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The Tryptase, Mouse Mast Cell Protease 7, Exhibits Anticoagulant Activity *in Vivo* and *in Vitro* Due to Its Ability to Degrade Fibrinogen in the Presence of the Diverse Array of Protease Inhibitors in Plasma*

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Mouse mast cell protease (mMCP) 7 is a tryptase of unknown function expressed by a subpopulation of mast cells that reside in numerous connective tissue sites. Because enzymatically active mMCP-7 is selectively released into the plasma of V3 mastocytosis mice undergoing passive systemic anaphylaxis, we used this *in vivo* model system to identify a physiologic substrate of the tryptase. Plasma samples taken from V3 mastocytosis mice that had been sensitized with immunoglobulin (Ig) E and challenged with antigen were found to contain substantial amounts of four 34–55-kDa peptides, all of which were derived from fibrinogen. To confirm the substrate specificity of mMCP-7, a pseudozymogen form of the recombinant tryptase was generated that could be activated after its purification. The resulting recombinant mMCP-7 exhibited potent anticoagulant activity in the presence of normal plasma and selectively cleaved the α-chain of fibrinogen to fragments of similar size as that seen in the plasma of the IgE/antigen-treated V3 mastocytosis mouse. Subsequent analysis of a tryptase-specific, phage display peptide library revealed that recombinant mMCP-7 preferentially cleaves an amino acid sequence that is nearly identical to that in the middle of the α-chain of rat fibrinogen. Because fibrinogen is a physiologic substrate of mMCP-7, this tryptase can regulate clot formation and fibrinogen/integrin-dependent cellular responses during mast cell-mediated inflammatory reactions.

Tryptases are major constituents of the secretory granules of mast cells in the mouse (1–4), rat (5–7), dog (8), gerbil (9), and human (10–13). In the mouse, two tryptases, designated mouse mast cell protease (mMCP)[1]-6 and mMCP-7, have been identified whose overall amino acid sequences are 71% identical. Although their physiologic substrates remain to be determined, the tryptase family of mouse mast cell proteases has been implicated in the pathobiology of certain airway responses elicited by the high affinity receptor for immunoglobulin (Ig) E. Tryptase inhibitors block antigen-induced airway constriction and tissue inflammatory responses in sheep sensitized with *Ascaris suum* (14). In addition, linkage analysis (15) has implicated the region of chromosome 17 where the mMCP-6 and mMCP-7 genes reside (16, 17) as one of the candidate loci for the inheritance of intrinsic airway hyper-responsiveness.

mMCP-6 and mMCP-7 are stored in granules in their mature, enzymatically active forms ionically bound to the glycosaminoglycan side chains of serglycin proteoglycans (1). Although mMCP-6 and mMCP-7 both have an overall negative charge at neutral pH, the two exocytosed tryptases differ in their ability to dissociate from serglycin proteoglycans outside the mast cell (18). Thus, they are metabolized quite differently in mice undergoing passive systemic anaphylaxis. Tongue, skin, spleen, and heart mast cells of normal BALB/c mice and spleen and liver mast cells of V3 mastocytosis mice all contain substantial amounts of mMCP-6 and mMCP-7 in their secretory granules. Ten min after antigen is administered to IgE-sensitized mice, protease-proteoglycan macromolecular complexes appear in the extracellular matrix adjacent to the activated tissue mast cells. These complexes can be readily stained by anti-mMCP-6 Ig but not by anti-mMCP-7 Ig. In V3 mastocytosis mice sensitized with IgE and challenged with antigen, exocytosed mMCP-7 rapidly makes its way into blood, where it circulates for >1 h. This plasma form of mMCP-7 has an intact N terminus. Moreover, it is properly folded, enzymatically active, and not degraded. Despite the fact that as much as 10% of the proteins in blood are protease inhibitors (19), plasma-localized mMCP-7 does not rapidly form covalent complexes with any protease inhibitor in the blood of V3 mastocytosis mice.

Modeling and site-directed mutagenesis of recombinant pro-mMCP-7 suggested that the natural form of the tryptase selectively dissociates from the macromolecular complex when exocytosed into a pH 7.0 environment because the glycosaminoglycan-binding domain on its surface consists predominately of a cluster of His residues rather than predominately Lys and Arg residues (20), as found in mMCP-6 (18) and all mast cell chymases (21). The prolonged retention of exocytosed mMCP-6 in the extracellular matrix around activated tissue mast cells suggests a local action, whereas the rapid dissipation of mMCP-7 from tissues and its poor ability to be inactivated by

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[1] The abbreviations used are: mMCP, mouse mast cell protease; EK, enterokinase; FLAG, the peptide whose amino acid sequence is Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; Ig, immunoglobulin; PAGE, polyacrylamide gel electrophoresis; *p*NA, *p*-nitroanilide; and pIII, the protein encoded by the phage gene designated gIII.

circulating protease inhibitors suggests that this tryptase cleaves proteins located at more distant sites.

Although mast cell tryptases have been purified from different species in an attempt to deduce their protein substrates, the number of mature mast cells that can be isolated from a mouse is inadequate to obtain enough mMCP-7 for in-depth study. No mast cell has been found that expresses just mMCP-7. For example, the mMCP-7$^+$ mast cells in the skin of the BALB/c mouse also express substantial amounts of similar sized mMCP-4, mMCP-5, mMCP-6, and carboxypeptidase A (22). Because all of these proteases are catalytic at neutral pH, the nearly impossible task of removing 100% of the other mMCPs from the starting preparations of mast cell lysates has prevented definitive studies that address the function of mMCP-7. A contributing problem is the fact that serine proteases like mMCP-7 tend to undergo inactivation during their isolation.

We now show that fibrinogen is preferentially degraded when mMCP-7 is released into the circulation of V3 mastocytosis mice that are undergoing systemic anaphylaxis. We show that recombinant mMCP-7 is not readily inhibited by any protease inhibitor in plasma, that this tryptase degrades fibrinogen in a manner comparable to that seen in the V3 mastocytosis mouse, and that the enzymatic activity of this tryptase is not heparin-dependent. Based on these *in vivo* and *in vitro* findings, we conclude that fibrinogen is a physiologic substrate of mMCP-7.

EXPERIMENTAL PROCEDURES

Identification of a Plasma Protein in the V3 Mastocytosis Mouse That Undergoes Rapid Degradation during Passive Systemic Anaphylaxis—V3 mastocytosis mice were created and systemically sensitized intraperitoneally with ~200 μg of anti-trinitrophenol IgE, as described (18, 23). Approximately 24 h later, ~300 μl of Hank's balanced salt solution alone or containing 10–1000 μg of trinitrophenol-bovine serum albumin was injected intraperitoneally into each mouse. Twenty min after antigen administration, 100–500 μl of blood was obtained from the retroorbital plexus with a Pasteur pipette pretreated with an anticoagulant (either 25 USP units of heparin glycosaminoglycan (Elkins-Sinn, Cherry Hill, NC) or 10 mM EDTA). After a 4-min centrifugation step at ~10,000 × *g* and at 4 °C, each 15-μl sample of plasma was subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The four prominent ~34–55-kDa peptides that preferentially appeared after the sensitization and antigen challenge were transferred to Immobilon-P membranes (Millipore, Bedford, MA) and subjected to N-terminal amino acid analysis.

Expression of Pro-enterokinase (EK)-mMCP-7 and Pro-EK-mMCP-7-FLAG in Insect Cells—Using a polymerase chain reaction approach, an oligonucleotide (5′-GACGACGATGACAAG-3′) encoding the EK-susceptible peptide Asp-Asp-Asp-Asp-Lys was inserted into the mMCP-7 cDNA (3) between the domain that encodes the pro-peptide and the N terminus of the mature tryptase. EK is a highly specific enzyme that cleaves the Lys-Ile bond in its Asp-Asp-Asp-Lys-Ile recognition motif (24). Because Ile is the essential N-terminal amino acid of mature mMCP-7 and because EK is a relatively stable enzyme at pH 5.0, it was anticipated that the secreted recombinant pseudozymogen could be activated under conditions where the generated tryptase would have very little enzymatic activity until the pH is raised to 7.0. The FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys), which consists of the EK-cleavage sequence C-terminal of a 3-residue linker, has been used by many to epitope-tag the N or C terminus of recombinant proteins (25). To facilitate the purification of the recombinant pseudozymogen with an anti-FLAG IgG antibody (26, 27), a second construct (pro-EK-mMCP-7-FLAG) was created that also contained the 8-residue FLAG peptide at its C terminus. These two cDNAs were inserted in the correct orientation into the multiple cloning site of pVL1393 (PharMingen, San Diego, CA) downstream of the promoter of the polyhedrin gene, as described for the expression of recombinant pro-mMCP-7 (20).

In each instance, purified plasmid DNA (~5 μg) was mixed with 0.5 μg of linearized BaculoGold™ DNA (PharMingen) and calcium phosphate. The resulting DNA solution was added to 3 × 10^6 adherent *Spodoptera frugiperda 9* insect cells (Invitrogen, San Diego, CA) that were in their log phase of growth, and infected cells were cultured for 7 days at 27 °C in medium (Invitrogen) supplemented with 10% heat-inactivated (56 °C, 30 min) fetal calf serum (Sigma). Recombinant virus particles (≥3 × 10^7) were added to a culture dish containing 6 × 10^6 *Trichoplusia ni* High Five™ insect cells (Invitrogen) in their log phase of growth, and the infected cells were cultured in serum-free, Xpress medium (BioWhittaker, Walkersville, MD). Four days later, the conditioned medium was centrifuged at 1500 × *g* for 15-min at room temperature. Under these conditions, recombinant pro-EK-mMCP-7 and pro-EK-mMCP-7-FLAG were recovered in the supernatants as soluble proteins.

Purification of Pro-EK-mMCP-7 and Pro-EK-mMCP-7-FLAG from Insect Cell-conditioned Medium, and EK Activation of the Recombinant Pseudozymogens—Recombinant pro-EK-mMCP-7 and pro-EK-mMCP-7-FLAG were purified by heparin-Sepharose chromatography, as described for pro-mMCP-7 (20). Alternatively, recombinant pro-EK-mMCP-7-FLAG was purified with a 2-ml column containing the mouse anti-FLAG M2 monoclonal antibody (International Biotechnol Inc., New Haven, CT). This anti-FLAG IgG affinity column was washed with 10 ml of 0.1 M glycine, pH 3.5, followed by 50 ml of 50 mM Tris-HCl and 150 mM NaCl, pH 7.4. After ~200 ml of insect cell-conditioned medium was passed through the affinity column, the resin was washed with 50 ml of the same pH 7.4 buffer. Bound pro-EK-mMCP-7-FLAG was eluted by washing the column with 0.1 M glycine, pH 3.5. The eluate was collected into tubes that contained 0.1 M Tris-HCl, pH 7.0, to minimize acid-mediated denaturation of the recombinant proteins. The final concentration of each recombinant protein was estimated by measuring the absorbence at 280 nm.

Purified pro-EK-mMCP-7 and pro-EK-mMCP-7-FLAG (~100 μg) was separately suspended in ~100 μl of 50 mM sodium acetate and 5 mM calcium chloride, pH 5.2. One μl of a solution containing 310 units of calf intestinal EK (Biozyme Laboratories, San Diego, CA) was added to each, and the mixture was incubated at 37 °C for ~3 h to allow EK to activate the zymogen. The spectrophotometric method of Svendsen and co-workers (28) was used to determine whether or not recombinant mMCP-7 and mMCP-7-FLAG were enzymatically active. A 1-μl sample of each activation mixture was placed in 1 ml of assay buffer (pH 7.4 buffer containing 25 mM sodium phosphate, 1 mM EDTA, and 50 μg/ml of a *p*-nitroanilide (*p*NA) substrate such as tosyl-Gly-Pro-Lys-*p*NA (Sigma)). The change in optical density at 405 nm was determined after a 3–5-min incubation at room temperature. In this assay, 1 unit of enzymatic activity is defined as a change in optical density at 405 nm of 0.001 per min. As noted under "Results," mMCP-7 exhibits an enzymatic activity of 175 to 50 units/μg when tosyl-Gly-Pro-Lys-*p*NA is the test substrate. However, analysis of a phage display peptide library revealed that tosyl-Gly-Pro-Lys-*p*NA is not the optimal substrate of mMCP-7. Thus, it is likely that the recombinant tryptase exhibits greater enzymatic activity against its physiologic substrate(s). One μg of bovine pancreatic trypsin (Sigma) is equivalent to ~350 units when tosyl-Gly-Pro-Lys-*p*NA is the test substrate. With the more widely used substrate N^{α}-benzoyl-L-Arg-ethyl ester, the reference preparation of trypsin employed in our study for comparative purposes has 11 units/μg. The ability of recombinant mMCP-7 and mMCP-7-FLAG to cleave the trypsin-susceptible substrates tosyl-Gly-Pro-Arg-*p*NA, benzoyl-Ile-Glu-Gly-Arg-*p*NA, benzoyl-Phe-Val-Arg-*p*NA, benzoyl-Pro-Phe-Arg-*p*NA, acetyl-Ile-Glu-Ala-Arg-*p*NA, benzoyl-Val-Gly-Arg-*p*NA, and D-Ile-Phe-Lys-*p*NA (Sigma) were also evaluated.

SDS-PAGE/Immunoblotting and N-terminal Amino Acid Analysis—Insect cell-conditioned medium (~20 μl) containing pro-mMCP-7, pro-EK-mMCP-7, pro-EK-mMCP-7-FLAG, or purified EK-activated mMCP-7 (~1 μl) was diluted in SDS-PAGE buffer (1% SDS, 5% β-mercaptoethanol, 0.1% bromphenol blue, and 500 mM Tris-HCl, pH 6.8) and boiled for 5 min before being loaded onto 12% polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie Blue or placed in a Bio-Rad (Richmond, CA) immunoblotting apparatus, and the resolved proteins were transferred for 2–4 h at 200 mA to Immobilon-P membranes in a solution consisting of 20% methanol, 16 mM Tris-HCl, and 120 mM glycine, pH 8.3. For analysis of the resulting protein blots, each membrane was incubated for 1 h in 5% non-fat milk and then for 1 h with a 1:500 dilution of affinity-purified rabbit anti-mMCP-7 Ig (29) in Tris-buffered saline with 0.01% Tween 20 (TBST buffer). After 3 washes in TBST buffer, the blots were incubated for 1 h in a 1:1000 dilution of anti-rabbit IgG alkaline phosphatase conjugate (~1 ng/ml final concentration) in TBST buffer. Immunoreactive proteins were visualized with nitro blue tetrazolium (0.2 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.1 mg/ml) as substrates. For N-terminal amino acid analysis, SDS-PAGE-resolved proteins were electroblotted onto membranes and briefly stained with 0.5% Ponceau S red (Sigma), and the relevant proteins/peptides were subjected to au-

tomated Edman degradation by the Harvard Microchemistry Facility (Harvard Biological Laboratories, Cambridge, MA).

Degradation of Mouse Fibrinogen by Recombinant mMCP-7 in the Presence and Absence of Plasma—Samples (50 μg or more) of purified mouse fibrinogen (Sigma) were suspended in 1 mM EDTA and 25 mM sodium phosphate, pH 7.4, containing 0.2–0.5 μg of recombinant mMCP-7-FLAG (10–25 units corresponding to 0.007–0.014 nmol of tryptase activated with 0.01 units of EK), ~0.5 μg of recombinant pro-EK-mMCP-7-FLAG, or 0.01 units of EK and then incubated at 37 °C for various time periods. The resulting digests were subjected to SDS-PAGE. In one experiment, the N-terminal amino acid sequences of the major fibrinogen fragments in an exhaustive digest were determined after a 3-h treatment of 100 μg of fibrinogen with 120 units of mMCP-7-FLAG. The ability of mMCP-7-FLAG, bovine trypsin, and bovine α-chymotrypsin (Sigma) to digest mouse fibrinogen in the presence and absence of the protease inhibitors found in normal mouse plasma was also evaluated. In these latter experiments, mMCP-7-FLAG (10 units), trypsin (10 units), and chymotrypsin (0.03 μg; an amount equal to that of trypsin on a weight basis) were suspended separately in 4 μl of 1 mM EDTA and 25 mM sodium phosphate, pH 7.4, lacking or containing 2.0–4.0% (v/v) mouse plasma for 30 min at room temperature. Thirty μl of reaction buffer containing 15 μg of fibrinogen was added and the samples were incubated for 1 h at 37 °C. The final fibrinogen:enzyme ratio in each of these experiments was at least 75:1. Comparable amounts of plasma was added to those samples that had not been exposed previously to plasma, and then the digests were subjected to SDS-PAGE. The resulting gels were stained with Coomassie Blue and the extent of digestion of each chain of fibrinogen by mMCP-7 in the presence and absence of plasma was evaluated by measuring the decrease in the optical density of SDS-PAGE separated, Coomassie Blue-stained α-, β-, and γ-chains by means of a densitometer (Molecular Dynamics, Sunnyvale, CA). To demonstrate that the α-chain of mouse fibrinogen is much more susceptible to digestion by mMCP-7-FLAG than either the β- or γ-chains, kinetic experiments also were carried in which 70 units of mMCP-7-FLAG was incubated at 37 °C for 1 min to 17 h with 15 μg of mouse fibrinogen in the presence or absence of 2% normal mouse plasma prior to the SDS-PAGE analysis.

A standard fibrinogenolysis assay (30) was used to detect mMCP-7 anticoagulant activity *in vitro*. Sodium citrate-treated, normal mouse plasma (100 μl/assay) was incubated for 30 min to 1 h at 37 °C in the absence or presence of ~4 μg (~200 units or 0.12 nmol) of EK-activated mMCP-7-FLAG or 10 USP units of heparin (~100 μg of the glycosaminoglycan). The time required for thrombin to clot the samples was then determined with a fibrometer. The plasma concentration of fibrinogen is ~3 mg/ml. Thus, even if 100% of the recombinant pseudozymogen was converted to active enzyme by EK treatment, there is ~75-fold more fibrinogen than mMCP-7 in the assay.

Generation and Screening of a Phage Display Peptide Library That Is Tryptase Specific—The genome of the Ff bacteriophage consists of the 11 genes designated gI to gXI. Although the protein (pIII) encoded by gIII is chymotrypsin-, thermolysin-, and subtilisin-susceptible, it is trypsin-resistant (31). Thus, phage display peptide libraries can give insight into the substrate specificities of certain proteases (32, 33). The N terminus of pIII extends out from the surface of the body of the filamentous phage. By taking advantage of the fact that pIII is trypsin-resistant and exhibits low valency, a phage display peptide library specific for tryptases was generated that encodes a pIII fusion protein containing at its N terminus the FLAG peptide followed by an 8-residue hypervariable peptide. The FLAG peptide was selected as the "tether" ligand so that those phage producing a bioengineered pIII could be readily isolated with the monoclonal anti-FLAG M1 antibody. To create the tryptase-specific library, two complementary single-stranded oligonucleotides (5′-CGGCCGACTACAAGGACGACGATGACAAG$(X)_{12}$A(A/G)G$(X)_9$GC-3′ and 5′-GGCCGC$(X)_9$C(T/C)T$(X)_{12}$CTTGTCATCGTCGTCCTTGTAGTCGGCCGGCT-3′, where "*X*" indicates a random nucleotide) were synthesized such that they could be annealed to one another *in vitro* to form short double-stranded DNAs that each contained *Sfi*I and *Not*I restriction sites at their 5′- and 3′-ends, respectively. Because it was found that recombinant mMCP-7 cleaves tosyl-Gly-Pro-Lys-*p*NA, the library was created such that the fifth residue in the hypervariable domain would be either Arg or Lys. The single-stranded oligonucleotides were mixed in approximately equal concentrations, heated to 94 °C for 1 min, and cooled to room temperature. The resulting double-stranded oligonucleotides were ligated into *Sfi*I/*Not*I-digested phagemid vector pCANTAB-5E (International Biotechnology). *Escherichia coli* (strain TG1), transformed by electroporation with the resulting constructs, were incubated for 1 h at 37 °C in 2 × YT medium (0.09 M NaCl containing 1.7% Bacto-tryptone (Difco Labs, Detroit, MI), and 1% Bacto-yeast extract (Difco Labs), pH 7.0) and 2% glucose. Ampicillin (50 μg/ml) and the M13 helper phage K (~10 phage/bacteria) were added, and the bacteria were incubated at 37 °C for another 1 h to induce the formation of recombinant phage. After the mixture was centrifuged at 2,000 × *g* for 20 min, the pellet was resuspended in 20 ml of 2 × YT medium containing 50 μg/ml ampicillin and 50 μg/ml kanamycin. Infected bacteria were incubated overnight at 37 °C and then subjected to a 20-min centrifugation at 2,000 × *g* to obtain the phage-enriched supernatant.

The resulting phage display peptide library was screened with bovine pancreatic trypsin to determine its suitability for substrate specificity studies. Because phage clones were obtained after two rounds of trypsin treatment that possessed different peptide sequences in the random portion of the pIII fusion protein, the library was screened with recombinant mMCP-7-FLAG. To purify the recombinant phage, 10 ml of the phage-enriched supernatant was added to 2 ml of 20% polyethylene glycol (8 kDa; Sigma) and 2.5 M NaCl and the mixture was incubated at 4 °C for 30 min. After a 30-min centrifugation of the mixture at 10,000 × *g*, the recombinant phage in the pellet were resuspended in 2 ml of 150 mM NaCl, 1 mM $CaCl_2$, and 10 mM sodium phosphate, pH 7.0, and applied to a 1-ml affinity column containing the anti-FLAG M1 monoclonal antibody. The column was washed three times with 10 ml of the same pH 7.0 buffer to remove unbound phage. Recombinant mMCP-7-FLAG or bovine pancreatic trypsin (~50 μg in 200 μl of the pH 7.0 buffer) was added, and the column was sealed and incubated at room temperature for 90 min. After treatment with protease, the column was washed with 2 ml of the pH 7.0 buffer to recover those phage that possessed protease-susceptible pIII fusion proteins. Log phase *E. coli* were infected with the obtained phage to produce phagemid. Bacteria were again grown in 2 × YT medium containing 2% glucose and the phagemid in the bacteria were converted to phage with the addition of helper phage. The selection procedure was repeated one to three additional times to isolate those phage that possessed the most protease-susceptible pIII fusion proteins.

E. coli were infected with phage that were susceptible to either trypsin or mMCP-7-FLAG to generate phagemids. The infected bacteria were seeded onto a plate containing 1.5% agar, 2% Bacto-tryptone, 0.5% Bacto-yeast extract, 2% glucose, 90 mM NaCl, 10 mM $MgCl_2$, and 50 μg/ml ampicillin. Individual clones were isolated and grown overnight at 37 °C in 2 ml of 2 × YT medium containing 2% glucose with 50 μg/ml ampicillin. Samples (50 μl) of the overnight cultures were centrifuged at ~12,000 × *g* for 5 min. The bacteria in the pellets were resuspended in 50 μl of water, boiled for 10 min, and again centrifuged. Each polymerase chain reaction was carried out on 2-μl samples of the supernatant with sense (5′-CCCAGCCGGCCGACTACAAGGACG-3′) and antisense (5′-TGTTCCTTTCTATGCGGCCCAGC-3′) primers. Each of the 35 cycles of the polymerase chain reaction consisted of a 1-min denaturing step at 94 °C, a 1-min annealing step at 60 °C, and a 1-min extension step at 72 °C. The polymerase chain reaction products were subjected to electrophoresis on a 1% agarose gel, and the nucleotide sequences that encode the 8-mer, protease-susceptible peptide domains in the pIII fusion proteins were determined.

RESULTS

Identification of a Plasma Protein in the V3 Mastocytosis Mouse That Undergoes Rapid Degradation during Passive Systemic Anaphylaxis—Relative to V3 mastocytosis mice sensitized with IgE but not challenged with antigen, the plasma from IgE/antigen-treated V3 mastocytosis mice contained large amounts of ~34-, 40-, and 55-kDa peptides, and lesser amounts of an ~42-kDa peptide (Fig. 1). Changes were also seen in at least three proteins whose molecular masses ranged from ~100 to 200 kDa. While we were not able to resolve the larger sized proteins well enough to determine their N termini, the ~34-, 40-, and 42-kDa peptides possessed the same N-terminal amino acid sequence of Thr-Asp-Thr-Glu-Asp-Lys-Gly-Glu-Phe-Leu-Ser-Glu-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg-Val-Val-Glu-Arg. In contrast, the ~55-kDa peptide possessed an N-terminal amino acid sequence of Try-Val-Ala-Thr-Arg-Asp-Asn-Cys-Cys-Ile-Leu-Asp-Glu.

Generation of Pro-EK-mMCP-7 and Pro-EK-mMCP-7-FLAG in Insect Cells, and EK Conversion of the Recombinant Pseudozymogens to Active Tryptases—Insect cells infected with the relevant baculovirus construct secreted substantial

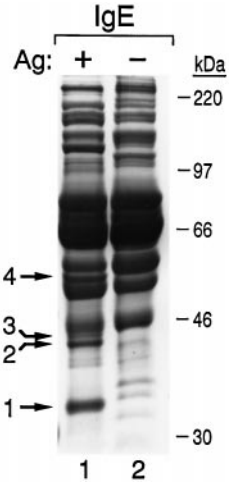

FIG. 1. **SDS-PAGE analysis of the plasma of V3 mastocytosis mice sensitized with IgE and either challenged with antigen (*lane 1*) or not challenged (*lane 2*).** *Arrows* on the *left* indicate the four prominent peptides of ~34, 40, 42, and 55 kDa that increase in concentration in the plasma of IgE/antigen-treated mice. Molecular weight markers are indicated on the *right*.

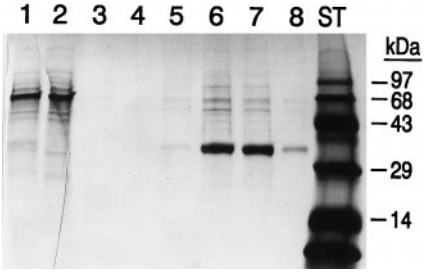

FIG. 2. **SDS-PAGE analysis of pro-EK-mMCP-7-FLAG purified from the insect cell conditioned medium.** SDS-PAGE analysis was carried out on the proteins in the insect cell-conditioned medium that was initially applied to the anti-FLAG IgG column (*lane 1*), the non-bound proteins (*lane 2*), and the bound proteins that were subsequently eluted with 0.1 M glycine, pH 3.5 (*lanes 3–8*). The gel was stained with Coomassie Blue. Molecular weight standards (*ST*) are shown on the *right*. The major ~32-kDa protein recovered in fractions 6 and 7 was recognized by anti-mMCP-7 Ig (data not shown).

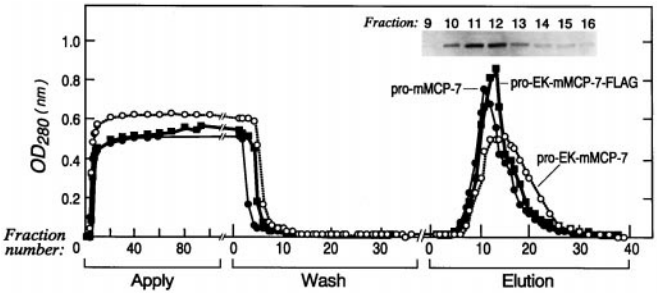

FIG. 3. **Heparin-Sepharose chromatography of varied recombinant forms of mMCP-7.** Conditioned medium (~400 ml) obtained from insect cells infected with baculovirus encoding pro-mMCP-7 (●), pro-EK-mMCP-7 (○), or pro-EK-mMCP-7-FLAG (■) was dialyzed against 0.1 M NaCl, 50 mM sodium acetate, pH 5.0, and then applied to replicate heparin-Sepharose columns (1 × 30 cm) equilibrated in the same buffer. Bound proteins were eluted on a linear gradient with the NaCl concentration in the buffer increasing to 2.1 M. The OD_{280} of the eluate of each 5-ml column fraction is depicted. Twenty microliters of each fraction was subjected to SDS-PAGE. The *inset* (*top right*) depicts the SDS-PAGE immunoblot analysis obtained with anti-mMCP-7 Ig on fractions 9–16 of the gradient eluate of the pro-EK-mMCP-7-FLAG-treated column.

amounts of pro-EK-mMCP-7 and pro-EK-mMCP-7-FLAG into the conditioned medium. These recombinant proteins could be purified from contaminating insect proteins by affinity column chromatography using anti-FLAG Ig (Fig. 2) or heparin-Sepharose (Fig. 3). Both recombinant proteins bound to a heparin-Sepharose column that had been equilibrated in 100 mM NaCl, 10 mM sodium phosphate, pH 5.5. Because they dissociated from the column when the NaCl concentration of the buffer was raised to ~300 mM, analogous to properly folded recombinant pro-mMCP-7, most of the insect cell-derived pro-EK-mMCP-7 and pro-EK-mMCP-7-FLAG secreted into the conditioned media are properly folded. As assessed by SDS-PAGE, both recombinant pseudozymogens decreased in size by ~2 kDa after treatment with EK (data not shown). Amino acid sequence analysis revealed that, after treatment with EK, both recombinant proteins possessed an N-terminal sequence of Ile-Val-Gly-Gly-Gln-*X*-Ala-*X*-Gly-Asn-Lys, which is identical to that of mature mMCP-7 deduced from its cDNA.

Recombinant mMCP-7 and mMCP-7-FLAG cleaved tosyl-Gly-Pro-Lys-*p*NA and tosyl-Gly-Pro-Arg-*p*NA at rates that were one-half to one-seventh as good as that obtained with pancreatic trypsin (Table I). Recombinant mMCP-7 and mMCP-7-FLAG also cleaved benzoyl-Val-Gly-Arg-*p*NA somewhat. However, unlike pancreatic trypsin, neither form of the mast cell tryptase effectively cleaved benzoyl-Ile-Glu-Gly-Arg-*p*NA, benzoyl-Phe-Val-Arg-*p*NA, benzoyl-Pro-Phe-Arg-*p*NA, acetyl-Ile-Glu-Ala-Arg-*p*NA, or D-Ile-Phe-Lys-*p*NA. The amount of tryptase activity was not increased substantially if heparin glycosaminoglycan was present during the EK activation step or during the incubation with the peptide substrates (data not shown). Recombinant mMCP-7 and mMCP-7-FLAG (Fig. 4*A*) possessed optimal enzymatic activity at pH 7.4, and both retained much of their enzymatic activities even after a 5-h incubation at 37 °C in the standard activation buffer (Fig. 4*B*).

Degradation of Mouse Fibrinogen by Recombinant mMCP-7 in the Absence and Presence of Plasma—Recombinant mMCP-7-FLAG was used in subsequent studies because it could be purified more easily by means of the anti-FLAG Ig column, it could be activated without heparin glycosaminoglycan, and its physical and biological properties were similar to those of mMCP-7. After an exhaustive 3-h digestion of mouse fibrinogen with mMCP-7-FLAG (Fig. 5*A, lane 1*), five prominent peptides of ~57, 42, 40, 38, and 34 kDa were obtained. The ~34-, 40-, and 42-kDa peptides possessed the same N-terminal amino acid sequence of Thr-Asp-Thr-Glu-Asp-Lys-Gly-Glu-Phe-Leu. In contrast, the N-terminal amino acid sequence of the 38-kDa peptide was Tyr-Val-Ala-Thr-Arg-Asp-Asn-*X*-*X*-Ile-Leu-Asp-Glu and that of the ~57-kDa peptide was Arg-Lys-Glu-Glu-Pro-(Pro)-Ser-Leu-Arg-Pro-Ala-Pro-Pro. Based on N-terminal amino acid analysis of the three chains of native mouse fibrinogen (Fig. 5*A, lanes 2* and *3*), the 34-, 38-, 40-, 42- and 57-kDa peptides in the digest are derived from the α-, γ-, α-, α-, and β-chains of mouse fibrinogen, respectively. In the absence of normal mouse plasma, mouse fibrinogen actually was more extensively degraded by trypsin and chymotrypsin than by mMCP-7. Nevertheless, neither pancreatic enzyme was able to degrade fibrinogen at 37 °C in the presence of plasma (Fig. 5*C*). Kinetic studies (Fig. 5*B*) revealed that mouse fibrinogen is rapidly cleaved by mMCP-7 and that the α-chain is most susceptible to the tryptase. Densitometric analyses of the SDS-PAGE gels of other experiments revealed that >50% of the α-chains in the samples were cleaved 1 and 3 min after 15 μg of mouse fibrinogen was incubated at 37 °C with 70 units of mMCP-7-FLAG in the absence ($n = 2$) or presence ($n = 1$) of 2.0% serum, respectively (data not shown). In contrast, even in the absence of plasma ($n = 2$), ~3 h of incubation at 37 °C was needed to obtain measurable degradation of the β- and γ-chains of mouse fibrinogen by mMCP-7-FLAG.

Despite the high concentration and diversity of protease

TABLE I

Comparison of the peptidolytic activities of trypsin, mMCP-7, and mMCP-7-FLAG

In each instance, recombinant mMCP-7-FLAG, recombinant mMCP-7, and trypsin (final concentrations of each = 0.4 μg/ml) were incubated separately for 3 min with a pNA substrate (final concentrations of each = 50 μg/ml) and then the optical density at 405 nm was measured. To obtain the activity ratio, the optical density data obtained with one of the recombinant enzymes was divided by the optical density data obtained with trypsin. Depicted are the optical density and the activity ratio data for each enzyme in one of the experiments. Depending on the preparation of recombinant enzyme used in the assay, trypsin cleaves tosyl-Gly-Pro-Lys-*p*NA at a rate 2–7-fold better than either mMCP-7 or mMCP-7-FLAG. Using benzoyl-Val-Gly-Arg-pNA as the substrate in reactions carried out at room temperature, the K_m, k_{cat}, and k_{cat}/K_m values of mMCP-7 and trypsin were 94 and 44 μM, 21 and 15 s^{-}, and 0.23 and 0.34 $\mu M^{-1}s^{-1}$, respectively.

Substrate	Trypsin	mMCP-7-FLAG	Activity ratio	mMCP-7	Activity ratio
Tosyl-Gly-Pro-Lys-*p*NA	0.0921	0.0497	0.54	0.0435	0.47
Tosyl-Gly-Pro-Arg-*p*NA	0.0950	0.0512	0.54	0.0450	0.47
Benzoyl-Val-Gly-Arg-*p*NA	0.1156	0.0155	0.13	0.0100	0.09
D-Ile-Phe-Lys-*p*NA	0.0272	0.0014	0.05	0.0010	0.04
Benzoyl-Phe-Val-Arg-*p*NA	0.0220	0.0009	0.04	0.0002	0.01
Acetyl-Ile-Glu-Ala-Arg-*p*NA	0.1445	0.0005	<0.01	0.0001	<0.01
Benzoyl-Ile-Glu-Gly-Arg-*p*NA	0.0198	0.0000	<0.01	0.0000	<0.01
Benzoyl-Pro-Phe-Arg-*p*NA	0.0052	0.0000	<0.01	0.0000	<0.01

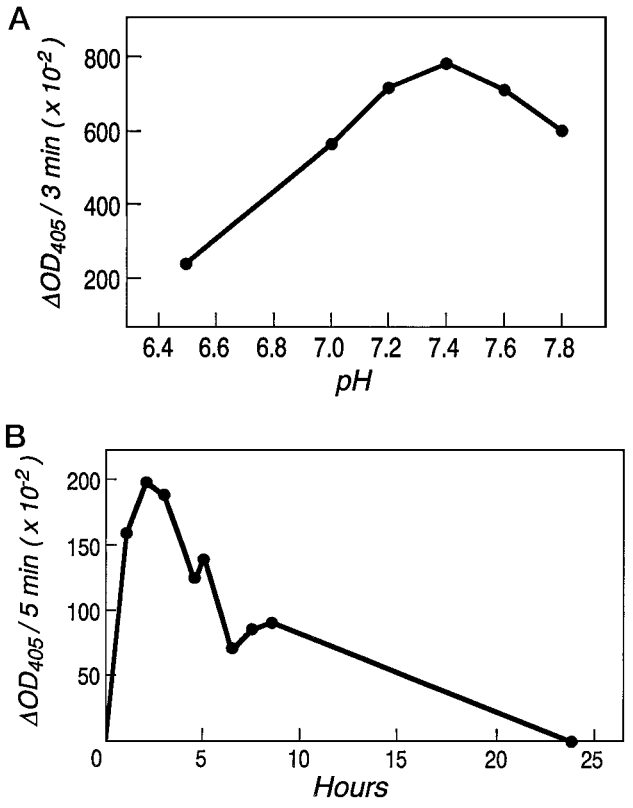

FIG. 4. **pH optimum and activation of the recombinant tryptase at pH 5.2.** In *panel A*, mMCP-7-FLAG was activated with EK at pH 5.2 in 50 mM sodium acetate and 5 mM calcium chloride. A sample of the resulting solution was suspended in a 1000-fold excess of assay buffer (25 mM sodium phosphate and 1 mM EDTA) whose pH ranged from 6.5 to 7.8, and the ability of the activated recombinant tryptase to cleave tosyl-Gly-Pro-Lys-*p*NA in the 3-min enzymatic assay was determined. In *panel B*, pro-EK-mMCP-7-FLAG was suspended in pH 5.2 buffer containing EK and the resulting solutions were incubated at 37 °C for 1 to 24 h. At the indicated times, samples were removed and analyzed for the ability to cleave tosyl-Gly-Pro-Lys-*p*NA at pH 7.4 in a 5-min enzymatic assay. The experiment depicted in *B* was carried out two additional times. Although small differences in the activation/inactivation profiles were noted in the other two experiments, in each instance a substantial increase in enzyme activity occurred during the first 3 h of incubation. This was followed by a gradual decline in enzymatic activity during the subsequent 21 h of incubation.

inhibitors in mouse plasma, the fibrinogenolysis assay carried out on whole plasma confirmed the *in vivo* and *in vitro* data that mMCP-7 is a potent anticoagulant. In control experiments, an excess of thrombin induced normal mouse plasma to clot within ~15 s. However, this same amount of thrombin was not able to induce the formation of a fibrin clot within 40 and 60 s in those plasma samples that had been pretreated for 1 h at 37 °C with 40 and 200 units of recombinant tryptase, respectively. On a weight basis, the anticoagulant activity of mMCP-7 in these assays was >20-fold higher than that of heparin glycosaminoglycan.

Screening of a Tryptase-specific, Phage Display Peptide Library—When the peptide library was subjected to 4 rounds of treatment with mMCP-7-FLAG, the only clone obtained had a peptide domain within the pIII fusion protein that consisted of Ser-Leu-Ser-Ser-Arg-Gln-Ser-Pro (Table II). When the library was subjected to only 2 rounds of treatment with mMCP-7-FLAG and 28 of the clones were arbitrarily sequenced, the peptide domains of 8 of the isolated clones possessed the same conserved sequence as that obtained after 4 rounds of treatment with mMCP-7-FLAG. Of the 20 remaining clones, 15 had at least one Ser or Thr residue. Ser and Thr were the favored residues at the P2 site. Moreover, 19 of the 28 clones had a Ser or Thr residue in either the putative P1′ or P2′ site. Although the preferred mMCP-7-susceptible peptide was found when the library was treated twice with bovine pancreatic trypsin, the other mMCP-7-susceptible peptides were not obtained.

DISCUSSION

Mouse mast cells express various combinations of at least nine serine proteases, two of which are tryptases. Although tryptases are major granule constituents of those mouse mast cells that reside in the peritoneal cavity, skin, skeletal muscle, and spleen (1–4, 18, 22, 23), their functions have not been determined. In the present study, we demonstrate that fibrinogen is a protein in plasma that is preferentially degraded by mMCP-7.

Because the plasma of the V3 mastocytosis mouse contains substantial amounts of enzymatically active mMCP-7 shortly after the IgE-sensitized animal is given antigen (18), this mouse model system was used to determine which plasma proteins, if any, are candidate substrates for mMCP-7 *in vivo*. Relative to mice that are sensitized with IgE but not challenged with antigen, the plasma from IgE/antigen-treated V3 mastocytosis mice contained large amounts of four peptides ranging from ~34 to 55 kDa (Fig. 1). The ~34-, 40-, and 42-kDa peptides all possessed the same N-terminal amino acid sequence. Although the complete amino acid sequences of the three chains of mouse fibrinogen have not been deduced, the last 13 residues of these three peptides are 100% identical to residues 11 to 23 of the α-chain of human fibrinogen (34). N-terminal amino acid analysis of purified mouse fibrinogen confirmed the conclusion that they were derived from the α-chain of fibrinogen. The ~55-kDa peptide in the plasma of the IgE/antigen-treated V3 mastocytosis mice possessed an N terminus that corresponds precisely with the N terminus of the γ-chain of

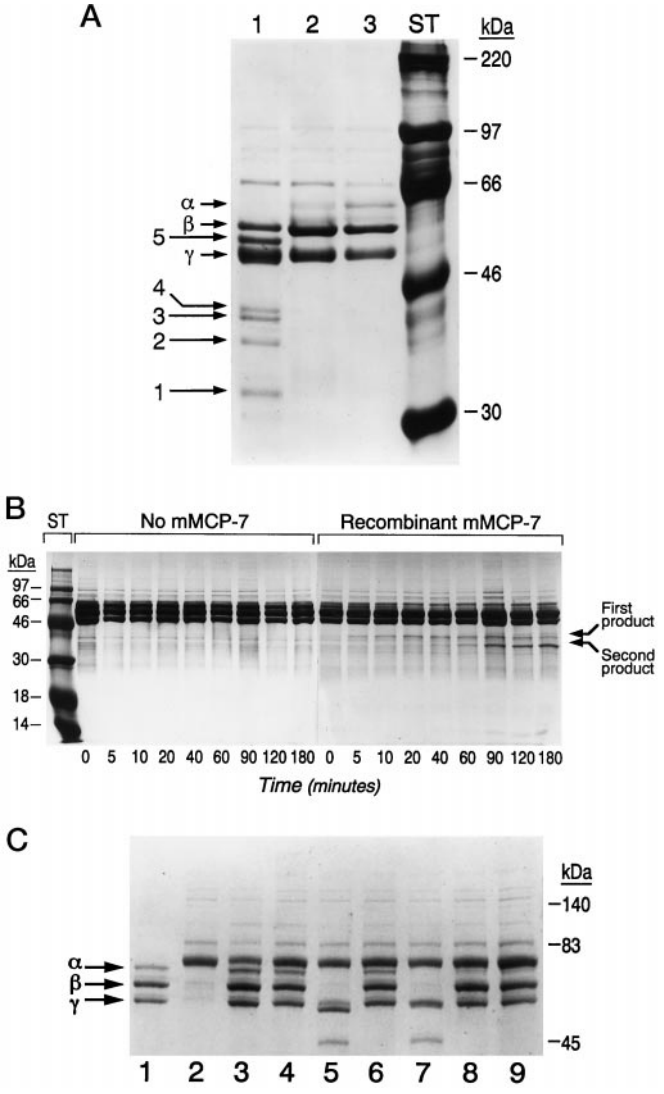

FIG. 5. **SDS-PAGE analysis of digests of mouse fibrinogen.** In *panel A*, mouse fibrinogen (100 μg) was incubated 3 h with EK-containing buffer alone (*lane 3*), pro-mMCP-7-FLAG (4 μg) (*lane 2*), or both (*lane 1*). The substrate:mMCP-7 (w/w) ratio in the depicted experiment is ~20:1. Molecular weight standards (*ST*) are shown on the *right*. *Arrows* on the *left* indicate the five prominent peptides of ~34, 38, 40, 42, and 57 kDa formed after the exhaustive tryptase digestion of the substrate. N-terminal amino acid analysis revealed that these peptides were derived from the α-, γ-, α-, α-, and β-chains of mouse fibrinogen, respectively. In the depicted kinetic study in *panel B*, 50 μg of mouse fibrinogen was incubated up to 3 h at 37 °C in the absence or presence of ~10 units (0.2 μg) of recombinant mMCP-7-FLAG. The substrate: enzyme (w/w) ratio used in depicted experiment is ~250:1. The *arrows* on the *right* indicate the two predominant digestion products that sequentially form during the incubation. Molecular weight standards (*ST*) are shown on the *left*. In *panel C*, 10 units of trypsin (*lanes 4* and *5*), chymotrypsin (*lanes 6* and *7*), and mMCP-7-FLAG (*lanes 8* and *9*) were incubated separately with 15 μg of mouse fibrinogen (substrate: enzyme (w/w) ratio is >75:1) before (*lanes 5, 7*, and *9*) and after (*lanes 4, 6*, and *8*) exposure to 4% plasma. Fibrinogen alone, plasma alone, and the combination of fibrinogen and plasma are depicted in *lanes 1–3*, respectively. The migration positions of three molecular weight standards are shown on the *right*. The *arrows* on the *left* indicate the α-, β-, and γ-chains of mouse fibrinogen. The major Coomassie Blue-stained protein in mouse plasma (*lane 2*) that is slightly larger than the α-chain of fibrinogen is mouse albumin. The SDS-PAGE gel was run considerably longer than the gel depicted in *panel B* to optimally resolve albumin and the α-chain of fibrinogen. The 34–42-kDa fragments of the α-chain of fibrinogen formed after mMCP-7 treatment are not detected because they have run off the gel. Whether the digestion was carried out for hours in the presence or absence of plasma, the major fragment of mouse fibrinogen that formed was the 34-kDa fragment that corresponded to the N terminus of the α-chain. Similar findings were obtained in three other experiments.

TABLE II
mMCP-7-susceptible peptides in pIII fusion proteins

The phage display peptide library was incubated 2 (A) or 4 (B) times with recombinant mMCP-7-FLAG, clones were isolated, and the deduced amino acid sequences of the peptides found in their protease-susceptible domains of the pIII fusion proteins were deduced.

No. of clones	Amino acid sequence of peptide
A. Two rounds of treatment	
8	Ser-Leu-Ser-Ser-Arg-Gln-Ser-Pro
1	Cys-Thr-Ser-Ser-Arg-Pro-Ser-Gly
1	Ser-Gly-Phe-Gly-Arg-Leu-Ser-Asp
1	Arg-Ser-Gln-Thr-Arg-Lys-Ser-Lys
1	Lys-Lys-Gln-Gly-Arg-Asp-Ser-Thr
1	Arg-Lys-Gln-Lys-Arg-Arg-Thr-Glu
1	Pro-Pro-Ser-Phe-Arg-Arg-Ser-Ser
1	Leu-Pro-Tyr-Gly-Arg-Ala-Thr-Thr
1	Asn-Thr-Pro-Thr-Lys-Leu-Ser-Pro
1	Arg-Arg-Pro-Thr-Lys-Lys-Asn-Thr[a]
1	Arg-Gly-Glu-Lys-Arg-Ser-Lys-Ser
1	Met-Leu-Leu-Ile-Arg-Thr-Trp-Glu
1	Val-Thr-Tyr-Ala-Arg-Leu-Cys-Try
1	Leu-Ser-Tyr-Arg-Lys-Leu-Arg-Phe
1	Gly-Thr-Arg-Arg-Arg-Glu-Glu-His
1	Asp-Arg-Lys-Gly-Arg-Gln-Gln-Gln
1	Arg-Tyr-Pro-Cys-Arg-Tyr-Gly-Leu
1	Lys-Glu-Glu-Asn-Arg-Lys-Asn-Asn
1	Phe-His-Pro-Ser-Arg-His-Pro-Pro
1	Ile-Ala-Arg-Glu-Lys-Gly-Gln-Gln
1	Ile-Cys-Pro-Pro-Arg-Leu-Leu-Gln
B. Four rounds of treatment	
12	Ser-Leu-Ser-Ser-Arg-Gln-Ser-Pro

[a] In peptides such as this one with more than one Arg or Lys residue, it is not clear which is the P1 residue in the peptide.

human fibrinogen (35), indicating that it, too, is derived from fibrinogen. Fibrinogen regulates endothelial cell adhesion and platelet aggregation via the $\alpha_V\beta_3$ and $\alpha_{IIb}\beta_3$ integrins, respectively. In the case of human fibrinogen, the varied integrin-binding motifs reside in the last half of the α- and γ-chains (36–41). The failure to detect the C-terminal peptides of the degraded α- and γ-chains of fibrinogen in the plasma of IgE/antigen-treated V3 mastocytosis mice suggests that they are rapidly cleared from the circulation via $\alpha_V\beta_3$ and/or $\alpha_{IIb}\beta_3$ integrin-mediated pathways.

mMCP-7 was the only enzymatically active protease that we consistently found in the plasma of those V3 mastocytosis mice that had underwent systemic anaphylaxis (18). Although the data obtained in the present studies from IgE/antigen-treated V3 mastocytosis mice suggested that fibrinogen is the physiologic substrate of mMCP-7, it was not possible to deduce using this *in vivo* model system alone whether the tryptase exerts its effect directly or indirectly. Although the substrate preference of a mMCP can be determined with recombinant protease, we and others have been unable to express large amounts of an enzymatically active protease like mMCP-7 in insect cells. To overcome these difficulties, we induced insect cells to express and secrete large amounts of two pseudozymogen forms of mMCP-7 that could be rapidly activated after their purification from conditioned medium (Figs. 1–4).

After an exhaustive *in vitro* incubation of mouse fibrinogen with recombinant mMCP-7-FLAG (Fig. 5*A*), five prominent peptides ranging from ~34 to 57 kDa were obtained. N-terminal amino acid analysis revealed that three of the peptides were derived from the α-chain; the other two were derived from the β- and γ-chains. The discovery that three of the major peptides found in the plasma of the V3 mastocytosis mouse (Fig. 1) were the same as those generated in the *in vitro* study (Fig. 5*A*) indicates that fibrinogen is a physiologic substrate of native mMCP-7 in the V3 mastocytosis mouse. In a comparative analysis, the anticoagulant activity of recombinant mMCP-7 was found to be substantially greater than that of

heparin glycosaminoglycan. Subsequent kinetic studies (Fig. 5*B*) revealed that the α-chain of fibrinogen is the chain most susceptible to degradation by mMCP-7. Based on N-terminal amino acid analysis of the resulting fragments in the digests, at least three mMCP-7-susceptible sites reside in the α-chain of fibrinogen. One resides in the middle of the chain; the others reside in the C-terminal portion of the chain.

Heparin-containing serglycin proteoglycans are required for human mast cell tryptases to exert optimal enzymatic activity (42). Since mMCP-7 readily cleaved tosyl-Gly-Pro-Lys-*p*NA in the absence of heparin glycosaminoglycan, the enzymatic activity of this mouse tryptase apparently is not controlled by serglycin proteoglycans outside of the mast cell. Furthermore, because the recombinant tryptase cleaves tosyl-Gly-Pro-Lys-*p*NA but not a number of other trypsin-susceptible *p*NA substrates (Table I), mMCP-7 possesses a more restricted substrate specificity than its homologous pancreatic enzyme. Because a prominent surface fold extends into its substrate-binding cleft (4, 20), it was previously predicted that mMCP-7 would not degrade a large number of proteins *in vivo*. We therefore anticipated that a phage display peptide library containing either a Lys or Arg at the P1 site would give insight as to what amino acid sequences are preferred by mMCP-7. The peptide library created in this study was screened with recombinant mMCP-7-FLAG rather than recombinant mMCP-7 because the former could be purified without the heparin-Sepharose chromatography step. When the peptide library was subjected to four rounds of treatment with mMCP-7-FLAG, the only clone obtained had a peptide domain in the pIII fusion protein that consisted of Ser-Leu-Ser-Ser-Arg-Gln-Ser-Pro (Table II). Even when the number of codons that encode Ser is taken into account, the representation of this amino acid in the mMCP-7-susceptible peptides is considerably higher than by chance. Since mMCP-7 is a tryptase, the Arg residue in the obtained octamer is the P1 residue. When the library was subjected to only 2 rounds of treatment with mMCP-7-FLAG, many of the clones possessed the conserved sequence. In addition, almost all clones had at least one Ser or Thr residue at the putative P2, P1′, and/or P2′ sites. Val and Ile were underrepresented in the mMCP-7-susceptible peptides.

A computer search of a protein data base with the sequence of Leu-Ser-Ser-Arg-Gln-Ser revealed that residues 309 to 314 in the middle of the α-chain of rat fibrinogen has the nearly identical sequence of Gly-Ser-Ser-Arg-Pro-Ser. If there is a comparable amino acid sequence in the middle of the α-chain of mouse fibrinogen, one would predict that a N-terminal 34-kDa fragment would be generated when mMCP-7 cleaves the protein. As noted in Figs. 1 and 5, *A* and *B*, this is precisely what is obtained *in vivo* and *in vitro*. Comparable amounts of the somewhat smaller C-terminal fragment apparently are not obtained *in vitro* because there are two other susceptible sites in this portion of the α-chain which cause it to be broken down further. It remains to be determined where these mMCP-7-susceptible sites reside. However, it is likely that they are located at two of the numerous Ser/Thr-rich sequences that reside in the C terminus of the chain which also have a basic residue (*e.g.* residues 371 to 377 in the α-chain of rat fibrinogen which has the sequence of Thr-Ser-Thr-Thr-Arg-Arg-Ser).

In general, the substrate-binding clefts of serine proteases are long enough to interact with 7 residues from P4 to P3′. The alignment of mMCP-7 with trypsin (Fig. 6) is consistent with the observed tryptic activity of recombinant mMCP-7, which is defined as its strong preference for a Lys or Arg residue at position P1. All 14 residues in trypsin that are in contact with the P1 Lys of bovine pancreatic trypsin inhibitor (45) are absolutely conserved in mMCP-7. The conservation of the S1 subsite is in contrast to the large differences between the other subsites in mMCP-7 and trypsin. Only 6 of the 42 residues in the substrate-binding loops that contribute to these subsites are conserved. In addition to residue differences relative to trypsin, the alignment of mMCP-7 and trypsin indicates residue insertions in three loops that form part of the substrate-binding cleft of mMCP-7. The insertions in mMCP-7 consist of 4 residues in loop A, 2 residues in loop B, and 9 residues in loop 3. Loops A, B, and 3 contribute to pockets S2′ to S3′, S2 to S1′, and S3, respectively. These insertions are likely to protrude out of the surface and make the substrate-binding cleft of mMCP-7 deeper than in trypsin, thereby restricting the substrate specificity of the former enzyme.

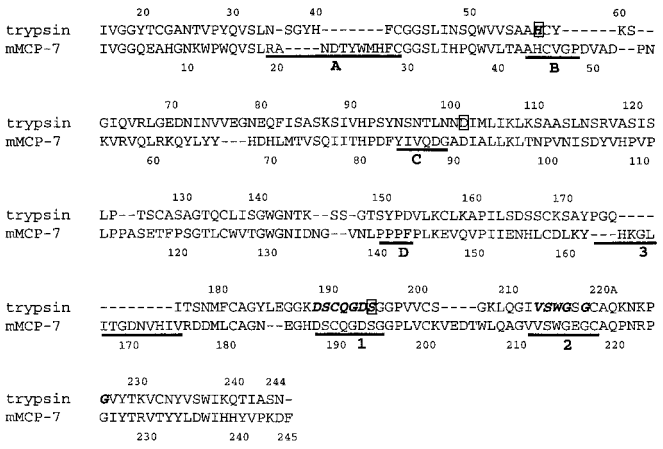

FIG. 6. **Amino acid alignment of trypsin and mMCP-7.** The *numbers* in the *top line* refer to the conventional numbering system of the trypsin structure. The *numbers* in the *bottom line* refer to the residues in mature mMCP-7. The 7 loops (designated *A-D* and *1–3*) forming the substrate-binding pocket of trypsin and the putative substrate-binding pocket of mMCP-7 are *underlined*. The amino acid residues in trypsin that have at least one atom within 4.5 Å of any atom of the P1 Lys residue of bovine pancreatic trypsin inhibitor are shown in *bold italic* style. The residues that form the active site in trypsin are *boxed*. *Dashes* indicate gaps in the amino acid sequences. The Modeller computer program (43, 44) was used to align the two serine proteases.

The observations that mast cell tryptases from other species can induce airway smooth muscle hyper-responsiveness in dogs (46), reverse airway smooth muscle relaxation induced by vasoactive intestinal peptide in ferrets (47), and induce proliferation and/or chemotaxis of cultured fibroblasts (48, 49) and epithelial cells (50) suggested that mast cell tryptases preferentially degrade or activate varied receptors (51) on the surfaces of cells. Nevertheless, the finding that a large number of proteins and/or biologically active peptides (*e.g.* high molecular weight kininogen (52), vasoactive intestinal peptide (53), vasopressin (5), neurotensin (5), insulin (5), calcitonin gene-related peptide (54), pro-opiomelanocortin (55), pro-atrial natriuretic factor (55), β-lipotropin (55), adrenocorticotropic hormone (55), pro-metalloproteinase-3 (56), pro-urokinase (57), complement protein C3 (58), and even fibrinogen (59)) are susceptible to degradation by mast cell tryptases *in vitro* raised the possibility that one of the mouse mast cell tryptases evolved primarily to degrade certain proteins residing in the extracellular matrix or plasma. When the peptide library was screened with recombinant mMCP-6, only 1 of the 30 arbitrary selected phage clones possessed the mMCP-7-susceptible peptide in its pIII fusion protein.[2] This functional difference between the two homologous tryptases emphasizes the importance of using recombinant protease in substrate specificity studies.

Fibrinogen, a plasma protein essential for blood coagulation,

[2] C. Huang and R. L. Stevens, unpublished data.

is a large sized glycoprotein consisting of two sets of three distinct polypeptide chains that are all disulfide bonded (60). When the Arg^{16}-Gly^{17} bond in the α-chain of a fibrinogen molecule is cleaved by thrombin, an N-terminal polymerization site is exposed, which in turn interacts with a complementary site on the γ-chain of the distal portion of another fibrinogen molecule to initiate the formation of the fibrous clot. Fibrinogen also plays a critical role in the aggregation of platelets during clot formation. Schwartz and co-workers (59) previously noted that a tryptase purified from human lung mast cells had poor fibrinogenolytic activity by itself but it could degrade fibrinogen *in vitro* in the presence of heparin under ideal conditions in a buffer lacking other plasma proteins. Unlike the human mast cell tryptase, mMCP-7 does not stay bound to heparin-containing serglycin proteoglycans *in vivo* after its exocytosis (18). Because mMCP-7 also can readily degrade fibrinogen in the absence of heparin, this mouse tryptase appears to be functionally different than the previously studied human tryptase.

While a large number of proteases can degrade fibrinogen *in vitro*, these proteases generally are not able to degrade fibrinogen *in vivo* because as much as 10% of the proteins in plasma consist of diverse types of protease inhibitors. The protein concentration of plasma is ~80 mg/ml, whereas the fibrinogen concentration generally is only ~3 mg/ml. mMCP-7 differs from other serine proteases in the mouse in its resistance to inactivation by known protease inhibitors (18). In the case of the V3 mastocytosis mouse, mMCP-7 quickly gets into the blood where it circulates for >1 h in its mature enzymatically active state. While it remains to be determined whether or not any human mast cell tryptase can selectively degrade fibrinogen in the presence of other plasma proteins, recombinant mMCP-7 is able to degrade fibrinogen in the presence of plasma protease inhibitors down to peptides that are similar in size to those seen in the circulation of IgE/antigen-treated V3 mastocytosis mice. These *in vitro* findings confirm the *in vivo* data from the V3 mastocytosis mouse (Fig. 1) which first suggested that fibrinogen is a physiologic substrate of mMCP-7.

SDS-PAGE analysis of the plasma of V3 mastocytosis mice that are undergoing systemic anaphylaxis (Fig. 1) raises the possibility that mMCP-7 digests additional substrates *in vivo*. Nevertheless, why mMCP-7 evolved to degrade fibrinogen is a matter of conjecture. Mast cells, as effector cells of the immune response that reside in tissues, are one of the first participants in inflammatory responses. When activated through their high-affinity IgE receptors, mast cells immediately release their preformed granule mediators and quickly generate and release different arachidonic acid metabolites. Within 30 min after IgE/antigen treatment of the cells, activated mast cells dramatically up-regulate their production of varied cytokines and chemokines. This complex immune response eventually results in vasodilation, edema, and an influx of hematopoietic cells into the inflammatory site. Surprisingly, one does not find large amounts of cross-linked fibrin in tissues after mast cell-mediated inflammatory responses. Aggregated platelets also are rarely seen. By quantitating the uptake of [^{125}I]fibrinogen into the skin of the mouse, Mekori and Galli (61) found that some plasma fibrinogen makes its way into the cutaneous site 2 h after the initiation of an IgE-dependent, immediate hypersensitivity reaction. However, 24 h after the initiation of the mast cell response, very little urea-insoluble [^{125}I]fibrin could be detected in the inflammatory site relative to that obtained in a T-cell dependent contact sensitivity reaction at a different cutaneous site in the same animal. Unlike the other mMCPs, mMCP-7 quickly dissociates from the protease-proteoglycan macromolecular complex after exocytosis (18, 20). Because it diffuses away from the inflammatory site, mMCP-7 can acutely inactivate fibrinogen at the endothelium/blood barrier before it has a chance to induce platelet aggregation and become cross-linked fibrin. Exocytosed mMCP-7 also could alter fibrinogen/integrin-mediated cellular responses during inflammatory reactions.

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