Rat Mast Cell Protease 4 Is a β-Chymase with Unusually Stringent Substrate Recognition Profile*

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Activated mast cells release a variety of potent inflammatory mediators including histamine, cytokines, proteoglycans, and serine proteases. The serine proteases belong to either the chymase (chymotrypsin-like substrate specificity) or trypstat (trypsin-like specificity) family. In this report we have investigated the substrate specificity of a recently identified mast cell protease, rat mast cell protease-4 (rMCP-4). Based on structural homology, rMCP-4 is predicted to belong to the chymase family, although rMCP-4 has previously not been characterized at the protein level. rMCP-4 was expressed with an N-terminal His tag followed by an enterokinase site substituting for the native activation peptide. The enterokinase-cleaved fusion protein was labeled by diisopropyl fluorophosphate, demonstrating that it is an active serine protease. Moreover, rMCP-4 hydrolyzed MeO-Suc-Arg-Ala-Tyr-pNA, thus verifying that this protease belongs to the chymase family. rMCP-4 bound to heparin, and the enzymatic activity toward MeO-Suc-Arg-Ala-Tyr-pNA was strongly enhanced in the presence of heparin. Detailed analysis of the substrate specificity was performed using peptide phage display technique. After six rounds of amplification a consensus sequence, Leu-Val-Trp-Phe-Arg-Gly, was obtained. The corresponding peptide was synthesized, and rMCP-4 was shown to cleave only the Phe-Arg bond in this peptide. This demonstrates that rMCP-4 displays a striking preference for bulky/aromatic amino acid residues in both the P1 and P2 positions.

Both types of rodent mast cells are activated by antigen cross-linking of surface-bound IgE and respond by rapidly releasing mediators stored in their granules. The inflammatory mediators include histamine, proteoglycans, prostaglandins, cytokines, and neutral proteases (4). Mast cells are a particularly rich source of serine proteases belonging to the trypstat family, the chymase family, and the recently identified mMCP-8 family. Proteases belonging to the mMCP-8 family have as yet unknown substrate specificity. The trypstat family of proteases cleaves their substrate at the C-terminal side of basic amino acids (Arg and Lys), whereas the chymase family has chymotrypsin-like substrate specificity, i.e. they cleave peptides after aromatic amino acids (Phe, Tyr, and Trp). Based on phylogenetic relationships, the chymase family can be further subdivided into two groups, the α- and the β-chymases (5). In nonrodent mammals only a single α-chymase can be found, whereas in rodents several β-chymases are expressed in addition to the α-chymase. At least 18 mammalian α- and β-chymases have been identified and cloned (6). However, only a few of them have been characterized in detail with regard to structure and cleavage specificity.

Five of the ten rat mast cell proteases (rMCP-1, rMCP-2, rMCP-3, rMCP-4, and rMCP-5) are considered to display chymase activity (7). rMCP-5 (initially designated as rMCP-3) is the rat α-chymase and the homologue to the single human chymase found so far. The rat mast cell β-chymases rMCP-1 and rMCP-2, expressed by connective tissue mast cells and MMC, respectively, have been extensively investigated with regard to tissue distribution, charge, heparin binding, substrate specificity, inhibitor susceptibility, and structure (7–10). Recent data suggest that MMC mediators, in particular rMCP-2, directly induce physiological changes in the gastrointestinal tract, such as an increase in epithelial permeability (11). This results in increased leukocyte migration and microbial product influx that potentially enhances the inflammatory response.

rMCP-3 and rMCP-4 (GenBank™ accession numbers U67888 and U67907, respectively) were initially cloned from the rat mucosal mast cell line RBL-1 (7) and group phylogenetically with the β-chymases. Both rMCP-3 and rMCP-4 have been suggested to be MMC proteases, with detectable mRNA levels primarily found during parasitic infections (12). However, neither rMCP-3 nor rMCP-4 has been characterized at the protein level. Chymases utilize extended substrate-binding sites to discriminate between substrates. Additional understanding of the biological role of a protease may be obtained by a detailed analysis of its substrate specificity. In the present article, rMCP-3 and rMCP-4 were characterized at the protein level.

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study, rMCP-4 has been characterized with regard to activity, heparin binding, and extended substrate specificity.

**EXPERIMENTAL PROCEDURES**

**Subcloning, Expression, and Purification of Recombinant rMCP-4** — The rMCP-4 expression construct was obtained by PCR amplification using full-length cDNA (7) as template. For purification purposes the 5'-primer contained a sequence encoding six histidine residues. The 5'-primer also introduced an enterokinase susceptible peptide (Asp-Asp-Asp-Lys) in replacement of the natural activation peptide. The enterokinase cleavage site enables subsequent removal of the histidine residues and thereby enables activation of the protease. The PCR product was inserted into the pCPE-Pu2 vector (13), and the nucleotide sequence of the final product was confirmed using the Thermo Sequenase-radiolabeled terminator cycle sequencing kit (Amersham Biosciences) and vector specific primers. Human embryonic kidney cells, 293-EBNA, were transfected with the pCPE-Pu2/rMCP-4 construct as previously described (13). Selection was initiated by the addition of 5 µg/ml puromycin to the cell culture medium (Dulbecco's modified Eagle's medium supplied with 10% fetal calf serum, 2 mM L-glutamine, and 50 µg/ml gentamicin). After 1 week of selection, the level of puromycin was decreased to 0.5 µg/ml. The conditioned medium was collected and centrifuged to collect cell debris, followed by the addition of 0.5 ml of Ni-NTA-agarose beads (Qiagen). After 6 h of incubation at 4 °C on a shaker, the beads were pelleted by centrifugation and transferred to 10-ml PolyPrep Chromatography columns (Bio-Rad). The beads were washed with PBS (pH 7.2) containing 1 M NaCl and 0.1% Tween 20, and the column was eluted with 100 mM imidazole in PBS.

Protein purity and concentration was determined on SDS-PAGE. The samples were mixed with sample buffer and 5% β-mercaptoethanol and run on a 12% gel. To visualize protein bands, the gel was stained with Coomassie Brilliant Blue.

**Activation and Active Site Titration of rMCP-4** — Recombinant rMCP-4 was digested for 5 h with EKMax™ (Invitrogen) enterokinase, one unit/10 µg of recombinant protease. A 10-ml PolyPrep Chromatography column containing 0.2 ml of heparin-Sepharose (Amersham Biosciences) was equilibrated with PBS (pH 7.2). 10 µg of EK-cleaved rMCP-4 in 125 µl of PBS (pH 7.2) was applied to the column, followed by washing with PBS (0.15 M NaCl) and stepwise elution with increasing NaCl concentrations from 0.25 to 1.15 M. The flow-through and eluted fractions were assayed by the chromogenic substrate S-2586 and run on SDS-PAGE to estimate the protein content.

Initial experiments showed that rMCP-4 bound to heparin-Sepharose, and the octapeptide (2-3) was eluted with 0.5 M NaCl. To remove the heparin-Sepharose, the column was washed with PBS (pH 7.2) and equilibrated with 1 M NaCl, 0.1% Tween 20 in PBS, pH 7.2. This was repeated until the flow-through was free of heparin. The purified EK-cleaved rMCP-4 was then dialyzed against PBS (pH 7.2) and stored at 4 °C for future use.

**Testing Phage Display Results in a Synthetic Peptide** — To verify that the sequences from the phage display selections were indeed substrates for the rMCP-4 protease, 24 h after phage addition, the bacteria lysed, and the phage sublibrary was added to fresh enterokinase-cleaved rMCP-4. After binding and washings, the sublibrary, a new round of selection was started. Following six rounds of selection, 35 plaques were arbitrarily isolated from LB plates, and each dissolved in phase display buffer (100 mM DFP (PerkinElmer Life Sciences) in 0.1% Tween 20 in PBS containing 0.5 M NaCl, 0.1% Tween 20) and plated on an SDS-PAGE gel. The phages lacking the histidine tag, 15 µl of fresh Ni-NTA-agarose beads were added to the phage suspension, and the mixture was agitated for 15 min followed by centrifugation to recover the supernatant. 10 µl of the supernatant was used to determine the amount of detached phases in each round of selection. The remaining 290 µl of the supernatant was added to a 10-ml culture (OD 0–0.61) of Escherichia coli (BLT5615). After amplification, the PCR fragments were purified using the QIAquick PCR purification kit (Qiagen). The purified PCR fragments were then sequenced using the Thermo Sequenase-radiolabeled terminator cycle sequencing kit (Amersham Biosciences).

**In a control experiment, to verify the completeness and functionality of the library, 1.5 µl (1 unit) of thrombin (Sigma) was used to pan the library, because the substrate specificity for thrombin is known (15). The protocol for the selections with thrombin was the same as for rMCP-4, except that the incubation time was decreased to 2 h, and only four rounds of selection were performed.**

**Testing Phase Display Results in a Synthetic Peptide** — To verify that the sequences from the phase display selections were indeed substrates for rMCP-4, and to confirm the cleavage site (P1–P1’ amino acids), a synthetic peptide was constructed from the aligned sequences. The peptide substrate, NH₂-LYWFGRG-OOOG (purchased from Åke Engström, Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden), was dissolved in 10 mM Tris-HCl buffer (pH 7.5). After 24 h of incubation, the samples were assayed by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue, incubated for 1 h in 1 M sodium salicylate, and dried in a vacuum dryer. To analyze the amount of labeled DFP covalently bound to the protease active site, an x-ray film was exposed to the gel for 2 weeks.

**Determination of Cleavage Recognition Profile by a Phage Display Approach** — A library of substrate peptides was generated by synthesizing degenerate oligonucleotides with the nucleotide sequence AATTCTCTC-ACTCAGGGGC-(NNK₆)₆-CATCACCACATCATAA (N represents any nucleotide, and K represents T or G) encoding a random nonamer followed by a histidine tag. The oligonucleotides were introduced into the coding region of the T7 phage capsid protein in the T7Select 1–1 vector (Novagen) and transferred to M13K07 packaging to produce M13K07/H9262 as helper bacteriophage to Cwirla et al. (14). The vector constructs were packaged into phage particles in vitro. The total number of T7 clones after packaging was 5.3 × 10⁷ plaque forming units. The phage library was amplified to a titer of 2 × 10⁷ plaque forming units/ml in the BLT5615 bacterial strain. Amplification is necessary for the expression of His-tagged inserts and their display on the surface of the phage particles. The His-tagged capsid protein is produced in very low numbers and is located on the upstream partial deletion of the capsid protein promoter region in the T7Select 1–1 vector. Hence, a His-tagged capsid protein is present on at least 10% of the assembled phage particles. The native capsid protein is expressed by the host bacterial strain, BLT5615, upon isopropyl-1-thio-β-D-galactopyranoside induction.

An aliquot of the amplified phages (~10⁶ plaque forming units) were added to 100 µl of Ni-NTA-agarose beads for 2 h while rotating gently. Unbound phages were removed by washing 10 times with 1.5 ml of 1 M NaCl, 0.1% Tween 20 in PBS, pH 7.2, further washed twice in 1.5 ml of PBS, finally resuspended in 100 µl of PBS. A control elution of the phages with 100 µl of 500 mM imidazole concluded that at least 1 × 10⁸ phages were attached to the matrix after washing. rMCP-4 was activated as previously described in the absence of heparin and then subsequently incubated with Ni-NTA-Sepharose. Phage elution from Ni-NTA-PBS containing 0.5 M NaCl, heparin was added to a 1:5 mass ratio of protease to heparin. The selection was started by adding 0.5 µg of protease (200 nM) or buffer without protease as a control to the tubes with the resuspended beads. The protease was allowed to digest susceptible phages at 37 °C overnight with gentle agitation. To recover released phages, the Ni-NTA-agarose beads were pelleted by centrifugation, and the phages in the supernatant were removed in a total volume of 3 × 100 µl of PBS (pH 7.2). To ensure that all recovered phages lacked the histidine tag, 15 µl of fresh Ni-NTA-agarose beads were added to the phage suspension, and the mixture was agitated for 15 min followed by centrifugation to recover the supernatant. 10 µl of the supernatant was used to determine the amount of detached phages in each round of selection. The remaining 290 µl of the supernatant was added to a 10-ml culture (OD 0–0.61) of Escherichia coli (BLT5615). The bacteria had been induced with 100 µM of IPTG and 100 µl of 1-MCl/1-phio-β-D-galactopyranoside 30 min before phage addition to ensure production of the phage capsid protein. Approximately 2 h after phage addition, the bacteria lysed, and the phage sublibrary was added to fresh agarose beads. After binding and washing the sublibrary, a new round of selection was started. Following six rounds of selection, 35 plaques were arbitrarily isolated from LB plates, and each dissolved in phase display buffer (100 mM DFP (PerkinElmer Life Sciences) in 0.1% Tween 20 in PBS containing 0.5 M NaCl, 0.1% Tween 20) and plated on an SDS-PAGE gel. The phages lacking the histidine tag, 15 µl of fresh Ni-NTA-agarose beads were added to the phage suspension, and the mixture was agitated for 15 min followed by centrifugation to recover the supernatant. 10 µl of the supernatant was used to determine the amount of detached phases in each round of selection. The remaining 290 µl of the supernatant was added to a 10-ml culture (OD 0–0.61) of Escherichia coli (BLT5615). After amplification, the PCR fragments were purified using the QIAquick PCR purification kit (Qiagen). The purified PCR fragments were then sequenced using the Thermo Sequenase-radiolabeled terminator cycle sequencing kit (Amersham Biosciences).

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enzyme. Enzymatic activity in the eluted fractions was measured with the chromogenic substrate S-2586. rMCP-4 was mixed with pig mucosal heparin at different mass (w/w) ratios. The enzymatic activity was measured with S-2586.

RESULTS

Expression, Purification, and Analysis of Recombinant rMCP-4—The coding region for rMCP-4 was PCR-amplified using the corresponding full-length cDNA clone as template. The construct contained an N-terminal His$_6$ tag followed by an EK site that replaced the natural activation peptide. The resulting fragment of 711 bp was inserted in the pCEP-Pu2 vector in frame with the BM40 signal sequence, which is a secreted protein, acidic and rich in cysteine), also referred to as osteonectin. The pCEP-Pu2 vector containing the protease construct was introduced into the 293-EBNA cell line by LipofectAMINE transfection. Recombinant rMCP-4 present in the culture medium was purified by Ni-NTA affinity chromatography and was subjected to enterokinase digestion. Many of the various isolated chymases show binding to heparin, mediated by positively charged amino acid residues located at the chymase surface (16–18). Experiments were therefore carried out to determine whether the recombinant rMCP-4 showed affinity for heparin. EK-digested rMCP-4 was loaded on columns of heparin-Sepharose, which were eluted stepwise with buffers containing increasing concentrations of NaCl. These experiments showed almost 100% binding of rMCP-4 to heparin-Sepharose at 0.15 M NaCl and elution of the protein at ~0.35 M NaCl (Fig. 1A). The heparin-Sepharose affinity was utilized for routine purification of rMCP-4 after the EK step, allowing separation of EK and other impurities from the EK-digested mature rMCP-4 protein.

The molecular mass of rMCP-4 before EK digestion, as determined by the migration on SDS-PAGE, was estimated to be 28 kDa (Fig. 2A). After enterokinase digestion a molecular mass of 26 kDa was observed for the main chymase found in rat connective tissue mast cells, rMCP-1 (19). rMCP-4 was indeed shown to hydrolyze S-2586. S-2586 hydrolysis was strongly enhanced by heparin in a dose-dependent manner and was barely detectable at suboptimal heparin concentrations (Fig. 1B). The specific activity toward S-2586 (at 1 mM concentration of S-2586) was ~60 pmol of hydrolyzed S-2586 s$^{-1}$/µg rMCP-4 at a 1:1 protease to heparin (w/w) ratio. Attempts were made to determine $K_m$ and $k_{cat}$ values of rMCP-4 for S-2586. However, no leveling out of the curve corresponding to the substrate cleaving rates was reached at S-2586 concentrations up to 3.5 mM. Above this concentration S-2586 was not soluble, and determination of kinetic parameters was therefore not possible.

The serine protease inhibitor DFP, which covalently binds to the serine residue in the catalytic triad, can be used to detect active serine proteases. EK-digested rMCP-4, with or without heparin, was incubated with $^3$H-labeled DFP as described under "Experimental Procedures." The purified protein sample was then cleaved with enterokinase to remove the purification tag. Subsequently, enterokinase-digested rMCP-4 was purified on heparin-Sepharose to remove the enterokinase. rMCP-4 digested with enterokinase (+EK) and one control sample without enterokinase digestion (–EK) were analyzed by SDS-PAGE (~2.5 µg of protein/lane) followed by staining with Coomassie Brilliant Blue. $B$, binding of DFP to recombinant rMCP-4. Approximately 2 µg of EK-cleaved rMCP-4 (+EK) with (+) or without (–) heparin and the same amount of uncleaved rMCP-4 (–EK) were incubated with $^3$HDFP as described under "Experimental Procedures." The samples were separated by SDS-PAGE (12% polyacrylamide), and $^3$HDFP-labeled bands were visualized by autoradiography.

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Active Site Titration of rMCP-4—EK-digested rMCP-4 was active site-titrated with $\alpha_1$-antichymotrypsin, a protease inhibitor belonging to the serpin family. Close to 100% inhibition of rMCP-4 was obtained at a molar ratio of inhibitor to protease of ~1:1, indicating that essentially all of the recombinant rMCP-4 molecules were correctly folded (Fig. 3).

Determination of Substrate Recognition Profile by Substrate Phage Selection—A phage display analysis was performed to determine the substrate specificity of rMCP-4 in more detail.
The C terminus of the capsid protein 10 of the T7 phage was manipulated to contain a nine-amino acid-long random peptide followed by a histidine tag. The histidine tag was used to anchor the phages to a matrix. Phages with a random peptide that is susceptible to protease cleavage will be detached from the matrix and can therefore be removed and be propagated. The release of phages after each protease digestion step was determined by titration and plating in top agarose. The number of cleaved phages after each selection compared with the control is shown in Fig. 4. After six rounds of selection, the addition of rMCP-4 to the amplified library gave a 20-fold higher release of phages than the control library with buffer only.

After six rounds of selection and propagation, 32 individual phage plaques were picked and sequenced (Table I). A consensus could be seen when aligning the phage sequences, especially at the N-terminal side of the scissile bond. The nomenclature by Schechter and Berger (20) is used to designate the amino acid residues in the substrate cleavage region, where P1–P1′ corresponds to the scissile bond. The obtained sequences (indicated in bold type in Table I) were aligned according to the presumed nomenclature by Schechter and Berger (20) to determine the specificities for rMCP-4, and the cleavage products were separated by HPLC. The major peak (and only visible peak except for the starting peptide and the flow-through of Me₃SO) was determined to be NH₂-Leu-Val-Trp-Phe-Gly-COOH by mass spectroscopy, demonstrating that the synthetic peptide was cleaved exclusively at the Phe-Arg bond (Fig. 6). The specificity toward NH₂-Leu-Val-Trp-Phe-Gly-COOH (substrate concentration, 1 mM) was 24 pmol of hydrolyzed peptide s⁻¹/µg rMCP-4 at a 1:1 prototype to heparin (w/w) ratio. Thus, the specific activity of rMCP-4 was 40-fold higher toward NH₂-Leu-Val-Trp-Phe-Gly-COOH than S-2586 at 1 mM substrate concentration. To determine the Kₘ values of rMCP-4 toward NH₂-Leu-Val-Trp-Phe-Gly-COOH, rMCP-4 was incubated with the peptide at increasing concentrations (0.1–1.0 mM), followed by quantification of formed cleavage products by HPLC analysis as above. Initial experiments showed that incubation of rMCP-4 with NH₂-Leu-Val-Trp-Phe-Gly-COOH (1 mM) gave a linear increase of formed cleavage product up to 1 h (not shown), and incubations were routinely interrupted within the linear phase of the enzymatic reaction (20 min). Based on these experiments, a Kₘ value of 1.6 ± 0.2 mM and a kₘ value of 1.5 ± 0.13 s⁻¹ for NH₂-Leu-Val-Trp-Phe-Gly-COOH were calculated. In contrast with S-2586 (see above), no difference in activity toward NH₂-Leu-Val-Trp-Phe-Gly-COOH could be seen when heparin was excluded.

A search in the SwissProt data base was performed to retrieve potential natural substrate proteins for rMCP-4. A degenerated consensus sequence, based on the results from the phage display experiments was used to perform a search with the ProteinProspector (University of California at San Francisco): P4 (Leu, Val, Trp, Tyr, and Ala), P3 (Val, Thr, Leu,
Biochemical Characterization of rMCP-4

TABLE I
Target sequences for rMCP-4

Amino acid sequences of the randomized nonamer regions present in rMCP-4 susceptible phages are shown. Susceptible phages were sequenced after six rounds of selection with rMCP-4. Phage clones (32) had their random sequence determined by sequencing with T7Select primers. The general structure of the amino acid sequences in the phage clones is PGGXH HHHHHH, where X indicates the randomized region. The sequences were aligned hypothetically into a P4–P2 consensus (shown in bold type) where cleavage is proposed to occur between the P1 and P1′ amino acid residue.

| Clone no. | P4 | P3 | P2 | P1 | P1′ | P2′ |
|-----------|----|----|----|----|-----|-----|
| 12        | Y  | I  | W  | F  | L   | G   |
| 13        | W  | I  | W  | Y  | N   | S   |
| 14        | W  | L  | F  | N  | G   | S   |
| 15        | W  | V  | F  | F  | S   | Y   |
| 16        | W  | V  | F  | F  | S   | Y   |
| 17        | W  | V  | F  | F  | S   | Y   |
| 18        | W  | V  | F  | F  | S   | Y   |
| 19        | W  | V  | F  | F  | S   | Y   |
| 20        | W  | V  | F  | F  | S   | Y   |
| 21        | W  | V  | F  | F  | S   | Y   |
| 22        | W  | V  | F  | F  | S   | Y   |
| 23        | W  | V  | F  | F  | S   | Y   |
| 24        | W  | V  | F  | F  | S   | Y   |
| 25        | W  | V  | F  | F  | S   | Y   |
| 26        | W  | V  | F  | F  | S   | Y   |
| 27        | W  | V  | F  | F  | S   | Y   |
| 28        | W  | V  | F  | F  | S   | Y   |
| 29        | W  | V  | F  | F  | S   | Y   |
| 30        | W  | V  | F  | F  | S   | Y   |
| 31        | W  | V  | F  | F  | S   | Y   |
| 32        | W  | V  | F  | F  | S   | Y   |
| 33        | W  | V  | F  | F  | S   | Y   |
| 34        | W  | V  | F  | F  | S   | Y   |
| 35        | W  | V  | F  | F  | S   | Y   |
| 36        | W  | V  | F  | F  | S   | Y   |

DISCUSSION

The large amounts of neutral serine proteases synthesized and stored by mast cells suggest an important role for these proteases in mast cell function. Some of the suggested functions of these proteases include activation of matrix metalloproteinases, stimulation of interleukin-8 release along with recruitment of neutrophils, angiotensin I conversion to angiotensin II (human chymase), angiotensin I degradation (rMCP-1), cleavage of vasoactive intestinal peptide, and inactivation of inflammatory neuropeptides (4, 5, 22). Several chymases from different species have been characterized previously, and they demonstrate wide variations with regard to charge, proteolytic binding properties, catalytic efficiency, and resistance to inhibitors. This indicates that the various related chymases each have different roles in mast cell function. In rodents, the expression pattern for chymases varies with mast cell subtype and location, further supporting distinct functions of the different chymases.

In this study, rat mast cell protease-4 was produced in a mammalian expression system. rMCP-4 is an active protease, as demonstrated by cleavage of a chromogenic chymotrypsin substrate and by binding of [3H]DFP, a potent serine protease inhibitor. Further, the expression system produced essentially 100% correctly folded enzyme, as indicated by active site titration of rMCP-4 with α1-antichymotrypsin.

Recombinant rMCP-4 bound to heparin with moderate affinity, and this interaction was essential to achieve hydrolysis of S-2586. This may be in agreement with previous findings for rMCP-1, where heparin was shown to stimulate S-2586 hydrolysis by lowering the Km for this substrate (19). Interestingly, only the cleavage of positively charged substrates, such as S-2586 (Arg at the P3 position) was stimulated by heparin, and it was suggested that heparin acts by masking positively charged groups on the chymase surface, thereby facilitating binding of positively charged substrates. However, whereas the stimulating effect of heparin on rMCP-1 was relatively modest, the presence of heparin is absolutely necessary for detection of rMCP-4-catalyzed hydrolysis of S-2586. This may reflect the presence of positively charged amino acid residues in the vicinity of the active site of rMCP-4, which cause strong repulsion toward positively charged substrates unless neutralized by binding to polyanions such as heparin. It is important to note that even in the presence of heparin, the activity of rMCP-4 is much lower (~700 times) than the activity of rMCP-1 toward the same substrate. This may reflect the highly stringent substrate specificity of rMCP-4 and that the structure of S-2586 differs significantly (except for the P1 position) from the consensus for recognition by rMCP-4 (see below).

Although chymases hydrolyze peptide bonds preferably after Phe or Tyr residues, the extended substrate specificity, and hence the preferred cleavage sites and target protein, can differ. To characterize the extended substrate specificity for
rMCP-4, a library of random peptides displayed on the T7 phage was screened for protease susceptibility. The phage display analysis revealed that rMCP-4 had surprisingly strict substrate specificity, even when using quite large amounts of protease and relatively long incubation times. The alignment of 32 sequenced phage clones revealed a consensus P4 – P2/H11032, Leu-Val-Trp-Phe-Arg-Gly. The corresponding peptide was synthesized, and this substrate was hydrolyzed 40 times more efficiently than S-2586 in the presence of heparin.

When comparing the results in the phage display analysis of rMCP-4 with the substrate specificity analysis for rMCP-2 performed by Powers et al. (8), several features of these enzymes were shown to be shared. The most preferred P3 amino acid for rMCP-2 was shown to be Val, followed by Leu, Met, Thr, and Phe, a result that correlates well with the phage display result for rMCP-4. Because no P2 aromatic amino acids were included in the rMCP-2 study, it is not possible to determine whether rMCP-2 and rMCP-4 have similar P2 residue preferences. The study by Powers et al. (8) also showed that the favored P1 residue was Phe. When substituting P1 Phe with Tyr, a 10-fold decrease of $k_{cat}/K_m$ was observed, and substituting to Trp resulted in a 100-fold lower $k_{cat}/K_m$. It was recently noted that the bovine chymotrypsin A and B differ in their catalytic activity toward substrates with P1 Trp (23). Bovine chymotrypsin A cleaved substrates with Trp or Phe with the same catalytic activity, whereas the B type chymotrypsin only cleaved P1 Phe substrates efficiently (23). This discrimination of substrates was shown to depend on a single amino acid, Ala226 in chymotrypsin B and Gly 226 in chymotrypsin A. By producing a chymotrypsin B A226G mutant, it was shown that the B type chymotrypsin could gain the same properties as that of the A type, i.e. to cleave substrates with P1 Trp. rMCP-4 has a high preference for P1 Phe or Tyr. Chymases generally have an Ala residue at position 226 (chymotrypsin numbering), but an Ile residue occupies position 226 in rMCP-4. This amino acid might restrict the S1 pocket even more and block activity against P1 Trp substrates.

In vitro, the substrate specificity of a protease might be quite well defined. However, in vivo, other factors such as localization and the expression level of the protease as well as substrate availability must be taken into consideration. To identify candidate in vivo substrates for rMCP-4, data base searches were...
made based on the consensus sequence for substrate recognition by rMCP-4. This strategy identified several potential substrate proteins for rMCP-4. One of these was procollagen C-proteinase enhancer protein. This protein potentiates the activity of procollagen C-proteinase, which removes the C-terminal propeptide of procollagen. The potential cleavage site for rMCP-4 is located in the first (most N-terminal) CUB domain, which is the motif with the enhancing capability. Thus, its cleavage by rMCP-4 may result in decreased amounts of collagen present at the site of mast cell degranulation. Another potential substrate in vivo may be plasminogen activator inhibitor-1. Destruction of plasminogen activator inhibitor-1 by rMCP-4 could lead to more plasmin being formed, which in turn may increase the proteolytic degradation of the extracellular matrix and result in increased cell migration and invasion. Further, the serine protease protein C was identified as a potential substrate for rMCP-4. Active protein C functions as a feedback inhibitor in the blood-clotting cascade. Inactivation of protein C could thus result in enhanced coagulation at sites of rMCP-4 release.

The biological function of the MMC proteases is uncertain. However, when we consider the large amounts of proteases stored within the MMC granules and their release under inflammatory conditions, it is likely that they fulfill an important role during MMC-mediated defense reactions in the gut. Direct evidence for involvement of MMC proteases in host defense was recently obtained by using a mouse strain with an engineered receptor type III, rat P27645 (218–221).

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**TABLE II**

**Potential substrates for rMCP-4**

| Target sequence | P4 | P3 | P2 | P1 | P1' | P2' |
|-----------------|----|----|----|----|----|----|
| V               | V  | F  | F  | R  | G  |    |
| L               | L  | W  | Y  | S  | G  |    |
| V               | M  | Y  | F  | N  | G  |    |
| V               | V  | Y  | F  | N  | G  |    |
| V               | V  | W  | Y  | S  | S  |    |
| A               | L  | Y  | F  | N  | G  |    |
| L               | V  | W  | F  | H  | A  |    |

Protein C precursor, rat P31394 (406–411)
Procollagen C-proteinase enhancer protein, rat O08628 (142–147)
Coagulation factor V, human P12259 (2015–2020)
TFG-β receptor type III, rat P26342 (518–527)
Cysteine-rich secretory protein-3, human P54108 (142–147)
Plasminogen activator inhibitor-1, rat P20961 (191–196)
Low affinity Ig γ Fc region receptor III, rat P26465 (218–221)
Rat Mast Cell Protease 4 Is a β-Chymase with Unusually Stringent Substrate Recognition Profile
Ulrika Karlson, Gunnar Pejler, Gunnar Fröman and Lars Hellman

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