Induction of a Selective and Persistent Extravasation of Neutrophils into the Peritoneal Cavity by Tryptase Mouse Mast Cell Protease 6[1]

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Recombinant mouse mast cell protease 6 (mMCP-6) was generated to study the role of this tryptase in inflammatory reactions. Seven to forty-eight hours after the i.p. injection of recombinant mMCP-6 into BALB/c, mast cell-deficient WCB6F$_1$-*Sl/Sl*^{d}, C5-deficient, or mMCP-5-null mice, the number of neutrophils in the peritoneal cavity of each animal increased significantly by >50-fold. The failure of the closely related recombinant tryptase mMCP-7 to induce a comparable peritonitis indicates that the substrate specificities of the two tryptases are very different. Unlike most forms of acute inflammation, the mMCP-6-mediated peritonitis was relatively long lasting and neutrophil specific. Mouse MCP-6 did not induce neutrophil chemotaxis directly in an in vitro assay, but did promote chemotaxis of the leukocyte in the presence of endothelial cells. Mouse MCP-6 did not induce cultured human endothelial cells to express TNF-α, RANTES, IL-1α, or IL-6. However, the tryptase induced endothelial cells to express large amounts of IL-8 continually over a 40-h period. Neither enzymatically active mMCP-7 nor enzymatically inactive pro-mMCP-6 was able to induce endothelial cells to increase their expression of IL-8. Although the mechanism by which mMCP-6 induces neutrophil accumulation in tissues remains to be determined, the finding that mMCP-6 induces cultured human endothelial cells to selectively release large amounts of IL-8 raises the possibility that this tryptase regulates the steady state levels of neutrophil-specific chemokines in vivo during mast cell-mediated inflammatory events. ***The Journal of Immunology*****, 1998, 160: 1910–1919.**

Mouse mast cells express two homologous tryptases (designated mouse mast cell protease (mMCP)[3] 6 and mMCP-7) (1–4) that have been implicated by linkage analysis (5) in the pathobiology of certain airway responses elicited by the high affinity receptor for IgE. Although the function of mMCP-6 has not been deduced, fibrinogen is a physiologic substrate of mMCP-7 (6). The genes that encode mMCP-6 and mMCP-7 both reside on chromosome 17 (7, 8) and are quite homologous (2, 3). Nevertheless, the expression of the two tryptases is differentially regulated in vitro and in vivo at the mRNA and protein levels (3, 9–11).

Like the mast cell tryptases in other species (12–18), mMCP-6 is initially translated as a zymogen that possesses a hydrophobic signal peptide and a 10-residue pro-peptide whose C-terminal amino acid is Gly (1, 2). After undergoing varied post-translational modification events, mMCP-6 is stored in the acidic granules of mouse mast cells in its mature, enzymatically active form ionically bound to serglycin proteoglycans. Exocytosed mMCP-6 and mMCP-7 are metabolized quite differently in mice undergoing passive systemic anaphylaxis because of the different rates of dissociation of these two tryptases from heparin-containing serglycin proteoglycans (19). Shortly after the relevant Ag is administered to IgE-sensitized mice, mMCP-6/proteoglycan macromolecular complexes appear in the extracellular matrix adjacent to activated mast cells in the tongue, skin, spleen, and heart of normal BALB/c mice and in the spleen and liver of V3 mastocytosis mice. Because exocytosed mMCP-6 does not dissociate readily from serglycin proteoglycans, this tryptase is retained in extracellular matrixes for >1 h. The prolonged retention of mMCP-6 in tissues around activated mast cells implies that mMCP-6 preferentially exerts its effects locally.

Mast cell tryptases have been purified from various species in an attempt to deduce their preferred substrates. Unfortunately, the number of mature mast cells that can be isolated from a mouse is inadequate to obtain enough mMCP-6 for study. The nearly impossible task of removing 100% of the other mMCPs from the starting preparations of mast cell lysates also has hindered studies that address the function of the tryptase. We now describe the derivation of a pseudozymogen form of recombinant mMCP-6 from baculovirus-infected insect cells that can be easily purified, activated ex vivo by enterokinase (EK), and then studied immediately. We show that recombinant mMCP-6 and mMCP-7 are functionally distinct tryptases by multiple critera and that mMCP-6 can selectively induce large numbers of neutrophils to extravasate into the peritoneal cavity of varied mouse strains. We also show that mMCP-6, but not mMCP-7 or pro-mMCP-6, induces cultured endothelial cells to selectively increase their expression of the neutrophil chemoattractant IL-8. Finally, we show that the substrate specificity of mMCP-6 is restricted by heparin.

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[3] Abbreviations used in this paper: mMCP, mouse mast cell protease; EK, enterokinase; FLAG, the peptide whose amino acid sequence is Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys; TBST buffer, Tris-buffered saline containing 0.01% Tween-20; RT, reverse transcriptase.

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Materials and Methods

Expression of pro-mMCP-6 and pro-EK-mMCP-6 in insect cells

The bioengineering approach recently developed to obtain a pseudozymogen form of mMCP-7 that could be activated with EK after its purification from the conditioned medium of insect cells (6) was used to obtain a similar pseudozymogen (pro-EK-mMCP-6) form of mMCP-6 as well as the nonmodified zymogen (pro-mMCP-6). Expressed pro-EK-mMCP-6 has an EK-susceptible peptide (Asp-Asp-Asp-Asp-Lys) between the domain that encodes the endogenous pro-peptide and the N-terminal Ile residue of the mature tryptase. It also has the eight-residue FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) (20) attached to its C-terminus to facilitate its purification. The relevant cDNA constructs, created with standard PCR approaches, 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. The nucleotide sequences of the resulting constructs were determined to rule out any PCR-induced artifact.

Insect cells were induced to express pro-mMCP-6 and pro-EK-mMCP-6, as described previously for pro-mMCP-7 (21). Briefly, purified plasmid DNA (~5 μg) was mixed with ~0.5 μg of linearized BaculoGold DNA (PharMingen) and calcium phosphate. Each resulting DNA solution was then added to 3×10^6 adherent *Spodoptera frugiperda* 9 insect cells (Invitrogen, San Diego, CA) that were in the log phase of growth, and the infected cells were cultured for 7 days at 27°C in medium (Invitrogen) supplemented with 10% heat-inactivated FCS (Sigma Chemcial Co., St. Louis, MO). Recombinant virus particles ($\geq 3 \times 10^7$) from these insect cells were added to a new culture dish containing 6×10^6 *Trichoplusia ni* High Five insect cells (Invitrogen) in the log phase of growth, and the infected cells were cultured in serum-free, Xpress medium (BioWhittaker, Walkersville, MD). Generally 4 days later, the conditioned medium was centrifuged at $1500 \times g$ for 15 min at room temperature before purification of the secreted recombinant protein was attempted.

Purification of pro-mMCP-6 and pro-EK-mMCP-6 from insect cell-conditioned medium and EK activation of the pseudozymogen

Pro-mMCP-6 and pro-EK-mMCP-6 were purified by heparin-Sepharose chromatography, with a modification of that described for recombinant pro-mMCP-7 (21). Conditioned medium obtained from insect cells infected with the relevant baculovirus was dialyzed against 0.1 M NaCl and 50 mM sodium acetate, pH 5.0, and then applied to replicate heparin-Sepharose columns that had been equilibrated in the same buffer. After the column was washed, bound proteins were eluted on a linear gradient, with the NaCl concentration in the buffer increasing to 3.1 M.

In most instances, the purification of pro-EK-mMCP-6 was conducted with an affinity column containing the anti-FLAG M2 mAb (International Biotechnology, New Haven, CT) (22, 23). This latter immunoaffinity column (2 ml) was washed with 0.1 M glycine, pH 3.5, and then with 50 mM Tris-HCl and 150 mM NaCl, pH 7.4. After application of the insect cell-conditioned medium, the column was washed briefly with the above pH 7.4 buffer, and then bound pro-EK-mMCP-6 was eluted 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 pro-EK-mMCP-6. The protein concentration of the eluate was estimated by measuring the absorbance at 280 nm.

Purified pro-EK-mMCP-6 (~100 μg in 100 μl) was separately mixed with 100 μl of a pH 6.0 buffer containing 10 mM Tris-HCl, 5 mM calcium chloride, 0.1% Triton X-100, and 50 μg of heparin. The commercial preparation of heparin (Sigma) used in the study was first suspended in 4 M GnHCl, 0.1 M sodium sulfate, and 0.1 M Tris-HCl, pH 7.0, and then subjected to a 48-h CsCl density gradient centrifugation step (24) to denature and remove any trace protein contaminants that might be present in Sigma's preparation of this glycosaminoglycan. In most cases, 1 μl of a solution containing 320 U of calf intestine EK (Biozyme, San Diego, CA) was added, and the mixture was incubated at room temperature for 17 h to allow EK to activate the mMCP-6 pseudozymogen. To determine the stability of the generated mMCP-6, in one experiment the mixture was incubated at 37°C for up to 30 h. At various times during the activation process, samples of the digest were evaluated for the ability of the activated mMCP-6 to cleave tosyl-Gly-Pro-Lys-*p*-nitroanilide in the 3-min assay described below. In some cases, mMCP-6 was activated with EK in the absence of heparin.

Insect cell-conditioned medium and column fractions (~20 μl) containing pro-mMCP-6, pro-EK-mMCP-6, or EK-activated mMCP-6 (~1 μl) were diluted in SDS-PAGE buffer (2% SDS, 1% 2-ME, 0.1% bromophenol blue, and 100 mM Tris-HCl, pH 6.8), boiled for 5 min, and loaded onto 12% polyacrylamide gels. After SDS-PAGE, the resolved proteins were stained with Coomassie blue or were transferred in 20 mM Tris-HCl and 150 mM glycine containing 20% methanol, pH 8.3, for 2 to 4 h at 200 mA to polyvinylidine difluoride membranes (Millipore Corp., Bedford, MA) with a Bio-Rad (Richmond, CA) immunoblotting apparatus. For immunoanalysis of the resulting protein blots, each membrane was sequentially incubated for 1 h in 5% nonfat milk for 1 h with a 1/500 dilution of affinity-purified rabbit anti-mMCP-6 Ig (19) in Tris-buffered saline containing 0.01% Tween-20 (TBST buffer), TBST buffer alone, and then for 1 h with a 1/1000 dilution of anti-rabbit IgG alkaline phosphatase conjugate (~1 ng/ml, final concentration) in TBST buffer. Immunoreactive proteins were visualized with nitroblue 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 polyvinylidine difluoride membranes and briefly stained with 0.5% Ponceau S red, and the relevant protein/peptide bands were subjected to automated Edman degradation by the Harvard Microchemistry Facility (Harvard Biologic Laboratories, Cambridge, MA).

Analysis of the substrate specificity of mMCP-6

The spectrophotometric method of Svendsen and co-workers (25) was used to determine whether mMCP-6 is enzymatically active in the absence or the presence of heparin glycosaminoglycan or normal mouse serum. Generally, 1-μl samples of each reaction mixture were placed in 1 ml of a pH 7.4 buffer containing 25 mM sodium phosphate, 1 mM EDTA, and 50 μg tosyl-Gly-Pro-Lys-*p*-nitroanilide. The change in optical density at 405 nm was determined after a 3-min incubation at room temperature. In this assay, 1 U of enzymatic activity is defined as a change in OD at 405 nm of 0.001/min. One microgram of bovine pancreatic trypsin (Sigma) is equivalent to ~350 U when tosyl-Gly-Pro-Lys-*p*-nitroanilide 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 U/μg. The ability of mMCP-6 to cleave the trypsin-susceptible substrates tosyl-Gly-Pro-Arg-*p*-nitroanilide, benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide, benzoyl-Pro-Phe-Arg-*p*-nitroanilide, and acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide was also evaluated.

A substrate phage display library that encodes an altered pIII containing at its N-terminus the FLAG peptide followed by an eight-residue hypervariable peptide was screened with recombinant mMCP-6 in the presence or the absence of heparin glycosaminoglycan to deduce which amino acid sequences are preferred for cleavage and whether heparin influences the substrate specificity of this tryptase. To prepare the phage column used in the screening process, 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 at $10{,}000 \times g$, 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 mAb. The column was washed three times with 10 ml of the same pH 7.0 buffer to remove unbound phage. EK-activated mMCP-6 (~50 μg in 200 μl buffer) in the absence or the presence of heparin glycosaminoglycan (~50 μg) was added, and the column was sealed and incubated at room temperature for 90 min. After protease treatment, 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 *Escherichia 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. This screening procedure was repeated up to four times to select those phage in the library whose pIII fusion proteins were most susceptible to degradation by mMCP-6. After cloning of the isolated phage, the nucleotide sequence that encodes the 8-mer, protease-susceptible peptide domains in the fusion protein of each isolated clone was then determined.

Mouse MCP-6-induced, neutrophil-specific peritonitis

Samples of purified mMCP-7 (2000 U), mMCP-6 (800 U), or pro-EK-mMCP-6 (amount equivalent to that of mature mMCP-6 on a weight basis) were injected separately into the peritoneal cavity of BALB/c, C5-deficient (B10.D2-*H2*^{d} *Tla*^{c} *Hc*^{0}/*oSn*) (26), mast cell-deficient WCB6F$_1$-*Sl*/*Sl*^{d} (also known as WCBF$_1$-*Mgf*^{Sl}/*Mgf*$^{Sl\text{-}d}$), and mMCP-5-null (27) mice. All mice, except mMCP-5-null mice, were obtained from The Jackson Laboratory (Bar Harbor, ME). Because mMCP-6 is bound to heparin-containing serglycin proteoglycans in vivo (1, 19) and because analysis of a tryptase-specific, phage display library (see below) revealed that heparin restricts the substrate specificity of mMCP-6, activated mMCP-6 was allowed to

form a 1/1 to 2/1 (w/w) macromolecular complex with heparin before being injected into the peritoneal cavity. Although heparin does not appear to influence the substrate specificity of mMCP-7 at neutral pH (6), this glycosaminoglycan also was present as a control when no mMCP, pro-EK-mMCP-6, or mMCP-7 was injected into the peritoneal cavity of separate mice. At specific time points (e.g., 36 h) after mMCP treatment, the mouse was killed by CO_2 asphyxiation, and 10 ml of Tyrode's buffer containing 0.1% (w/v) gelatin was injected into its peritoneum. The injected fluid was aspirated ~30 s later. The cells in the peritoneal lavage fluid were counted with a hemocytometer and then were subjected to a 5-min centrifugation at 28 × *g* onto glass slides with a Cytospin 2 cytocentrifuge (Shandon, Pittsburgh, PA). The resulting slides were stained with Diff-Quik (Baxter Healthcare Corp., McGaw Park, IL). In one experiment, the peritoneal membrane from a BALB/c mouse exposed to mMCP-6 for 36 h was removed, spread on a glass slide, and stained with methylene blue. The number of membrane-bound neutrophils was compared with that of a peritoneal membrane isolated from a control BALB/c mouse exposed to medium alone.

In vitro neutrophil chemotaxis assay, and evaluation of cytokine and chemokine expression by human endothelial cells exposed to mMCP-6

ChemoTx microplates (Neuro Probe, Cabin John, MD) containing membranes with 8-μm standard pore sizes were used to determine whether mMCP-6 is a chemotactic factor that can act directly or indirectly on mouse peripheral blood neutrophils. In the first experimental protocol, 29-μl portions of DMEM containing 2% FCS with or without FMLP (5.4 μM; Sigma), mature mMCP-7 (200 U), mature mMCP-6 (90 U), or pro-EK-mMCP-6 (amount equivalent to that of mature mMCP-6 on a weight basis) were added to separate wells in the bottom chambers of the assay plate. In the second experimental protocol, HUVEC (Clonetics, San Diego, CA) were placed in the bottom chambers of the assay plate 24 h before the addition of the test agent to confluent monolayers. Because FMLP is a strong chemoattractant for neutrophils (28), this three-residue peptide was used as a positive control in these in vitro experiments. Peripheral blood was collected from the retro-orbital plexus of BALB/c mice into pipettes containing EDTA anticoagulant, and the neutrophils were enriched to a purity of >75% by gradient centrifugation technology with the NIM-2 leukocyte isolation kit from Cardinal Associates (Santa Fe, NM). Samples of DMEM (25 μl) containing 2% FCS and a constant number of freshly isolated neutrophils (3,000–10,000 cells depending on the experiment) were then placed into each top well of the assay plate. After a 30-min incubation at 37°C, the membrane separating the two chambers was removed and placed in Diff-Quik stain, and the neutrophils that had migrated through it were counted.

ELISA kits from Endogen (Cambridge, MA) were used as described by the manufacturer to quantitate the amounts of human TNF-α, IL-1α, IL-6, and IL-8 in samples (1–10 μl) of the conditioned medium of replicate endothelial cell cultures exposed for various periods of time to no mMCP, mature mMCP-7 (200 U), mature mMCP-6 (90 U), or pro-EK-mMCP-6 (amount equivalent to that of mature mMCP-6 on a weight basis). Total RNA was isolated according to the method of Chomczynski and Sacchi (29) from endothelial cells exposed for 2 h to no mMCP, mMCP-6, pro-EK-mMCP-6, or mMCP-7. The RNA samples were denatured in formaldehyde/formamide, electrophoresed in 1.3% formaldehyde-agarose gels, and transferred to nylon membranes (Micron Separations, Inc., Westboro, MA). The resulting blots were incubated in QuickHyb buffer (Stratagene Cloning Systems, La Jolla, CA) containing a radiolabeled, gene-specific probe for either IL-8 (30) or β-actin (31). After the RNA blots were washed at 60°C in 0.1× SSC buffer and 0.1% SDS, they were analyzed by autoradiography. Reverse transcriptase (RT)-PCR also was performed with assay kits from BioSource International (Camarillo, CA) to evaluate the presence of IL-8, RANTES, and TNF-α transcripts in human endothelial cells that had been exposed to mMCP-6 for 1, 2, or 3 h. The IL-8-, RANTES-, and TNF-α-specific primers used in these assays will result in the generation of 227-, 197-, and 342-bp DNA fragments if the respective chemokine/cytokine transcripts are present in a cell lysate.

Results

Generation of pro-mMCP-6 and pro-EK-mMCP-6 in insect cells and EK conversion of the pseudozymogen to enzymatically active tryptase

Insect cells infected with the relevant construct secreted forms of pro-mMCP-6 and pro-EK-mMCP-6 (Fig. 1) into the conditioned medium that could be purified by heparin-Sepharose chromatography (Fig. 2). Like pro-mMCP-6, pro-EK-mMCP-6 bound to a heparin-Sepharose column that had been equilibrated in 100 mM NaCl/10 mM sodium phosphate, pH 5.5. Because both recombinant proteins dissociated from the heparin-Sepharose column when the NaCl concentration of the buffer was raised to 1.4 M, it was concluded that most of the pro-mMCP-6 and pro-EK-mMCP-6 secreted by the insect cells into the conditioned medium were properly folded. Pro-EK-mMCP-6 also could be purified with the anti-FLAG immunoaffinity column (Fig. 3*A*).

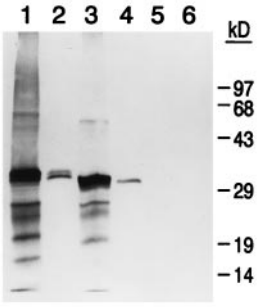

FIGURE 1. Expression of pro-EK-mMCP-6 and pro-mMCP-6 in insect cells. Samples of conditioned medium (10 μl; *lanes 2, 4,* and *6*) or cell lysates (1 μl; ~10^7 cells/ml; *lanes 1, 3,* and *5*) from insect cells infected with wild-type virus (*lanes 5* and *6*) or virus that encodes either pro-EK-mMCP-6 (*lanes 1* and *2*) or pro-mMCP-6 (*lanes 3* and *4*) were applied to individual wells of a polyacrylamide gel. After electrophoresis of the gel, a protein blot of the separated proteins was prepared and stained with anti-mMCP-6 Ig. Shown on the *right* are the migration positions of the standard m.w. markers.

As assessed by SDS-PAGE, the molecular mass of the recombinant protein decreased by ~2 kDa when the mMCP-6 pseudozymogen was incubated for 3 h with EK (data not shown). Even if exposed to EK at pH 6.0 for up to 24 h, the recombinant protein did not undergo substantial autolysis. Amino acid sequence analysis revealed that the resulting product possessed an N-terminal sequence of Ile-Val-Gly-Gly-His-Glu-Ala-Ser-Glu, which is identical with that of mature, native mMCP-6 (1, 2). Mouse MCP-6 exhibited good enzymatic activity after a 2-h incubation with EK (Fig. 3*B*) and optimal enzymatic activity at pH 7.2 to 7.6 (data not shown), and readily cleaved tosyl-Gly-Pro-Lys-*p*-nitroanilide and tosyl-Gly-Pro-Arg-*p*-nitroanilide in the absence of heparin glycosaminoglycan. However, in the absence or the presence of heparin, mMCP-6 did not effectively cleave benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide, benzoyl-Pro-Phe-Arg-*p*-nitroanilide, or acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide. When a tryptase-specific, phage display library was subjected to four rounds of treatment with enzymatically active mMCP-6 in the absence of heparin glycosaminoglycan, no specific peptide sequence in the hypervariable domain of the pIII fusion protein was obtained in the 30 arbitrarily selected clones (Table I). The two most prominent features of the peptides obtained by screening the library with mMCP-6 alone were the over- and under-representation of positively and negatively charged residues, respectively. One-half of the selected clones had three or more Lys and/or Arg residues in the susceptible peptide, and two of the clones actually had five positively charged residues. When the same library was subjected to two to four rounds of treatment with mMCP-6 in the presence of an equal amount of heparin glycosaminoglycan, a much more limited number of sequences was obtained (Table II).

Mouse MCP-6 was susceptible to inactivation by factors in normal mouse serum. In contrast to mMCP-7, mMCP-6 failed to cleave tosyl-Gly-Pro-Lys-*p*-nitroanilide after a 30-min incubation

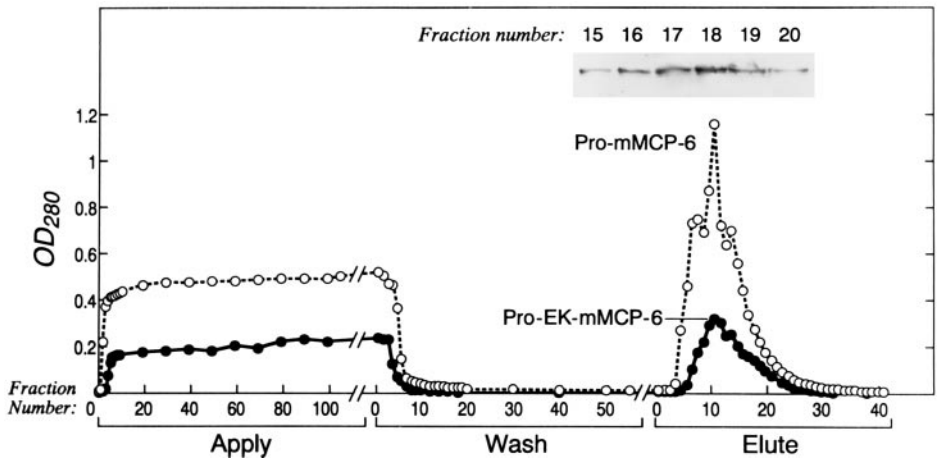

FIGURE 2. Heparin-Sepharose chromatography of pro-mMCP-6 and pro-EK-mMCP-6. Samples of conditioned medium (400 ml) from insect cells that express either pro-mMCP-6 (○) or pro-EK-mMCP-6 (●) were applied to replicate 18-ml heparin-Sepharose columns. The columns were washed, and the bound proteins were eluted with a salt gradient. Depicted is the OD_{280} of the eluate 5-ml fractions of each column. The inset (*top right*) depicts the SDS-PAGE-immunoblot analysis obtained with anti-mMCP-6 Ig on samples (20 μl) of fractions 15 to 20 of the gradient eluate of the pro-EK-mMCP-6-treated column. Similar findings were obtained in two other experiments with pro-mMCP-6.

at 37°C or a 1.5-h incubation at room temperature in the presence of 50% mouse serum. Either the inhibitory factor(s) in mouse serum is relatively species dependent or the concentration of this factor is substantially higher in mouse serum than in FCS, because mMCP-6 was inhibited by only 33% after a 1.5-h incubation at room temperature in the presence of 50% FCS.

Mouse MCP-6-induced, neutrophil-specific peritonitis

Neutrophils represented only 1 ± 1% (mean ± SD; $n = 3$) of the $1.0 \pm 0.2 \times 10^7$ cells in the peritoneal lavage fluid of untreated BALB/c mice. Thirty-six hours after 800 U of enzymatically active mMCP-6 (an amount that corresponds to ~2 μg or 0.6 nM trypsin) was injected i.p. into BALB/c mice, the number of cells in the exudates was comparable to those in untreated, mMCP-7-treated, and pro-EK-mMCP-6-treated BALB/c mice. Nevertheless, neutrophils now represented 54 ± 9% (mean ± SD; $n = 6$) of the total cells in the exudates of the mMCP-6-treated mice. Thus, mMCP-6 induced a significant ($p < 0.001$) >50-fold increase in the number of neutrophils in the peritoneal cavity of this mouse strain. Not buffer alone, pro-EK-mMCP-6, or mature mMCP-7 could induce substantial numbers of neutrophils to accumulate in the peritoneal cavity of a BALB/c mouse. Neutrophils represented only 1 ± 1, 6 ± 2, and 4 ± 4% (mean ± SD or ½ range; $n = 2$ or 3) of the total cells in the peritoneal cavity exudates of BALB/c mice exposed for 36 h to medium alone, medium containing pro-EK-mMCP-6, and medium containing mMCP-7, respectively. The mMCP-6-induced neutrophil accumulation in BALB/c mice was prominent after 7 h, was maximal after 1 day, and persisted for at least 2 days (Fig. 4). As shown in Figure 5, the peritoneal membrane of a BALB/c mouse examined 36 h after mMCP-6 treatment also contained 58-fold more neutrophils than that of the control mouse. When three other mouse strains were examined 36 h after 800 U of mMCP-6 was injected once into the peritoneal cavity of each animal, neutrophils represented 64 ± 1, 50 ± 2, and 50 ± 17% (mean ± ½ range), respectively, of the cells in the peritoneal exudates of mMCP-5-null ($n = 2$), C5-deficient ($n = 2$), and mast cell-deficient $WCB6F_1$-*Sl/Sl*^{d} ($n = 2$) mice. At no time were large numbers of eosinophils, basophils, or platelets detected in the peritoneal exudate of any mMCP-6-treated mouse. Mast cells are normal resident cells of the peritoneal cavity, and their numbers in the lavage fluids were similar in all treated mice.

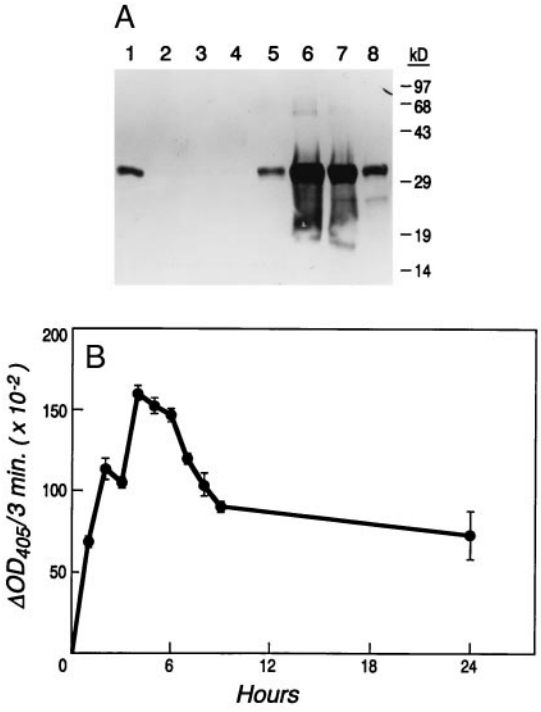

FIGURE 3. Immunoaffinity chromatography and activation of pro-EK-mMCP-6. In *A*, insect cell-conditioned medium (400 ml; *lane 1*) was applied to a 1-ml anti-FLAG IgG column. After the immunoaffinity column was washed to remove nonbound proteins (*lane 2*), the bound pseudozymogen was eluted with 0.1 M glycine, pH 3.0 (lanes 3–8). Samples (10 μl) of the resulting 1.5-ml fractions were subjected to SDS-PAGE/immunoblot analysis with anti-mMCP-6 Ig. Shown on the *right* are the migration positions of the standard m.w. markers. In the activation/stability assay depicted in *B*, pro-EK-mMCP-6 was suspended in pH 5.2 buffer containing EK, and the resulting solution was incubated at 37°C for 1 to 24 h. At the indicated times (●), samples were removed and analyzed for the ability of this recombinant tryptase to cleave tosyl-Gly-Pro-Lys-*p*-nitroanilide at pH 7.4 in a 3-min enzymatic assay. The depicted data are the mean ± range of two experiments.

Table I. *mMCP-6-susceptible peptides obtained in the absence of heparin*[a]

Clones (No.)	Amino Acid Sequence of Peptide
2	Val-Arg-Pro-Val-Lys-Ser-Phe-Arg
1[b]	Ser-Leu-Ser-Ser-Arg-Gln-Ser-Pro
1	Ser-Pro-Arg-Pro-Arg-Ser-Thr-Pro
1	Gln-Arg-Thr-Lys-Arg-Lys-His-Asn
1	Gly-Pro-Arg-Leu-Arg-His-Pro-Arg
1	Asn-Leu-Arg-Lys-Arg-Lys-Ile-Lys
1	Asn-Ser-Thr-Val-Arg-Lys-Arg-Lys
1	Pro-Pro-Pro-Phe-Arg-Arg-Ser-Ser
1	Pro-Leu-Ile-Leu-Arg-Ser-Arg-Ala
1	Lys-Lys-Ile-Glu-Arg-Arg-Asn-Thr
1	Gln-Lys-Arg-Gly-Arg-Glu-Pro-Arg
1	Glu-Glu-Lys-Lys-Lys-His-Lys-Lys
1	Arg-Gln-Asn-Arg-Arg-Pro-Ser-Asn
1	Val-Arg-Pro-Ala-Arg-Ala-Leu-His
1	Leu-Ile-Ala-Leu-Arg-Ser-Thr-Thr
1	Pro-Thr-Pro-Leu-Lys-His-Pro-Arg
1	Pro-Tyr-Pro-Pro-Lys-Arg-Thr-Pro
1	Leu-Ser-Thr-Ser-Arg-Ala-Ser-Ile
1	Thr-Gly-Val-His-Lys-Pro-Ser-Thr
1	Leu-Cys-Ala-Lys-Arg-Leu-Tyr-Arg
1	Arg-Lys-Pro-Thr-Lys-Lys-Asn-Ser
1	Glu-Cys-Arg-Gln-Arg-His-Thr-Arg
1	Ser-Leu-Ala-Leu-Arg-Val-Trp-Arg
1	Gly-Pro-Arg-Leu-Arg-His-Pro-Arg
1	Phe-Ile-Ser-Arg-Arg-Val-Cys-Arg
1	Pro-Asp-Asn-Gln-Arg-Tyr-Ile-Thr
1	Pro-Leu-Pro-Cys-Lys-Leu-Asp-Ala
1	Ile-Arg-Phe-Ala-Arg-Ser-Gln-Ala
1	Pro-Thr-Pro-Leu-Lys-His-Pro-Arg

[a] The tryptase-specific, phage-display library was incubated four times with recombinant mMCP-6 in the absence of heparin. Clones were isolated, and the deduced amino acid sequences of the peptides found in the protease-susceptible domains of the pIII fusion protein were deduced.

[b] This clone was the only one obtained when the library was screened four times with recombinant mMCP-7 (6).

In vitro neutrophil chemotaxis assay and evaluation of cytokine and chemokine expression by human endothelial cells exposed to mMCP-6

Freshly isolated mouse peripheral blood neutrophils could not be induced to migrate within 30 min through a membrane with 8-μm pores when they were exposed briefly to buffer, mMCP-6, mMCP-7, endothelial cells, or the combination of endothelial cells and mMCP-7. However, migration through the membrane did occur when neutrophils were exposed to FMLP or were concomitantly exposed to mMCP-6 and human endothelial cells. In two experiments, 75- and 100-fold more neutrophils were induced to migrate through the membrane when the endothelial cells in the

Table II. *mMCP-6-susceptible peptides obtained in the presence of heparin*[a]

Clones (No.)	Amino Acid Sequence of Peptide
	Two Rounds of Treatment
1	Pro-Phe-Thr-His-Lys-Ser-Leu-Ser
1	Ser-Val-Leu-Pro-Lys-Leu-Arg-Ile
1	Pro-Lys-Glu-Thr-Lys-Gln-Thr-Asn
3	Ser-Leu-Ser-Ser-Arg-Gln-Ser-Pro
5	Thr-Pro-Leu-Leu-Lys-Ser-Trp-Leu
11	Arg-Asn-Arg-Gln-Lys-Thr-Asn-Asn
	Four Rounds of Treatment
1	Pro-Lys-Glu-Thr-Lys-Gln-Thr-Asn
1	Ser-Val-Leu-Pro-Lys-Leu-Arg-Ile
2	Ser-Leu-Ser-Ser-Arg-Gln-Ser-Pro
4	Arg-Asn-Arg-Gln-Lys-Thr-Asn-Asn
7	Thr-Pro-Leu-Leu-Lys-Ser-Trp-Leu

[a] The tryptase-specific, phage-display library was incubated two or four times with recombinant mMCP-6 in the presence of an equal weight amount of heparin. Clones were isolated, and the deduced amino acid sequences of the peptides found in the protease-susceptible domains of the pIII fusion protein were deduced.

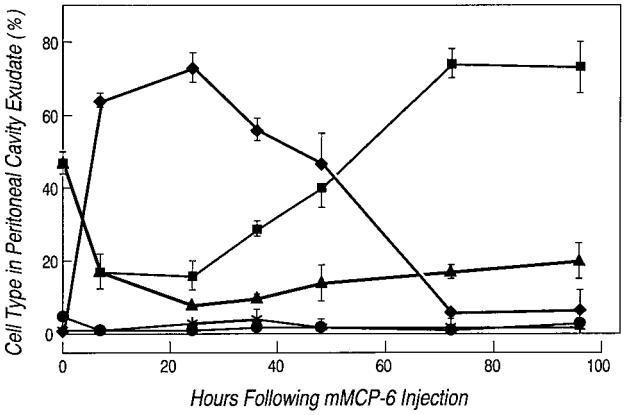

FIGURE 4. Quantitation of neutrophils in the peritoneal cavity of BALB/c mice at various times after treatment with mMCP-6. Mouse MCP-6 (800 U) was injected into the peritoneal cavity of 12 mice. Two mice were killed at each time point, and slides of their peritoneal exudates were prepared. Three separate fields were examined to determine the relative percentages of cells in the exudate of each specimen that were neutrophils (♦), lymphocytes (■), macrophages (▲), mast cells (●), and eosinophils (∗). In each mouse, >300 cells in the peritoneal lavage fluid were examined. The depicted data are the mean of the results (±range) obtained from the two animals at each time point, and from three untreated mice (time zero). As a control, two additional BALB/c mice were given medium alone. When these control mice were examined 36 h later, neutrophils, lymphocytes, macrophages, mast cells, and eosinophils represented 1 ± 1, 59 ± 6, 36 ± 6, 3 ± 1, and 2 ± 1%, respectively, of the cells in their peritoneal lavage fluid.

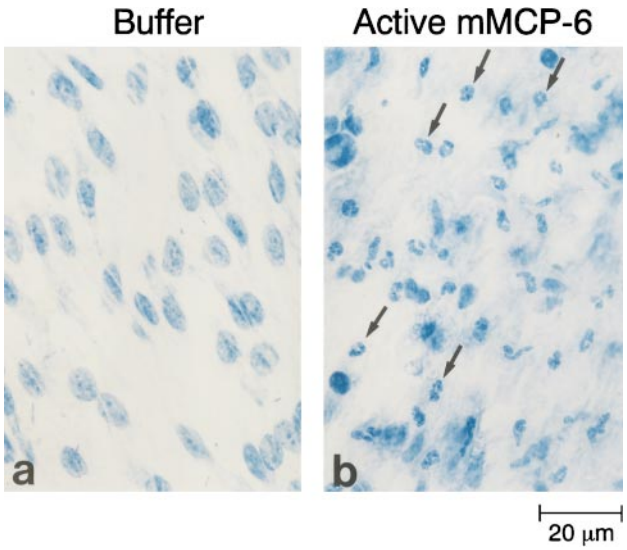

FIGURE 5. Mouse MCP-6-induced attachment of neutrophils to the peritoneal membrane. Buffer (*a*) or 800 U of mMCP-6 (*b*) was injected into BALB/c mice, and 36 h later the peritoneal membrane was removed from each animal and analyzed histochemically. The arrows in *b* point to a few of the many neutrophils bound to the membrane of the mMCP-6-treated mouse. When five separate fields were examined with a ×50 objective, the number of neutrophils per 100 mesothelial cells was 0.7 ± 0.4 (mean ± SD) in the buffer control and 41 ± 6 in the mMCP-6-treated animals.

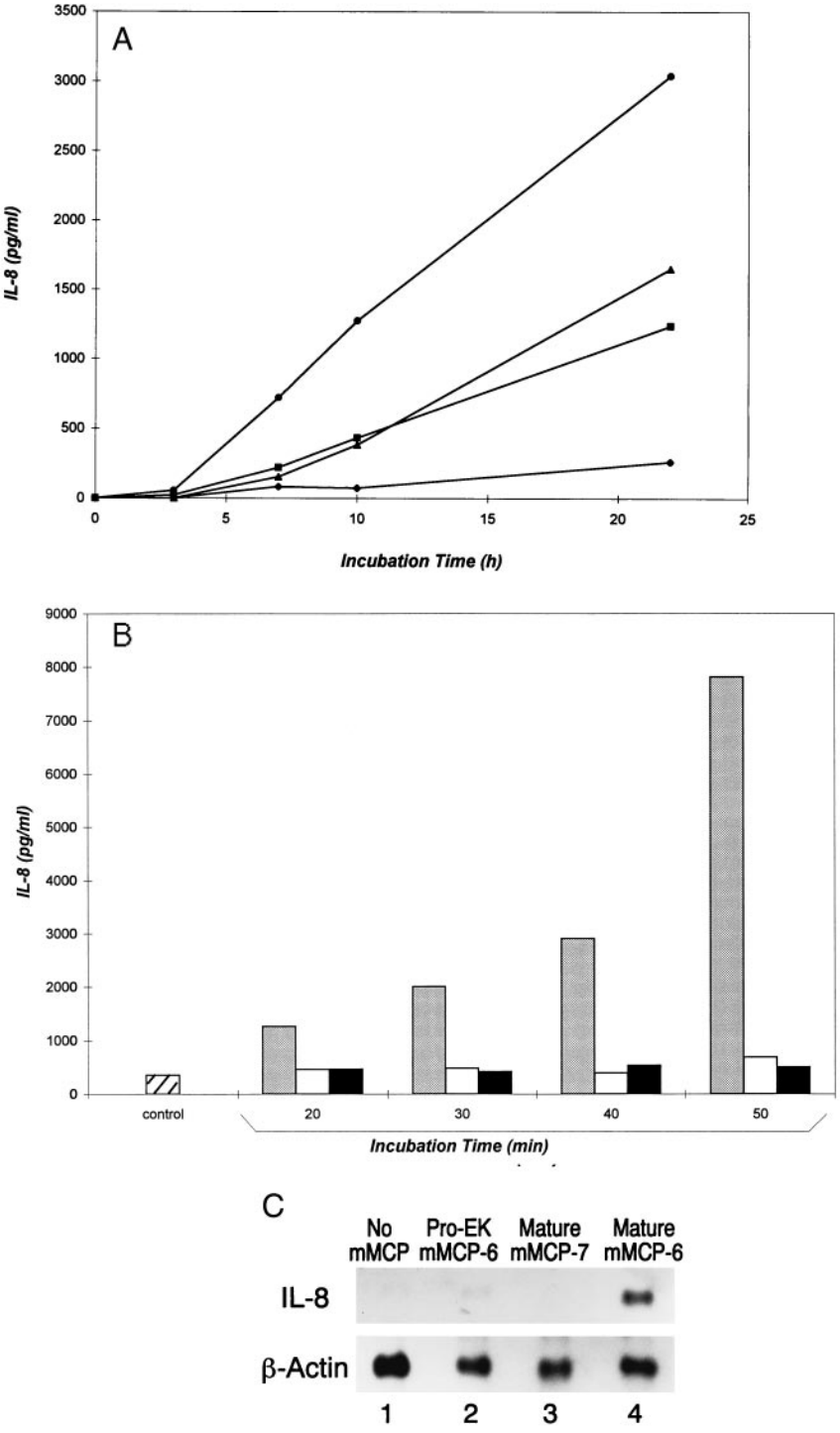

FIGURE 6. Expression of IL-8 by mMCP-6-treated human endothelial cells. *A*, Confluent monolayers of endothelial cells were continually exposed to 0.9 (♦), 4.4 (■), 17 (▲), or 87 (●) U of mMCP-6 for up to 22 h. At the indicated times, the amount of IL-8 protein in the conditioned medium of each culture was determined by ELISA. *B*, Confluent monolayers of endothelial cells were exposed to no mMCP (hatched box), mMCP-6 (shaded boxes), mMCP-7 (open boxes), or pro-EK-mMCP-6 (black boxes) for 20 to 50 min. The mMCP-containing medium was removed from each culture, fresh mMCP-free medium was added, and the cells were incubated for an additional 10 h. Depicted are the levels of IL-8 protein in the resulting 10-h conditioned medium from the different cultures. When the conditioned medium was replaced every 10 h in a separate experiment, the level of IL-8 in the conditioned medium obtained during the 30- to 40-h period after the initial 50-min exposure to mMCP-6 was still >10-fold higher than that of nontreated cells (data not shown). *C*, Blots containing total RNA isolated from human endothelial cells exposed for 2 h to no mMCP (*lane 1*), pro-EK-mMCP-6 (*lane 2*), mMCP-7 (*lane 3*), or mMCP-6 (*lane 4*) were probed with the relevant cDNA to compare the levels of IL-8 mRNA in the four populations of cells relative to those of the β-actin transcript. Similar findings were obtained in a second experiment. The data depicted in *A*, *B*, and *C* are derived from different experiments.

bottom well were exposed to enzymatically active mMCP-6 than when they were exposed to the inactive zymogen.

As assessed by ELISA, the levels of TNF-α, IL-1α, and IL-6 protein produced by the mMCP-6-treated human endothelial cells all were below the limit of detection. However, mMCP-6-treated cells dramatically increased their expression of immunoreactive IL-8 in a dose-dependent manner (Fig. 6*A*). To up-regulate their expression of this chemokine, the endothelial cells only had to be exposed to mMCP-6 for 20 to 50 min (Fig. 6*B*). Moreover, the endothelial cells continued to produce large amounts of immunoreactive IL-8 40 h after the initial 20- to 50-min exposure to mMCP-6. Neither pro-EK-mMCP-6 nor mMCP-7 was able to induce these endothelial cells to increase their expression of the neutrophil chemotactic factor (Fig. 6*B*). No RANTES- or TNF-α-specific DNA fragments were generated from mMCP-6-treated endothelial cells even when the RNA samples were reverse transcribed and subjected to 35 cycles of PCR. In contrast, the IL-8-specific, 227-bp DNA fragment was readily generated when the RT-PCR assay was performed on endothelial cells that had been exposed to mMCP-6 for 1 ($n = 1$), 2 ($n = 2$), or 3 ($n = 1$) h (data

not shown). Furthermore, as assessed by RNA blot analysis (Fig. 6*C*), the level of IL-8 mRNA in mMCP-6-treated cells was considerably higher than that in replicate cells exposed to medium alone, pro-EK-mMCP-6, or mature mMCP-7.

Discussion

A bioengineering approach was used to obtain enzymatically active, recombinant mMCP-6 for study of its function in vivo. By injecting an extremely small amount of mMCP-6 one time into the peritoneal cavity of different mouse strains, we were able to induce a large and selective accumulation of neutrophils that persisted for >2 days. Mouse MCP-6 did not induce neutrophil chemotaxis directly. Rather, it induced the expression of IL-8-like chemokines, which, in turn, induced chemotaxis of neutrophils.

Insect cells infected with the relevant construct secreted pro-EK-mMCP-6 and pro-mMCP-6 (Fig. 1) into the conditioned medium. As assessed by heparin-Sepharose chromatography (Fig. 2), most of the mMCP-6 pseudozymogen secreted into the conditioned medium by the insect cell is properly folded. Although recombinant (6) and native (19) mMCP-7 exhibit good catalytic activity in the absence of heparin, it has been reported that a human mast cell tryptase purified from the lung does not exhibit substantial enzymatic activity unless this glycosaminoglycan is present in the assay (32, 33). The ability to purify pro-EK-mMCP-6 from the conditioned medium by means of the immunoaffinity column (Fig. 3) allowed us to determine whether the mouse tryptase exhibits enzymatic activity in the absence of heparin. The finding that the EK-activated tryptase readily cleaved tosyl-Gly-Pro-Lys-*p*-nitroanilide and tosyl-Gly-Pro-Arg-*p*-nitroanilide in the absence of heparin indicates that the broad catalytic activity of this tryptase is not dependent on heparin-containing serglycin proteoglycans.

The observation that recombinant mMCP-6 cleaves acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide less efficiently than recombinant mMCP-7 in vitro suggested that the two mouse tryptases might have different substrate specificities even though their overall amino acid sequences are 71% identical. Thus, the substrate phage display library that helped us identify a physiologic substrate of mMCP-7 (6) was used to identify mMCP-6-preferred peptide substrates. When the library was subjected to four rounds of treatment with enzymatically active mMCP-6 in the absence of heparin glycosaminoglycan, no specific peptide sequence in the hypervariable domain of the pIII fusion protein was obtained in the 30 arbitrarily selected clones (Table I). Nevertheless, the observation that only one of these mMCP-6-susceptible clones had the preferred mMCP-7-susceptible sequence in its pIII fusion protein was further evidence that the two homologous tryptases degrade very different substrates. The two most prominent features of the peptides obtained by screening the library with mMCP-6 alone were the over- and under-representation of positively and negatively charged residues, respectively. These findings are consistent with the electrostatic properties of mMCP-6, which predicted that the substrate-binding pocket of mMCP-6 is more negatively charged than that in mMCP-7 (19). The difference in the electrostatic potential of the pocket is due primarily to loop 3 which has a −3 net charge in mMCP-6 and a 0 net charge in mMCP-7.

When the substrate phage display library was screened with a mMCP-6/heparin binary complex, a more limited number of sequences was obtained (Table II). Surprisingly, the two clones that were obtained repeatedly had dissimilar sequences of Arg-Asn-Arg-Gln-Lys-Thr-Asn-Asn and Thr-Pro-Leu-Leu-Lys-Ser-Trp-Leu. The latter favored peptide and the other less favored peptides obtained in the selection process with the mMCP-6/heparin complex were similar in that each had a Pro residue, at least one Thr or Ser residue, and a net charge of only +1 or +2. The discovery that the favored peptide in this series had a Pro residue at its P4 site is of interest because Cromlish and co-workers (34) noted that a human mast cell tryptase purified from the pituitary will cleave three prohormones ex vivo that have Pro residues at their P4 sites and Lys/Arg residues at their P1 sites.

Despite these findings, the other favored peptide from the substrate phage display library that possesses a +3 charge might be more physiologically relevant because its overall charge is more similar to that obtained when the library was screened with mMCP-6 alone. Why only one peptide with a +3 charge was obtained and why this peptide was not present in the original 30 clones isolated when the library was screened with just mMCP-6 remains to be determined experimentally. However, the electrostatic potential of the three-dimensional model of mMCP-6 suggests that the putative heparin binding domain on the surface of this tryptase resides closer to its active site than in all other mMCPs. Thus, it is likely that heparin glycosaminoglycan sterically restricts the substrate binding cleft of mMCP-6 by directly influencing one or more of the seven loops that form the pocket. The discovery that the substrate specificity of a rat mast cell chymase is also altered by heparin glycosaminoglycan (35) emphasizes the importance of the glycosaminoglycan side chains of serglycin proteoglycans in fine-tuning the substrate specificities of certain members of the chromosome 14 and chromosome 17 families of serine proteases. Although it remains to be determined which proteins are the physiologic substrates of mMCP-6, the phage display library data document that mMCP-6 and mMCP-7 have different substrate specificities. Because analysis of the substrate phage display library revealed that heparin can influence the substrate specificity of mMCP-6, the biologic activity of mMCP-6 in most experiments was evaluated in the presence of heparin. Like mMCP-7, mMCP-6 exhibited optimal enzymatic activity at approximately pH 7.4.

We previously noted that mMCP-7 is resistant to inactivation by the diverse array of protease inhibitors in the blood of the mouse (19). The finding that the enzymatic activity of mMCP-6 was inhibited completely after a 30-min incubation at 37°C or a 1.5-h incubation at room temperature in the presence of 50% mouse serum confirms other data indicating that mMCP-6 and mMCP-7 are functionally different enzymes. This finding also suggests that even if some exocytosed mMCP-6 is able to leave an inflamed tissue site, it probably would not stay enzymatically active for long in the circulation. Although it remains to be determined what are the inhibitors in mouse serum that selectively inactivate mMCP-6, the rat homologue of mMCP-6 readily binds the inter-α-trypsin inhibitor (36, 37) and α_1m (38) families of protease inhibitors.

The mast cells that reside in the peritoneal cavity of the BALB/c mouse express mMCP-6, but not mMCP-7 (9). Because this observation suggested that mMCP-6 cleaves specific proteins that reside in the peritoneal cavity or its membrane, enzymatically active mMCP-6 was injected into the peritoneal cavity of the BALB/c mouse to assess whether the recombinant tryptase could induce a detectable biologic response in vivo. Seven hours after mMCP-6 administration, a pronounced accumulation of neutrophils occurred in the peritoneal cavity of this mouse strain, as typically seen in acute inflammatory responses (39). However, unlike inflammatory responses induced by LPS, the mMCP-6-induced accumulation of neutrophils persisted for >2 days (Fig. 4). Thus, mMCP-6 induced a selective and persistent accumulation of neutrophils. The fact that pro-EK-mMCP-6 could not induce the peritonitis indicates that the inflammatory reaction is dependent on

enzymatically active mMCP-6. The finding that very few neutrophils extravasated into the peritoneal cavity of BALB/c mice exposed to 2.5-fold more mMCP-7 than mMCP-6 documents the specificity of the tryptase effect. The reason that large numbers of lymphocytes, monocytes, and/or eosinophils do not extravasate into the peritoneal cavity of mMCP-6-treated mice could be the proteolytic inactivation of the chemokines that recruit these three populations of cells. Alternatively, mMCP-6 could induce the local expression of a factor that selectively attracts neutrophils or a factor (e.g., a binding protein) that selectively inactivates all chemoattractants except those that recruit neutrophils.

Neutrophils generally undergo apoptosis within 24 h after they extravasate into an inflammatory site (39). The presence of large numbers of neutrophils in the peritoneal cavity 2 days after a single injection of mMCP-6 probably is due to the persistent release of neutrophil-specific chemotatic factors from endothelial cells in the blood vessels lining the peritoneal membrane, from the mesothelial cells in the peritoneal cavity, or from the resident macrophages in the peritoneal cavity. However, it is also possible that the extravasated neutrophils persist in the mMCP-6-treated mice in part because their apoptosis is delayed. Coxon and co-workers (40) recently demonstrated that the neutrophils that extravasate into the peritoneal cavity of thioglycolate-treated CD11b-null mice do not undergo rapid apoptosis. Using Ab specific for β_2 integrins, these investigators demonstrated that phagocytosis and generation of an oxidative burst through the CD11b/CD18 integrin is needed for the efficient induction of apoptosis of this leukocyte. In our model, the neutrophils that extravasated into the peritoneal cavity of mMCP-6-treated mice might be longer lived because of their failure to come in contact with opsonized particles that bind to the CD11b/CD18 integrin on the surface of the neutrophil.

Mouse MCP-6 induced similar numbers of neutrophils to extravasate into the peritoneal cavity of BALB/c, mMCP-5-null, C5-deficient, and mast cell-deficient WCB6F$_1$-*Sl/Sl*^{d} mice. Certain populations of mouse mast cells activated through their high affinity Fc receptors for IgE express leukotriene B_4 (41), platelet-activating factor (42), and a large number of cytokines and chemokines (43–47). That mMCP-6, by itself, can induce neutrophil extravasation into the peritoneum of a mast cell-deficient mouse strain indicates that the tryptase does not have to induce resident mast cells to release neutrophil-specific chemoattractants. C5a anaphylatoxin is a potent neutrophil chemotactic factor (48) that is normally generated by a tryptic cleavage at Arg^{751} of its 190-kDa precursor protein by C5 convertase. The finding that neutrophils also accumulate in the peritoneal cavity of the mMCP-6-treated, C5-deficient mouse indicates that mMCP-6 is not functioning primarily as a C5 convertase.

In vitro studies revealed that in the absence of endothelial cells, mMCP-6 was unable to induce freshly isolated mouse neutrophils to migrate within 30 min through a membrane with 8-μm pores. This observation coupled with the findings that mMCP-6 is inactivated by factors in the blood and that very little free or complexed mMCP-6 can be found in the blood of mice undergoing systemic anaphylaxis (19) suggest that the mMCP-6-induced extravasation of neutrophils into the peritoneal cavity of the BALB/c mouse is not primarily a consequence of the tryptase acting directly on the circulating neutrophil. By themselves, the endothelial cells used in the in vitro assay did not induce neutrophil chemotaxis. However, chemotaxis did occur when neutrophils were concomitantly exposed in vitro to mMCP-6 and endothelial cells.

Generally, neutrophil extravasation into tissues is dependent on the CXC family of chemokines (30, 49–52). While IL-8/NAP-1 is the most potent neutrophil chemoattractant in humans, other CXC chemokines (e.g., neutrophil-activating peptide-2, epithelial cell-derived neutrophil-activating peptide-78, and growth-related oncogene α, β, and γ) also are potent chemoattractants for human neutrophils. One human chemokine receptor is relatively IL-8 specific, whereas the other recognizes numerous members of the IL-8 chemokine family (53). The precise number of IL-8-like chemokines in the mouse has not been determined, but there appears to be only one IL-8-like receptor in this species, and the acute migration of neutrophils into the thioglycolate-treated peritoneum is severely compromised in transgenic mice lacking this chemokine receptor (54).

Because mouse IL-8 has not been cloned and because ELISAs to this and related mouse chemokines are not currently available, we evaluated the ability of mMCP-6 to induce human endothelial cells to increase their expression of an IL-8-like chemokine that would interact with the only receptor on the surface of the mouse neutrophil that reacts with this family of chemokines. As assessed by ELISA and/or RT-PCR, mMCP-6-treated endothelial cells did not produce TNF-α, RANTES, IL-1α, or IL-6. Nevertheless, these treated cells dramatically increased their expression of IL-8 protein and mRNA (Fig. 6). Cairns and Walls (55) noted that a mast cell tryptase preparation isolated from human lung tissue also can induce a cultured human epithelial cell line to increase its expression of IL-8. Four cDNAs have been isolated by two groups (13–15) that encode highly homologous human mast cell tryptases (designated tryptases I, II/β, III, and α). While it remains to be determined whether these human tryptases are derived from distinct genes or are allelic variants of a smaller number of genes, two distinct mast cell tryptase genes exist in varied inbred mouse strains (2, 3, 7–9). Even though the amino acid sequences of mMCP-6 and mMCP-7 are 71% identical, mMCP-7 was unable to induce neutrophil extravasation and was unable to induce human endothelial cells to produce IL-8. Because mMCP-6 and mMCP-7 often are coexpressed in the same mast cell in many tissues (2, 3, 9, 10, 19), caution now must be exercised in drawing conclusions about the function of a mast cell tryptase that has not been evaluated in its recombinant form.

Mouse MCP-6 could cleave receptors on the plasma membrane of the endothelial cell (e.g., the thrombin receptor) (56) or receptor-associated proteins in the matrix (e.g., fibronectin) (57), thereby inducing a signal transduction cascade that ultimately results in the increased transcription of the IL-8 gene or the increased stabilization of its transcript. Because human IL-8 often is activated after a tryptic-like cleavage of its precursor at Arg^{28}, mMCP-6 also could act directly on the precursor of this chemokine. Whatever the mechanism(s), the finding that levels of IL-8 are increased in the lungs of patients with asthma and other lung disorders (58–61) now raises the possibility that the level of this chemokine in certain allergic situations is regulated in part by the human homologue of mMCP-6. Because mMCP-6 induces neutrophil extravasation and because mMCP-7 inhibits the formation of fibrin/platelet clots, these two homologous tryptases appear to have evolved in the mouse to work in concert with mast cell-derived TNF-α (62) and leukotriene B_4 (41) to facilitate the influx of neutrophils into inflammatory sites.

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