

Evaluation of the Substrate Specificity of Human Mast Cell Trypsase β I and Demonstration of Its Importance in Bacterial Infections of the Lung*

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Human pulmonary mast cells (MCs) express tryptases α and β I, and both granule serine proteases are exocytosed during inflammatory events. Recombinant forms of these tryptases were generated for the first time to evaluate their substrate specificities at the biochemical level and then to address their physiologic roles in pulmonary inflammation. Analysis of a tryptase-specific, phage display peptide library revealed that tryptase β I prefers to cleave peptides with 1 or more Pro residues flanked by 2 positively charged residues. Although recombinant tryptase β I was unable to activate cultured cells that express different types of protease-activated receptors, the numbers of neutrophils increased >100-fold when enzymatically active tryptase β I was instilled into the lungs of mice. In contrast, the numbers of lymphocytes and eosinophils in the airspaces did not change significantly. More important, the tryptase β I-treated mice exhibited normal airway responsiveness. Neutrophils did not extravasate into the lungs of tryptase α -treated mice. Thus, this is the first study to demonstrate that the two nearly identical human MC tryptases are functionally distinct *in vivo*. When MC-deficient W/W^v mice were given enzymatically active tryptase β I or its inactive zymogen before pulmonary infection with *Klebsiella pneumoniae*, tryptase β I-treated W/W^v mice had fewer viable bacteria in their lungs relative to zymogen-treated W/W^v mice. Because neutrophils are required to combat bacterial infections, human tryptase β I plays a critical role in the antibacterial host defenses of the lung by recruiting neutrophils in a manner that does not alter airway reactivity.

Mast cells (MCs),¹ which reside in connective tissue matrices and epithelial surfaces, are effector cells that participate in innate (1–4) and acquired immunity. All populations of MCs examined to date store in their secretory granules various combinations of carboxypeptidase A, chymases, and tryptases ionically bound to serglycin proteoglycans that contain either heparin or highly sulfated chondroitin glycosaminoglycans. Human MCs express at least five distinct tryptases, and the cDNAs and genes have been isolated that encode human tryptases α , β I, β II, and β III, as well as a less homologous transmembrane tryptase (5–10). Mapping and sequencing analysis of the region of human chromosome 16, where these genes reside, has revealed the presence of additional genes in the family (9, 11), but it remains to be determined whether or not any of these genes encode enzymatically active proteases. Splice variants of different human tryptase transcripts also have been identified that are predicted to encode proteases with altered substrate-binding clefts and therefore different specificities (12). Three homologous tryptases (designated mouse MC protease (mMCP) 6 (13, 14), mMCP-7 (15), and transmembrane tryptase (8)) have been identified in mouse MCs. The cDNAs and genes that encode these neutral proteases reside in the region of mouse chromosome 17 (8, 16, 17) syntenic to human chromosome 16. A fourth member of this family of mouse tryptases has been cloned (18), but has yet to be identified in any MC population.

On the basis of *in vitro* studies, protease-activated receptor (PAR) 2 (19–23) and more than a dozen other proteins have been proposed to be the physiologic substrates of what was previously thought to be only one enzymatically active human tryptase. Although the primary function(s) of each human tryptase remains to be determined, it is apparent that the individual mouse tryptases are metabolized differently during Fc ϵ receptor I-mediated inflammatory reactions (24) and are functionally distinct (25, 26). The discovery that mMCP-6 and mMCP-7 can form homotypic and heterotypic tetramer complexes (27) alters the interpretation of earlier functional studies carried out with non-recombinant tryptases because heterotypic and homotypic tetramers probably degrade extracellular proteins quite differently.

In 1996, two groups (1, 2) independently used MC-deficient WBB6F1/J-Kit^W/Kit^{W^v} (W/W^v) mice to demonstrate the global importance of MCs in combating chronic, life-threatening bac-

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¹ The abbreviations used are: MC, mast cell; mMCP, mouse mast cell protease; PAR, protease-activated receptor; IL, interleukin; IL-8R, interleukin-8 receptor; BAL, bronchoalveolar lavage.

terial infections of the lung and peritoneal cavity. The cytokine tumor necrosis factor- α was reported to be the primary MC-derived factor in this innate immunologic response. Nevertheless, it quickly became apparent that other undefined factors that are more restricted to MCs had to be involved because many cell types produce tumor necrosis factor- α , because immunologically activated MCs produce substantial amounts of tumor necrosis factor- α for only a brief period of time (28–30), and because MC activation improves host defenses against bacterial infections even in tumor necrosis factor- α -deficient mice (31).

The MCs in the peritoneal cavity store substantial amounts of the tryptase mMCP-6 in their secretory granules (13). In 1998, we reported that large numbers of neutrophils are preferentially recruited into the peritoneal cavities of mice given a single dose of ~ 0.2 nmol of insect cell-derived, recombinant mMCP-6 (26). Hallgren *et al.* (32) reported similar findings recently with recombinant mMCP-6 generated in the human kidney cell line 293-EBNA. No factor has been found to be as selective, potent, and long lasting as mMCP-6 in terms of its ability to induce neutrophil influx into the peritoneal cavities of mice. The primary function of neutrophils is to kill bacteria. Although not proven experimentally, the finding that mMCP-6 regulates neutrophil recruitment *in vivo* implies that this tryptase participates in the early stages of the body's global defense against bacteria. Using the mouse as an experimental model and using recombinant material, we now demonstrate that human tryptase β I also selectively recruits neutrophils into the lung. The ability of human tryptase β I to rescue the pulmonary antibacterial deficiency of MC-deficient mice in a manner that does not compromise airway reactivity documents for the first time the functional significance of the tryptase β I-mediated neutrophil extravasation.

EXPERIMENTAL PROCEDURES

Generation of Recombinant Human Tryptases α and β I and Analysis of the Substrate Specificity of Homotypic Tetramer Complexes of Human Tryptase β I Bound to Heparin—The human tryptases α and β I cDNAs were isolated by reverse transcriptase-polymerase chain reaction treatment of a commercial source of whole lung RNA derived from a single individual. Expression constructs were then prepared that encode pseudo-zymogen forms of these two tryptases that both have a 5-residue enterokinase-susceptible peptide between the natural propeptide and the mature portion of the enzyme and an 8-residue FLAG peptide attached to the C terminus. The resulting cDNAs were subcloned into the expression vector pVL1393, and the recombinant pseudo-zymogens were expressed in baculovirus-infected High Five[®] insect cells as previously described for human tryptase α , mMCP-6, and mMCP-7 (25, 26, 33). After their purification, the mature forms of the two human tryptases were obtained by proteolytic removal of the bioengineered propeptides. The enzymatic activities of the resulting recombinant tryptases were monitored in preliminary studies with the chromogenic substrate tosyl-Gly-Pro-Lys-p-nitroanilide and by measuring the incorporation of [³H]diisopropyl fluorophosphate (Amersham Pharmacia Biotech) into each activated enzyme (33).

As noted below, tryptase β I functions in a manner similar to mMCP-6 in the lung, but not in the peritoneal cavity. Therefore, a phage display peptide library screening approach (25, 26) was used to more precisely define the substrate specificity of recombinant human tryptase β I when it is bound to heparin glycosaminoglycan as a homotypic tetramer. A new library was created with the approach we previously described (25) to evaluate the substrate specificities of recombinant mMCP-6 and mMCP-7. Each phage in the library has a modified pIII protein on its surface that contains at its N terminus the FLAG peptide followed by an 8-residue hypervariable peptide. To make the library more tryptase-specific, the fifth residue in the bait peptide was fixed as either Lys or Arg. The FLAG peptide was again selected as the tether ligand so that those phage that contained the bioengineered pIII protein could be isolated readily with an immunoadfinity column containing anti-FLAG M1 antibody. After five rounds of screening, *Escherichia coli* bacteria were infected with the resulting tryptase β I-susceptible phage to generate phagemids. DNA were isolated from 22

arbitrarily selected clones. A polymerase chain reaction/sequencing approach was then used to determine the nucleotide sequence that encodes the 8-mer tryptase β I-susceptible peptide domain in each pIII fusion protein.

Immunoblot and N-terminal Amino Acid Analysis—Samples of conditioned medium from insect cells infected with the relevant baculovirus construct or the protein purified from the affinity column were diluted in SDS-polyacrylamide gel electrophoresis buffer (1% SDS, 5% β -mercaptoethanol, 0.1% bromophenol blue, and 500 mM Tris-HCl, pH 6.8) and boiled for 5 min before being loaded onto a 12% polyacrylamide gel. After electrophoresis, the gels were stained with Coomassie Blue or were placed in a Bio-Rad immunoblotting apparatus. The resolved proteins were transferred to an Immobilon-P membrane, and the protein blots were probed with affinity-purified mouse anti-FLAG M2 antibody, as previously described for other recombinant tryptases (25). For N-terminal amino acid analysis, SDS-polyacrylamide gel electrophoresis protein blots were briefly stained with Ponceau S red (Sigma). The visualized protein bands were isolated and then subjected to automated Edman degradation.

Biologic Effect of Recombinant Human and Mouse Tryptases in the Peritoneal Cavity and Lung—Samples (100 μ l each) of purified recombinant mMCP-6, mMCP-7, human tryptase α , or human tryptase β I (~ 2000 units, corresponding to ~ 50 μ g of protein or ~ 1 nmol of active enzyme) or their inactive pseudo-zymogens (50 μ g of protein) bound to heparin ($\sim 10:1$ (w/w) tryptase/heparin) were injected separately into the peritoneal cavities of BALB/c mice, C57BL/6 mice, or transgenic mice unable to express an interleukin (IL)-8 receptor homolog (34) (Jackson Laboratories, Bar Harbor, ME). The commercial heparin used in this study had been subjected to an additional CsCl density gradient centrifugation purification step (26) to denature and remove any trace protein and lipid contaminants that might be present in Sigma's preparation of this glycosaminoglycan. 24–48 h after tryptase treatment, the mice were killed by CO₂ asphyxiation, and 10 ml of Tyrode's buffer containing 0.1% (w/v) gelatin was injected into the peritoneal cavity of each mouse. The injected fluid was aspirated; the cells in the peritoneal lavage fluid were counted with a hemocytometer; and 100- μ l samples of the fluid were centrifuged for 5 min at $28 \times g$ onto glass slides. The slides were stained with Diff-Quik[®] (Dade Behring, Newark, DE).

For the instillation of a recombinant tryptase or vehicle control into the lungs, mice were anesthetized with an intramuscular injection of ketamine hydrochloride (40–60 mg/kg) and xylazine (5–10 mg/kg) (35, 36). When an acceptable stage of anesthesia was achieved, the area was disinfected with an iodine-based antiseptic, and a midline incision of the neck was performed to expose the trachea. A 50- μ l bolus of phosphate-buffered saline containing 0.2 nmol of either control (tryptase zymogen) or active mature tryptase was instilled into the trachea with a 100- μ l Hamilton syringe. The margins of the incision were then closed with surgical clips. Mice used as negative controls received intratracheal instillations of buffer alone or buffer containing protryptase β I bound to heparin glycosaminoglycan. 24–48 h after administration of the relevant mouse or human tryptase, each mouse was anesthetized, and its trachea was cannulated. After the mouse was killed by exsanguination, its lungs were lavaged with 2 ml of phosphate-buffered saline. Each lung was fixed with paraformaldehyde, embedded in paraffin, and sectioned. The resulting sections and cytospins of the bronchoalveolar lavage (BAL) fluids were evaluated histochemically for their granulocytes and lymphocytes. To ensure that the instilled liquid bolus would distribute itself relatively evenly throughout the lung, a control experiment was carried out in which 50 μ l of a 0.4% solution of trypan blue was injected into the trachea of a mouse. Analysis of tissue sections revealed that the dye was distributed throughout the lungs.

*Analysis of the Airway Reactivity of Tryptase β I-treated Mice and Evaluation of the Ability of Tryptase β I-treated W/W^o Mice to Combat Infection with *Klebsiella pneumoniae**—The airway responsiveness of tryptase-treated mice was measured as previously described (35, 36) 1 day after airway challenge. Methacholine dose-response curves were obtained by intravenous administration of sequentially increasing doses of methacholine (33–1000 μ g/kg) in 20–35- μ l volumes. Each dose-response curve was log-transformed and then subjected to regression analysis to interpolate the dose required for a 2-fold increase in lung resistance (R_L) (log ED₂₀₀ R_L). This dose, referred to as the effective dose required to increase R_L to 200% of control values (ED₂₀₀ R_L), was used as an index of airway responsiveness.

MC-deficient W/W^o and MC-sufficient C57BL/6 mice (Jackson Laboratories) were also used to study the antimicrobial effects of human tryptase β I in the lungs. Mice were anesthetized by intramuscular injection of ketamine hydrochloride (100 mg/kg) and acepromazine maleate (5 mg/kg); the trachea was surgically exposed; a catheter was

inserted through the trachea into the left bronchus; and a mixture containing 25 μ l of a tryptase preparation (\sim 10 μ g of either inactive proenzyme or active enzyme) and 25 μ l of sterile saline was instilled. A second instillation, 24 h after the first, contained 25 μ l of the same enzyme preparation and 25 μ l of *K. pneumoniae* (strain KP1415) suspended in sterile saline at a density of 4.8×10^8 colony-forming units/ml. This bacterial strain was specifically chosen because it had been used in a previous study by others to evaluate the role of MCs in innate immunity (2). Control studies demonstrated that none of the tryptase preparations, at the doses used in the *in vivo* bacterial infection studies, directly affected the viability of KP1415 bacteria *in vitro*. 6 h after the bacteria were instilled, the mice were killed by a lethal overdose of halothane anesthesia. The lungs were excised, homogenized, serially diluted, and plated on 5% sheep blood agar. After an overnight incubation, colonies were counted, and the resulting viable bacteria were expressed as colony-forming units/lung.

Evaluation of the Ability of Recombinant Human Tryptase β I to Activate Cells via PAR1, PAR2, PAR3, and/or PAR4—Cells that differ in their expression of the four known PARs were used to determine whether or not recombinant human tryptase β I can activate cells via one of these surface receptors. HEK-293 human kidney fibroblasts express the transcripts that encode PAR1, PAR2, and PAR3, but not PAR4 (37). These cells also are responsive to PAR1 and PAR2 peptide agonists (38). HEK-293T cells are derivatives of HEK-293 cells and have been induced to express the simian virus 40 large tumor antigen (39, 40). These cells were chosen for study because fluorescence-activated cell sorter analysis revealed that HEK-293T cells express more PAR2 on their surface than do the parent cells. HEK-293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Dami cells (human megakaryoblastic cells (41) that express PAR1, PAR3, and PAR4, but not PAR2 (42, 43)) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin.

For determination of the agonist-mediated changes in the cytosolic levels of Ca^{2+} in HEK-293T cells, nearly confluent cultures were loaded with 5 μ M Fura-2/AM (Molecular Probes, Inc., Eugene, OR) in complete medium for 1 h. The labeled cells were released from the culture dishes in a non-proteolytic manner with 1 mM EDTA in phosphate-buffered saline. The detached cells were washed, resuspended in Dulbecco's modified Eagle's medium, incubated for 15 min, washed again, and resuspended at a density of 1×10^6 cells/ml in RPMI 1640 medium lacking phenol red. The level of cytosolic Ca^{2+} in Dami cells was quantitated as described for HEL cells (44). Changes in Fura-2 fluorescence were measured with an SLM-AMINCO Model AB2 fluorescence spectrophotometer within 30 min (45). Reagents were added serially to the cuvette in those experiments in which cells were sequentially treated with multiple agonists and/or antagonists. Cell lines were exposed to thrombin (20 nM); the PAR1/PAR2 agonist peptide Ser-Phe-Leu-Leu-Arg-Asn (100 μ M); comparable amounts (33 units) of enzymatically active recombinant tryptase β I, tryptase α , mMCP-6 (26), mMCP-7 (25), or trypsin (Sigma); or similar weight amounts of enzymatically inactive protryptase β I or protryptase α . In some experiments, HEK-293T cells were exposed to PAR1-blocking antibodies (43) or irrelevant control antibodies before treatment with thrombin. Calcium was measured with prewarmed reagents, and incubations were carried out at 37 $^{\circ}$ C.

RESULTS

Generation of Recombinant Human Tryptases α and β I and Evaluation of the Substrate Specificity of Tryptase β I—Recombinant pseudo-zymogen forms of human tryptases α and β I were expressed in insect cells and activated by enterokinase after their purification. Recombinant protryptase α (data not shown) and protryptase β I (Fig. 1a) were both secreted into the conditioned medium as properly folded monomers on the basis of their ability to bind to heparin-Sepharose in a conformation-dependent manner (46). The fact that both secreted proteins possess an N-terminal amino acid sequence of Ala-Pro-Gly-Gln-Ala-Leu-Gln indicated that the natural hydrophobic signal peptides of the two human tryptase pseudo-zymogens were properly removed by the insect cells. Like the recombinant mouse tryptases we previously generated, the bioengineered propeptides were readily removed after enterokinase treatment to create functional human tryptases. However, unlike recombinant mMCP-6 (26) and mMCP-7 (25), heparin glycosaminogly-

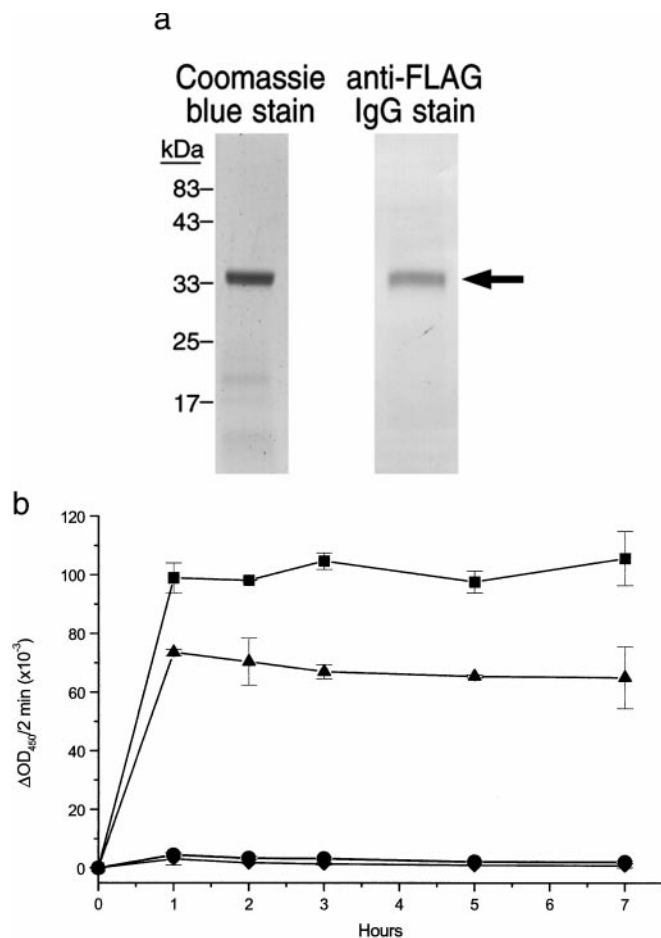


FIG. 1. Biochemical and functional analysis of recombinant human tryptase β I. *a*, expression of recombinant human tryptase β I. Samples of conditioned medium from insect cells infected with the construct that encodes recombinant tryptase β I were applied to individual wells of replicate polyacrylamide gels. After SDS-polyacrylamide gel electrophoresis, one gel was stained with Coomassie Blue. The protein blot prepared from the duplicate gel was stained with anti-FLAG antibody. Prestained protein molecular mass markers are shown on the left. The arrow on the right indicates human tryptase β I monomer. Although some immunoreactive fragments of this tryptase were found inside the expressing insect cells (data not shown), only intact human tryptase β I was secreted. *b*, evaluation of the enzymatic activity of recombinant human tryptase β I. Recombinant human tryptase β I was activated at 37 $^{\circ}$ C (● and ▲) or 22 $^{\circ}$ C (■ and ◆) for up to 7 h in the presence (■ and ▲) or absence (● and ◆) of heparin glycosaminoglycan. The ability of each preparation of the recombinant tryptase to cleave the colorimetric substrate in the 2-min assay was then evaluated by monitoring the change in absorbance at 450 nm (ΔOD_{450}). Two analyses were performed at each time with each sample; the depicted data are the means \pm range. In the absence of heparin, the amount of enzymatically active tryptase β I generated at 37 $^{\circ}$ C was virtually identical to that generated at 22 $^{\circ}$ C at all time points.

can was required for the generation of enzymatically active human tryptase β I and for the maintenance of its activity for >7 h (Fig. 1b).

Analysis of a tryptase-specific, phage display peptide library revealed that the homotypic tetramer form of tryptase β I bound to heparin prefers to cleave peptides with 1 or more Pro residues flanked by 2 positively charged residues (Table I). 16 of the 22 susceptible peptides obtained from the library had a Pro residue at the P2, P3, P4, or P5 site. The exact position of the Pro residue in the N-terminal portion of the susceptible peptide does not appear to be critically important. However, the fact that the susceptible peptides lack a positively or negatively charged residue at the P2 site implies that additional residues also influence substrate susceptibility. These data are in agree-

TABLE I
Human tryptase β I-susceptible peptides isolated from a phage display peptide library

The phage display peptide library was incubated five times with recombinant human tryptase β I in the presence of an approximately equal weight amount of heparin glycosaminoglycan. 22 susceptible clones were arbitrarily selected after this screening process, and then the amino acid sequences of the 8-mer peptides present in the bait regions of their pIII fusion proteins were determined. The bait regions in two of the clones were identical. The conserved Lys residue at the end of the FLAG peptide is depicted, as well as the conserved Ala residue at the beginning of pIII. In those peptides that contain more than 1 basic amino acid (shown in boldface) in the bait region, it is not clear which is the P1 residue. Nevertheless, the peptides are aligned on the basis of the likely site of cleavage, assuming that the Pro residue (shown in italics) resides at the P2, P3, P4, or P5 site.

Frequency	Probable P1 residue ↓
(1)	FLAG- Lys -Ala-Gly-Ser-Pro- Lys -Glu-Pro-Phe-Ala-pIII protein
(1)	FLAG- Lys -Leu-Pro-Pro- Arg - Lys -Ala-Ser-Leu-Ala-pIII protein
(2)	FLAG- Lys - Arg -Cys-Pro-Ser- Lys -Gly-Asp-Glu-Ala-pIII protein
(1)	FLAG- Lys -Thr-Leu-Pro-Leu- Arg -Leu-Gln-Phe-Ala-pIII protein
(1)	FLAG- Lys -Cys-Pro-Thr- Lys - Lys -Pro- Arg -Phe-Ala-pIII protein
(1)	FLAG- Lys -Gln-Thr-Gln-Met- Lys -Pro-Gln- Lys -Ala-pIII protein
(1)	FLAG- Lys -Glu- Arg -Pro-Tyr- Lys -Trp- Lys -Phe-Ala-pIII protein
(1)	FLAG- Lys -Asp-Leu-Pro-Leu- Arg -Ala-Ala-Thr-Ala-pIII protein
(1)	FLAG- Lys - Lys -Pro-Pro-Gly- Arg -Ala-Val-Ile-Ala-pIII protein
(1)	FLAG- Lys -Val-Pro-Phe-Pro- Lys -Ile-Ala-His-Ala-pIII protein
(1)	FLAG- Lys -Leu-Pro-Val-Ser- Arg -Asn-His-Ser-Ala-pIII protein
(1)	FLAG- Lys -Leu-Pro-Ile-Tyr- Arg -Thr-Asn-Pro-Ala-pIII protein
(1)	FLAG- Lys -Pro-Phe-Thr-Trp- Arg -Pro-Ser-Ala-Ala-pIII protein
(1)	FLAG- Lys -Pro-Asn-Phe-Leu- Lys -Leu-Gly-Thr-Ala-pIII protein
(1)	FLAG- Lys -Pro-Thr-Ile-Ala- Lys -Ile-Pro-Ser-Ala-pIII protein
(1)	FLAG- Lys -Gly-Thr-Gly-Tyr- Lys -Ala-Gly- Arg -Ala-pIII protein
(1)	FLAG- Lys - Arg -Ser-Ile-Ser- Lys -Gly-Ala-His-Ala-pIII protein
(1)	FLAG- Lys -Asn-Leu-Asn-His- Arg -Ser- Arg -Val-Ala-pIII protein
(1)	FLAG- Lys -Met-Gly-His-Gly- Arg -Trp-Glu- Arg -Ala-pIII protein
(1)	FLAG- Lys -Val-Gly-Thr-Leu- Arg -Leu-Ser-Gln-Ala-pIII protein
(1)	FLAG- Lys -Leu-Ala-Thr-Asn- Arg -Ala-Glu-Leu-Ala-pIII protein

ment with those obtained for nine different commercially available, trypsin-susceptible colorimetric substrates.

Evaluation of the Ability of Recombinant Human Tryptase β I to Activate Two Cell Lines via PAR1, PAR2, PAR3, and/or PAR4—Three of the known PARs have a Pro residue one to two positions N-terminal of the susceptible basic residue in its activation domain. Thus, we examined whether or not recombinant human tryptase β I could activate a cell via one of the four known PARs. HEK-293T cells were responsive to thrombin, the PAR2 agonist peptide, and trypsin (Fig. 2). The thrombin-mediated increase in the intracellular levels of calcium in HEK-293T cells could be completely inhibited by preincubation of these cells with PAR1-blocking antibodies (Fig. 2B), but not with irrelevant control antibodies (Fig. 2A). In contrast, the PAR1-specific antibodies had no effect on the robust PAR2-mediated increase in intracellular levels of calcium elicited by the PAR2 agonist peptide (Fig. 2B). Thus, although HEK-293T cells could be activated via PAR1 or PAR2, these cells could not be induced to increase their intracellular levels of calcium by recombinant mMCP-6, mMCP-7, protryptase α , or tryptase α (data not shown); protryptase β I (Fig. 2C); or tryptase β I (Fig. 2D). The failure of tryptase β I to activate HEK-293T cells was not a consequence of receptor desensitization because tryptase β I-treated HEK-293T cells responded to trypsin (Fig. 2D) and thrombin (data not shown) in the same manner as cells that had not been pre-exposed to this MC protease. PAR1⁺/PAR2⁻/PAR3⁺/PAR4⁺ Dami cells also were not responsive to recombinant human tryptase β I at the protease concentrations used in these studies (data not shown). The fact that tryptase β I pretreatment of Dami cells actually led to a modest increased Ca²⁺ response when these cells were subsequently exposed to thrombin relative to control cells (data not shown) was further

evidence that tryptase β I does not desensitize cells in terms of their PAR response.

Tryptase-mediated Extravasation of Granulocytes into the Peritoneal Cavity and Lung—Intraperitoneal administration of mMCP-7 to mice resulted in the selective extravasation of eosinophils (Fig. 3b), whereas mMCP-6 treatment resulted in the selective extravasation of neutrophils (Fig. 3a). When instilled into the trachea, mMCP-6 also induced a pronounced and selective extravasation of neutrophils into the lung (data not shown). There was no significant change in the number of neutrophils, eosinophils, or lymphocytes in the peritoneal cavities of mice given a macromolecular complex of heparin glycosaminoglycan and recombinant human tryptase α (data not shown), tryptase β I (Fig. 3c), or protryptase β I (Fig. 3d). However, when ~0.2 nmol of human tryptase β I was instilled into the tracheas of BALB/c or C57BL/6 (Fig. 4) mice, there was a >100-fold increase in the number of neutrophils in the BAL fluid 24–48 h later. The numbers of neutrophils (exclusive of other white blood cells) were also increased in tissue sections of lung parenchyma, and these granulocytes resided in the alveolar spaces rather than the conducting airways. Intratracheal administration of comparable amounts of active tryptase α or inactive tryptase β I zymogen did not result in neutrophil extravasation. Thus, tryptases α and β I are functionally distinct in the lung. Moreover, the ability of tryptase β I to recruit neutrophils is highly dependent on the enzymatically active form of this protease.

The fact that eosinophils and lymphocytes were not recruited into the lungs of the tryptase β I-treated mice raised the possibility that this serine protease induces a local expression of a neutrophil-specific chemokine in the airways that recognizes the only IL-8 receptor (designated IL-8RB) mice are presently

FIG. 2. Activation of HEK-293T cells. HEK-293T cells were loaded with Fura-2 and treated as indicated in each panel, and then the changes in the intracellular levels of Ca^{2+} (Ca_{i2+}) were determined at each time interval. In *A*, cells were exposed to irrelevant antibodies before thrombin treatment. In *B*, cells were sequentially exposed to the PAR1-blocking antibodies, thrombin, and the indicated PAR2 agonist peptide. In *C* and *D*, cells were exposed to inactive protryptase β I and active tryptase β I, respectively, followed by trypsin. In this latter experiment, HEK-293T cells were exposed to the same amounts of tryptase β I and trypsin in terms of their ability to cleave the colorimetric substrate tosyl-Gly-Pro-Lys-*p*-nitroanilide. *A* and *B* are single experiments that are representative of two separate experiments; *C* and *D* are single experiments that are representative of three separate experiments.

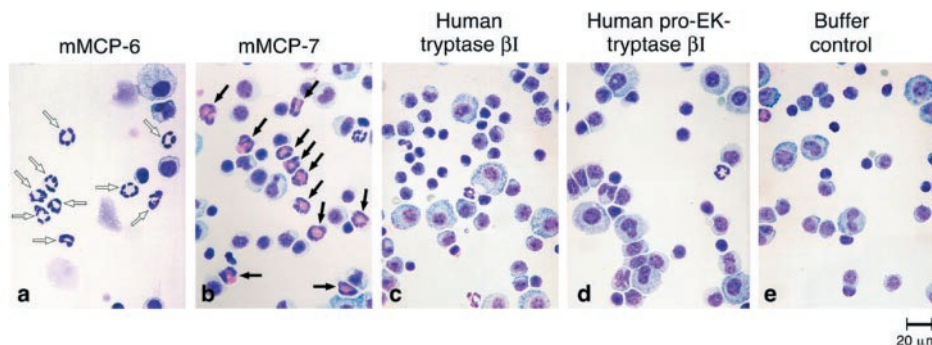
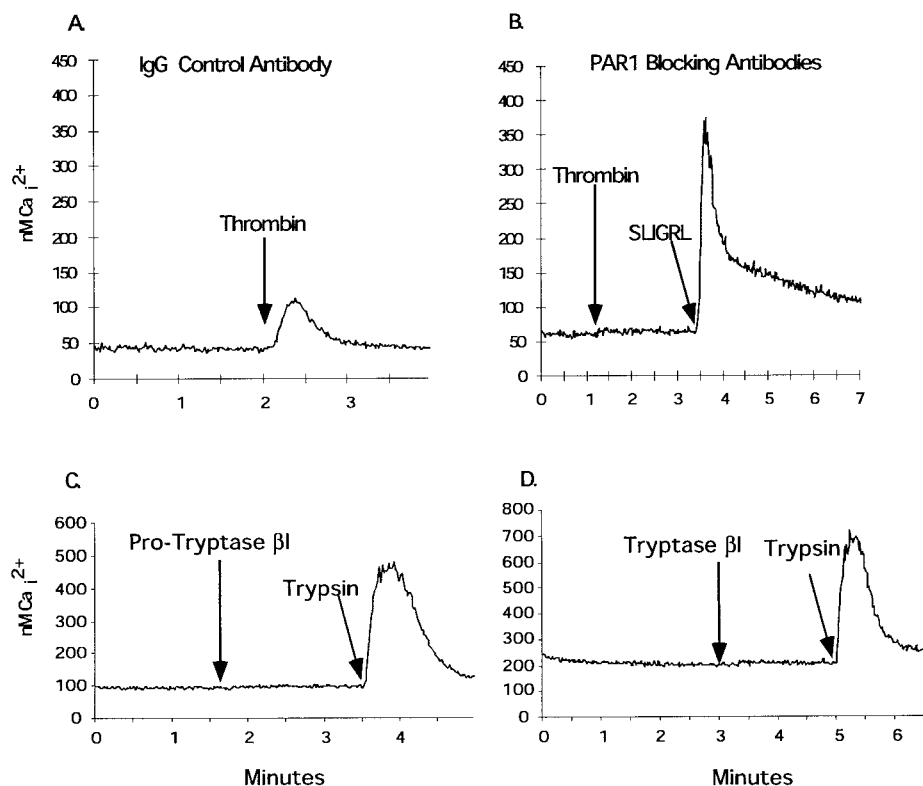


FIG. 3. Tryptase-mediated extravasation of granulocytes into the mouse peritoneal cavity. Buffer containing mature mMCP-6 (*a*), mMCP-7 (*b*), or human tryptase β I (*c*) was injected into the peritoneal cavities of individual mice. The colorimetric substrate tosyl-Gly-Pro-Lys-*p*-nitroanilide was used to confirm that all three mature tryptases were enzymatically active before their administration. For negative controls, other mice were given buffer alone (*e*) or comparative amounts of enzymatically inactive protryptase β I (*d*). 36–48 h later, the mice were killed, and cytocentrifugation preparations of the peritoneal cavity lavage fluids were stained with Diff-Quik. The *white* and *black* arrows point to the large number of neutrophils and eosinophils recruited into the peritoneal cavity by mMCP-6 and mMCP-7, respectively. *EK*, enterokinase.

known to express. Analogous to its effect on normal BALB/c and C57BL/6 mice, recombinant tryptase β I induced large numbers of neutrophils to extravasate into the lungs of mice harboring a targeted deletion of the IL-8RB gene ($n = 8$) (data not shown). Thus, neutrophil extravasation into the lungs of tryptase β I-treated mice occurs in an IL-8/IL-8RB-independent manner.

Functional Consequences of Human Tryptase β I in the Lung—Because tryptase β I treatment resulted in increased numbers of neutrophils in the lung and because pulmonary functions can be acutely affected during a neutrophil-mediated inflammatory reaction, we investigated whether or not methacholine-induced bronchoconstriction was altered in tryptase β I-treated mice. The dose of methacholine required to elicit a 2-fold increase in R_L was not significantly affected by tryptase β I treatment of the mice (Fig. 5). Thus, even though substantial numbers of neutrophils were recruited into the lungs of tryptase β I-treated mice, this acute immunologic response did not result in increased airway responsiveness.

Because the primary function of neutrophils is to kill bacteria and because MC-deficient W/W^v mice are unable to combat a pulmonary *K. pneumoniae* infection effectively, the functional consequence of the tryptase β I-elicited neutrophil response was evaluated during a bacterial infection. Preliminary studies confirmed that MC-deficient W/W^v mice possess a greatly diminished ability to kill KP1415 bacteria relative to MC-sufficient WBB6F1^{+/+} mice or C57BL/6 mice (data not shown). It has been shown previously that the MCs in WBB6F1^{+/+} and C57BL/6 mice differ in their expression of mMCP-7, but not of mMCP-6 (47–49). The finding that WBB6F1^{+/+} and C57BL/6 mice do not differ significantly in their ability to combat a KP1415 bacterial infection now indicates that the primary immunologic defect in MC-deficient W/W^v mice is not a consequence of their failure to express mMCP-7. As noted above, recombinant mMCP-6 and human tryptase β I are functionally the same in the lung in terms of their ability to recruit neutrophils. Thus, the ability of tryptase β I-treated W/W^v mice to combat a KP1415 bacterial infection was evaluated next. When

FIG. 4. Trypsin β I-mediated extravasation of neutrophils into the lungs of C57BL/6 mice. Samples of buffer alone (a and d), buffer containing enzymatically inactive protrypsin β I (b and e), or buffer containing enzymatically active mature trypsin β I (c and f) were instilled into the tracheas of individual mice. 24h later, the mice were killed; their BAL fluids were obtained (a–c), and tissue sections of the washed lungs were prepared (d–f). The tissue sections and cytocentrifugation preparations of the BAL fluids were stained with Diff-Quik. Arrows indicate neutrophils that had selectively extravasated into the lungs of the trypsin β I-treated mice.

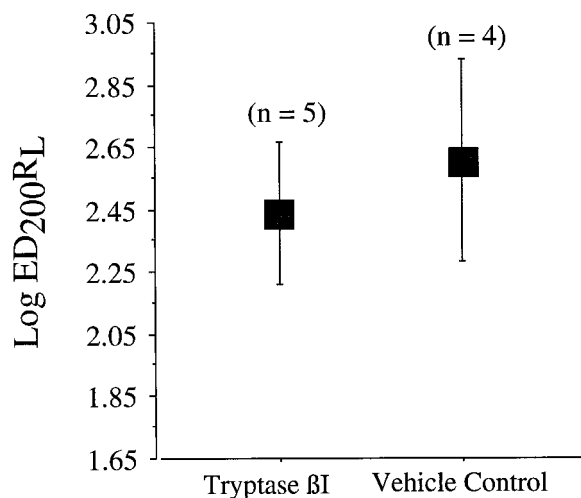
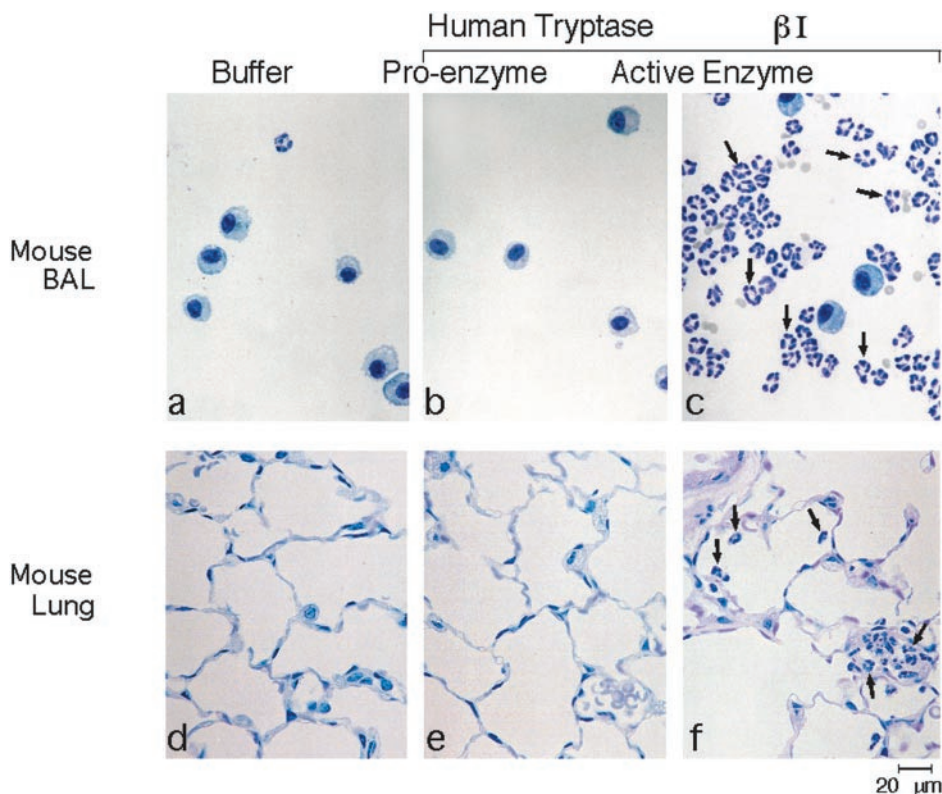


FIG. 5. Airway reactivity. Values are the means \pm S.E. of log ED₂₀₀RL in vehicle control and trypsin β I-treated C57BL/6 mice. There was no statistical difference.

MC-deficient W/W^o mice received mature trypsin β I, their ability to fight the infectious agent improved significantly (Fig. 6). However, because replicate mice exposed to the zymogen form of this protease were unable to combat the infectious agent (Fig. 6), human trypsin β I must be enzymatically active to exert its host defense effect in the lung.

DISCUSSION

Trypsins α and β I are major constituents of the secretory granules of human lung MCs (5, 7). In this study, we generated recombinant forms of these two human MC trypsinases (Fig. 1) and demonstrated that they are functionally different *in vivo* despite their overall 93% amino acid sequence identity. When small amounts of enzymatically active trypsin β I were instilled into the mouse trachea, there was a >100-fold increase in the number of neutrophils in the BAL fluid within 24 h (Fig.

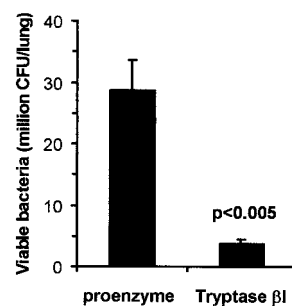


FIG. 6. Trypsin β I-mediated rescue of the antimicrobial deficiency of MC-deficient W/W^o mice. Six W/W^o mice were pre-exposed to the enzymatically inactive zymogen form of recombinant human trypsin β I. At the same time, six other W/W^o mice were pre-exposed to comparable weight amounts of the enzymatically active mature form of human trypsin β I. The next day, *K. pneumoniae* cells (12×10^6 colony-forming units (CFU)) were administered to each animal. 6 h later, the animals were killed, and the total number of colony-forming units in the lungs of each animal was quantitated. The depicted results are the means \pm S.E.

4). Because neutrophils did not extravasate into the lungs of mice given inactive pseudo-zymogen, trypsin β I must be catalytically active to elicit this biologic response. The fact that trypsin β I and protrypsin β I were unable to induce neutrophil extravasation in the peritoneal cavity, coupled with the fact that protrypsin β I was unable to induce neutrophil extravasation into the lung, indicated that the trypsin β I-mediated effect in the lung was not endotoxin-induced.

Essentially no eosinophils extravasated into the lungs of animals that had received recombinant human trypsin β I. He *et al.* (50) observed that a preparation of human trypsin purified from skin induced eosinophil extravasation when injected into guinea pig skin. The finding that mMCP-7 selectively induced eosinophil extravasation into the peritoneal cavity (Fig. 3), coupled with the existence of at least one human trypsin gene on chromosome 16 with substantial homology to mMCP-7 (9), raises the possibility that the non-recombinant

material used in the earlier study (50) contained a mixture of human tryptases that individually exhibit different biologic effects *in vivo*.

Recombinant mMCP-6 and human tryptase β I elicited similar biologic responses in the lung, but different biologic responses in the peritoneal cavity (Fig. 3). Because different adhesion proteins regulate the extravasation of neutrophils into the lungs and peritoneal cavities of mice (51), the tissue-specific effects of tryptase β I are not without precedent. The mechanism that dictates this site-selective action of tryptase β I is not known, but a number of possibilities exist. Because recombinant mouse tryptases differ in their ability to be inactivated by factors in serum (25, 26), it is possible that the concentration of a yet to be identified inhibitor of tryptase β I is greater in the peritoneal cavity than in the lung. Tryptase β I is more dependent on heparin (Fig. 1) than is mMCP-6 (26). Thus, it is also possible that tryptase β I is unable to induce neutrophils to extravasate into the peritoneal cavity because the physical environment (*e.g.* pH or cation concentration) of this site causes the rapid dissociation of the active tetramers into inactive monomers. However, a more likely explanation is that the substrate specificities of mMCP-6 and human tryptase β I are similar, but not identical. Analogous to other serine proteases (52), seven loops form the substrate-binding cleft of human tryptase β II (53) and therefore its related protease, human tryptase β I. A comparison of the residues in the substrate-binding clefts of mMCP-6 and human tryptase β I revealed substantial differences in loops A, C, D, and 3 (8). In agreement with those findings, the peptide library data (Table I) indicated that the substrate specificity of recombinant human tryptase β I is unique even though it is more similar to that of recombinant mMCP-6 (26) than recombinant mMCP-7 (25). 7 of the 15 mMCP-6-susceptible peptides isolated in our previous mouse tryptase study contained Pro at the P4 site (26). Although the substrate specificity of human tryptase β I is different, this serine protease also cleaves a peptide sequence containing a Pro residue at the P4 site (Table I). The current peptide library data therefore predict that a protein in the lungs with a (Lys/Arg)-Pro-X-(Lys/Arg) sequence (where X can be 0 to 3 non-charged amino acids) is cleaved by human tryptase β I to initiate the signaling pathway that ultimately results in the selective extravasation of neutrophils. In support of our peptide library data with recombinant material, Stack and Johnson (54) found that an enriched human MC tryptase preparation preferentially cleaved the Lys¹⁵⁸-Ile¹⁵⁹ bond in the Arg-Pro-Arg-Phe-Lys-Ile sequence in pro-urokinase. The crystal structure of human tryptase β II (53) predicts that human tryptase β I exists *in vivo* as a ring-like tetramer with the four catalytic sites residing in the central pore. Pro residues make peptide sequences more rigid. Thus, Pro at the P5, P4, P3, and/or P2 site may be needed to facilitate the penetration of the extended substrate into the central pore of the tetramer.

In vitro studies have been carried out during the last 6 years with tryptase preparations from different species to ascertain whether or not any MC tryptase can activate a cell via one of the four known PARs (19, 20, 22, 55, 56). Unfortunately, nearly all of these earlier studies used non-recombinant tryptase. Because of the nearly impossible task of separating human tryptases that are 93–99% identical, because human MCs often express more than one tryptase, and because of the recent discovery of different splice variants of the same tryptase, most of the preparations used in the earlier studies undoubtedly contained functionally distinct tryptases. Although Mirza *et al.* (21) used recombinant tryptases in their *in vitro* PAR study, they reported that recombinant human tryptases α and β II activate PAR2⁺ BaF3 cells in a similar manner. Tryptases β I

and β II probably cleave the same proteins because the loops that compose the substrate-binding clefts of these two MC proteases are predicted to be identical. However, the loops that compose the substrate-binding clefts of tryptases α and β II are very different. Thus, it is highly unlikely that these two human tryptases are functionally identical *in vivo*. Using an expression/site-directed mutagenesis approach, we demonstrated that the ability of recombinant tryptase α to cleave fibrinogen and various colorimetric substrates *in vitro* differs from that of other mouse and human tryptases due, in part, to a single Asp/Gly difference in loop 2 (33).

Neutrophils express PAR2 and can be activated by trypsin and PAR2 receptor agonists (57), and the constituents of this cell's secretory granules contribute to airway hyperreactivity in various *in vivo* model systems of inflammation (58, 59). The neutrophils that extravasated into the lungs of tryptase β I-treated mice (Fig. 4) appeared to retain their granules. Moreover, the airway reactivity of the tryptase β I-treated mice was similar to that of control mice (Fig. 5) despite the large number of neutrophils in the lungs (Fig. 4). Based on these findings, it appeared highly unlikely that tryptase β I was activating any cell type in the lung via PAR2. The phage display peptide library data revealed that human tryptase β I prefers to cleave basic peptides containing Pro at the P2–P5 residues. The fact that no Pro amino acid is present in the activation domain of PAR2 supports our conclusion that tryptase β I is not inducing neutrophil extravasation *in vivo* via PAR2. Nevertheless, the Pro residue resides at the P2 or P3 cleavage site in the activation domains of PAR1, PAR3, and PAR4. Because the finding of Mirza *et al.* (21) are difficult to reconcile with those of others and because mouse PAR2 (60) and human PAR2 (61) lack the tryptase β I-preferred sequences identified in our peptide library (Table I) and in the tryptase-susceptible proteins identified by Stack and Johnson (54) and Cromlish *et al.* (62), we reinvestigated this issue with trypsin- and/or thrombin-responsive cell lines that express different combinations of PARs. Intracellular Ca²⁺ was measured because an increased level of this cation is a hallmark response of PAR activation (63). In agreement with the peptide library data, recombinant tryptase β I by itself was unable to increase the cytosolic levels of Ca²⁺ in PAR1⁺/PAR2⁺/PAR3⁺ HEK-293T cells and in PAR1⁺/PAR3⁺/PAR4⁺ Dami cells (Fig. 2) at the protease concentrations used to induce neutrophil extravasation *in vivo* (Fig. 4). Significant changes in the cytosolic concentration of Ca²⁺ in Dami or HEK-293T cells could not be induced by recombinant pro-mMCP-6, mature mMCP-6, pro-mMCP-7, mature mMCP-7, protryptase α , mature tryptase α , protryptase β I, or mature tryptase β I (Fig. 2). Because tryptase β I-treated HEK-293T cells responded to both trypsin and thrombin in a manner indistinguishable from that of the control cells, we concluded that the unresponsiveness of these cells to tryptase β I was not a consequence of proteolytic removal of PAR1 or PAR2 from the cell's plasma membrane. These data now suggest that the individual human tryptases and/or their splice variants are functionally distinct in terms of their ability to activate PAR2.

We have generated heparin-null mice by targeted disruption of the *N*-deacetylase/*N*-sulfotransferase 2 gene (64). Although the heparin⁻/chondroitin sulfate E⁺ MCs developed *in vitro* from these mice with IL-3 cannot store carboxypeptidase A and mMCP-5 in their granules, the levels of mMCP-6 and mMCP-7 in this MC population are relatively normal. These findings indicate that tryptases can be packaged in secretory granules of mouse MCs bound to serglycin proteoglycans that have either heparin or chondroitin sulfate E chains. Because the substrate specificity of mMCP-6 is restricted when this tryptase is bound to heparin (26) and because chondroitin sulfate E is

present in abundance in human lung MCs (65, 66) and the HMC-1 cell line (67), an alternate possibility to explain the PAR2 data of others is that the type of glycosaminoglycan bound to trypsinase β I plays a critical role in dictating whether or not this human protease can directly activate PAR2 on the surface of a cell.

By itself, mMCP-6 is not an efficient chemotactic factor for mouse neutrophils *in vitro* (26). The ability of mMCP-6 to induce cultured human endothelial cells to produce large amounts of IL-8 suggested that mMCP-6 regulates neutrophil extravasation in the peritoneal cavity, in part, by indirectly inducing bystander cells to release neutrophil-specific chemokines. Although the particular trypsinase has not been identified, one or more human trypsinases also induce cultured human epithelial (68) and endothelial (69) cells to increase their expression of IL-8. Human neutrophils express two distinct IL-8 receptors (designated IL-8RA or CXCR1 and IL-8RB or CXCR2), whereas mouse neutrophils express only IL-8RB. IL-8RB-null mice contain tremendous numbers of granulocytes in their peripheral blood (34, 70). As has been demonstrated with CD18-deficient mice (71, 72), this finding can confound analyses of the roles of IL-8RB in mediating neutrophil emigration. Nevertheless, our studies with IL-8RB-null mice indicate that trypsinase β I probably induces neutrophil recruitment into the lungs in an IL-8RB-independent manner. In agreement with this conclusion, a preliminary GeneChip analysis of the transcript profile of cultured human pulmonary endothelial and epithelial cells exposed for 2 h to recombinant human trypsinase β I revealed no major change in the level of the transcripts that encode the neutrophil regulatory chemokines IL-8, ENA-78, GRO- γ , IP-10, and NAP-2 relative to cells exposed to inactive protrypsinase β I.²

Neutrophils and their granular constituents have been reported to contribute to airway hyperreactivity in various *in vivo* model systems of inflammation (58, 59). Although the mechanism by which human trypsinase β I regulates neutrophil extravasation into the lung remains to be determined at the molecular level, airway responsiveness to methacholine surprisingly was unchanged relative to vehicle-treated control mice (Fig. 5). This unexpected finding indicates that trypsinase β I has the ability to selectively recruit neutrophils *in vivo* in a manner that does not compromise airway reactivity. The fact that recombinant trypsinase β I, by itself, could rescue the antimicrobial defect in MC-deficient *W/W^v* mice (Fig. 6) now documents the functional importance of human trypsinase β I and its homolog mMCP-6 in innate immunity.

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