1% SDS, 1 mM EDTA, and 10 mg/ml of salmon sperm DNA carrier, pH 7.0). Two individual KiSV-MC-derived clones, designated cDNA-2 and cDNA-4, were isolated. The KiSV-MC DNA library was rescreened with cDNA-2 to isolate cDNA-14. The three cDNAs (designated cDNA-2, cDNA-4, and cDNA-14) were then digested with HindIII and cloned into the BamHI site of pSV-β-gal (Amersham Corp.) and sequenced by the deoxyxil chain termination method of Sanger et al. (33). Both strands of cDNA-2 were sequenced.
Characterization of a Mouse Mast Cell Protease

Protease (as determined by direct amino acid sequencing of the isolated protein) lacks the 2 glutamic acid residues carboxyl-terminal to the predicted cleavage site of the signal peptide, indicating that the enzyme is probably translated in a "pre-pro" form. The Mr of the mature form of the protein is 24,700, and it consists of 224 amino acids. A potential N-linked glycosylation site is present at amino acid residue 24. Based on the number of basic (Arg + Lys = 27) and acidic (Asp + Glu = 20) amino acids, the mature form of the mouse mast cell enzyme is basic in charge at pH 7.4. A comparison of the deduced amino acid sequence of cDNA-2 with the amino acid sequences of other rat and mouse mast cell secretory granule proteases is shown in Fig. 2. The deduced amino acid sequence of the mature form of the mouse mast cell protease MMCP-2 was found to be 65% homologous to MMCP, 63% homologous to RMCP-I, and 60% homologous to RMCP-II. As in the case of other serine proteases, MMCP-2 possesses the charge relay system of histidine, aspartic acid, and serine at amino acid residues 45, 99, and 182, respectively. The mature protein contains 6 cysteine residues, and thus MMCP-2 has no more than three intrachain disulfide bonds.

DNA and RNA Blot Analyses—Samples of mouse genomic liver DNA were digested with a variety of restriction endonucleases, and the DNA fragments were separated in agarose gels and blotted onto Zetabind. When the DNA blots were probed with the full-length cDNA-2 under conditions of high stringency, multiple DNA fragments were detected regardless of which restriction enzyme was used (Fig. 3A). When the same DNA blot was reprobed with the KpnI+3' fragment of cDNA-2 (Fig. 3B), hybridization to a single DNA fragment was seen in each lane, indicating that this 3' fragment could be used as a gene-specific probe.

Blots containing total RNA from KiSV-MC5, from the proximal small intestines of N. brasiliensis-infected mice (where mucosal mast cells are increased markedly) (20, 21), and from the proximal small intestines of uninfected mice were probed with the gene-specific KpnI+3' fragment of cDNA-2. As shown in the representative experiment in Fig. 4A, the 1.0-kb mRNA transcript for MMCP-2 was detected in the intestine of the nematode-infected mouse but not in the intestine of the uninfected control mouse. Similar results were obtained for three other pairs of N. brasiliensis-infected and sham-infected mice. RNA blot analysis of total RNA probed with the gene-specific KpnI+3' fragment of cDNA-2 under conditions of high stringency revealed that the gene for MMCP-2 was not expressed in mouse peritoneal CTMC, DMMC, WEHI-3 cells, 3T3 fibroblasts, or KiSV-infected 3T3 fibroblasts. Nevertheless, when probed with the full-length cDNA-2, a 1.0-kb transcript was detected in RNA from CTMC (Fig. 4B). This latter finding indicates that mouse CTMC express a distinct but homologous serine protease transcript. No hybridization was seen when a blot containing 40 µg of total RNA from protease peptidase-elicted inflammatory cells (obtained from the peritoneal cavities of mice that were infected with S. mansoni) was probed with the gene-specific KpnI+3' fragment of cDNA-2 (data not shown).

**DISCUSSION**

A 28,000 Mr, DFP-binding protein was found to be a major constituent of the secretory granules of KiSV-MC. NH2-terminal amino acid analysis (Fig. 1A) revealed this protein to be novel, and thus we have used the designation MMCP-2 to distinguish it from the only other mouse mast cell serine protease, MMCP, for which an amino acid sequence is known (13, 14). Because a cDNA that encodes RMCP-II (9) hybrid-
FIG. 1. A, NH$_2$-terminal amino acid sequence of a 28,000 M$_r$ protein isolated from KiSV-MC. B, restriction enzyme map and sequencing strategy for cDNA-2, cDNA-4, and cDNA-14. C, nucleotide sequence and deduced amino acid sequence of MMCP-2. The 23-amino acid portion of the deduced amino acid sequence that is boxed corresponds to the amino acid sequence obtained directly from the protein. The two arrows indicate the putative sites for cleavage of the signal peptide (37) and the pro-peptide. Stop represents the stop codon, and asterisks (***), underline the potential glycosylation site. The three charge-relay amino acids are circled. The polyadenylation signal nucleotide sequence is underlined. The numbers on the right and on the left indicate the amino acid and nucleotide positions in the respective sequences. The most 3' nucleotide before the poly(A) tail is displayed in parentheses because it was present in cDNA-2 and cDNA-4, but not in cDNA-14.

A

\begin{align*}
\text{NH$_2$-terminal amino acid sequence} & \quad \text{(10)} \\
\text{Ser-Arg-Pro-Tyr-Met-Ala-Tyr-Leu-Lys-Phen} & \quad \text{(20)} \\
\text{The The Lys} & \quad \text{(30)}
\end{align*}

B

\begin{align*}
\text{cDNA-2} & \\
& \rightarrow \\
& \rightarrow \\
& \rightarrow \\
& \rightarrow \\
& \rightarrow \\
& \rightarrow \\
\text{cDNA-4} & \\
& \rightarrow \\
& \rightarrow \\
& \rightarrow \\
& \rightarrow \\
\text{cDNA-14} & \\
& \rightarrow \\
& \rightarrow \\
& \rightarrow \\
& \rightarrow
\end{align*}

C

\begin{align*}
\text{cDNA-2} & \\
& \rightarrow \\
& \rightarrow \\
\text{cDNA-4} & \\
& \rightarrow \\
& \rightarrow \\
\text{cDNA-14} & \\
& \rightarrow \\
& \rightarrow \\
& \rightarrow
\end{align*}
Characterization of a Mouse Mast Cell Protease

A.

Comparison of the predicted amino acid sequences of the mouse 28,000 M, serine protease (MMCP-2) with MMCP (13), RMCP-I (6), and RMCP-II (3, 9). The amino acid sequences of MMCP-2 (Fig. 1C) and RMCP-II (9) are deduced based on the nucleotide sequences of their respective cDNAs. The MMCP (13) and RMCP-I (6) amino acid sequences are determined from the mature proteins. The numbering is based on the sequence of MMCP-2 with position 1 assigned to the first isoleucine of the mature form of the enzyme.

ized under conditions of low stringency to a 1.0-kb mRNA transcript present in KISV-MC1 (data not shown), a cDNA library was prepared from KISV-MC1 and screened under conditions of low stringency with the rat probe to obtain a 953-bp cDNA and a 956-bp cDNA that encode MMCP-2 (Fig. 1B). A third cDNA (cDNA-14), which contained a poly(A) tail, was isolated when the library was screened using cDNA-2 as the probe. The deduced amino acid sequence of these three cDNAs (Fig. 1C) revealed that MMCP-2 contained the same histidine-aspartic acid-serine charge relay system present in the active site of all serine proteases. Although no stop codon was found in the 5' untranslated region of either cDNA, it was concluded that translation begins at the first ATG codon because it is in the same position as the translation-initiation codon of RMCP-II and the subsequent nucleotide sequence encodes a hydrophobic signal peptide with an amino acid sequence identical to that of RMCP-II (Figs. 1C and 2).

The deduced amino acid sequence of the mouse protease predicts that the pre-pro form of the enzyme is 26,700 M, and consists of 244 amino acids. Based on the -3, -1 rule for cleavage of signal peptides (37), the 18-amino acid signal peptide would be predicted to be removed between amino acid residues -2 and -3, resulting in a pro form of the enzyme that consists of 226 amino acids. Since the NH2-terminal sequence of the mature protein begins with an isoleucine (Fig. 2), the 2 glutamic acids at -1 and -2 (Fig. 1C) are apparently removed from the NH2 terminus of the pro form of the enzyme during its subsequent post-translational processing.

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chymotrypsinogen, the NH₂-terminal isoleucine of the mature protease forms an ion pair with aspartic acid 194, which is adjacent to the “charge relay” serine 186, resulting in activation of the chymotrypsin (3). Since an aspartic acid residue (position 181) is also adjacent to the charge relay serine (position 182) in MMCP-2, it is likely that removal of the di glutamic acid pro-peptide activates this mast cell enzyme. The mature form of the enzyme is predicted to have 224 amino acids and a 24,700 Mᵣ. Since amino acid residue 24 of the mature enzyme is a potential N-linked glycosylation site, it is likely that the mature serine protease has a larger Mᵣ, because it contains an oligosaccharide.

MMCP-2 has a serine residue at position 176 (Fig. 2), which is conserved in RMCP-1 (Fig. 2), rat chymotrypsin B (position 189) (38), and bovine chymotrypsins A and B (position 189 in both) (39). This serine residue is located in the substrate-binding region of the rat and bovine enzymes and confers the preference for hydrophobic residues to be positioned at the site of substrate cleavage (6, 8, 40). In bovine trypsin (39) and rat trypsins I and II (41), the corresponding residue in the substrate-binding region is an aspartic acid, which confers the preference for basic residues to be positioned at the site of substrate cleavage. Thus, it is likely that the substrate preference for MMCP-2 is chymotryptic rather than tryptic.

Based on its deduced amino acid sequence, MMCP-2 has only 3 intrachain disulfide bonds, and the positions of the cysteines are identical to those in MMCP, RMCP-I, and RMCP-II (Fig. 2). The presence of 3 intrachain disulfide bonds is also characteristic of other secretory granule serine proteases from hematopoietic cells such as mouse cystytic T lymphocyte granzymes (42-46) and human neutrophil cathepsin G (47), and thus differs from pancreatic and plasma serine proteases which contain 4 such bonds (3, 38, 39). In addition, MMCP-2 (Fig. 1) and other secretory granule proteases of hematopoietic cells such as RMCP-II, (9), mouse cystytic T lymphocyte granzymes (42-46), and human neutrophil cathepsin G (47), all have acidic amino acid pro-peptides. In contrast, pancreatic serine proteases (39) such as rat chymotrypsin B (38), trypsin I (41), and trypsin II (41) have 15-, 8-, and 8-amino acid pro-peptides, respectively, which are cleaved from the mature protease at either a lysine or an arginine residue.

DNA blot analysis revealed that 3 to 4 mouse genomic DNA restriction enzyme fragments hybridized to the full-length cDNA-2 regardless of which nuclease was used to digest the genomic DNA (Fig. 3A). This finding indicates that there are 3 to 4 genes in the mouse that encode this and homologous proteins or that there are a more limited number of genes which contain introns susceptible to all of these restriction enzymes. Because the KpnI-3′ fragment of MMCP-2 cDNA recognized only one mouse genomic DNA fragment (Fig. 3B), this 3′ fragment of the cDNA could be used as a specific probe for mRNA encoding MMCP-2. RNA blots probed under conditions of high stringency with the gene-specific portion of cDNA-2 indicated that the gene that encodes the 28,000 Mᵣ, serine protease was expressed in KISVC5 and in the small intestines of mice infected with N. brasiliensis (Fig. 4A), where mucosal mast cells are prominent (20, 21). The gene was not expressed in the proximal small intestines of sham-infected mice, or in mouse peritoneal CTMC, BMMC, or WEHI-3 myelomonocytic cells (Fig. 4). The MMCP-2 transcript was also not detected in uninfected or KISV-infected 3T3 fibroblasts, or in peritoneal exudate cells from S. mansoni-infected mice which were predominantly eosinophils, lymphocytes, and macrophages (data not shown). Because hybridization was readily seen when the full-length cDNA-2 was used to probe a blot containing mouse CTMC RNA (Fig. 4B), it was concluded that mouse CTMC express a homologous but distinct serine protease.

Nakano and co-workers (45) have demonstrated, based on histochemical criteria, that BMMC can give rise to both CTMC and mucosal mast cells because both populations are reconstituted in mast cell-deficient (W/Wv) mice by the administration of interleukin-3-dependent BMMC. Because the gene-specific portion of cDNA-2 failed to hybridize to RNA from mouse BMMC or from CTMC (data not shown), the gene for MMCP-2 appears to be expressed relatively late and selectively in the differentiation of mast cell progenitors to the mucosal mast cell type.

The recent chemical and immunochemical characterization of a mouse mucosal mast cell protease (MMCP) (12, 14) that possesses an amino acid sequence distinct (13) from the cloned protease reported in this manuscript (Fig. 2), suggests that mouse mucosal mast cells contain at least 2 serine proteases with chymotryptic-like substrate preferences. The additional finding of a homologous but distinct protease mRNA in CTMC, by hybridization with the full-length cDNA-2 but not its gene-specific portion, argues that mast cells will express a family of related proteases but with a selective distribution to members of a particular mast cell subclass. Although subclass-related distribution of two proteases has been previously recognized in the rat (4, 7), the evidence in the mouse for a larger mast cell serine protease family of genes, and the lack of transcription of the MMCP-2 gene in the relatively immature interleukin-3-dependent BMMC, has implications for the differentiation, function, and heterogeneity of mast cells.

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