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# Biochemical and Functional Characterization of Human Transmembrane Tryptase (TMT)/Tryptase γ

TMT IS AN EXOCYTOSED MAST CELL PROTEASE THAT INDUCES AIRWAY HYPERRESPONSIVENESS *IN VIVO* VIA AN INTERLEUKIN-13/INTERLEUKIN-4 RECEPTOR α/SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION (STAT) 6-DEPENDENT PATHWAY*[S]

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**Transmembrane tryptase (TMT)/tryptase γ is a membrane-bound serine protease stored in the secretory granules of human and mouse lung mast cells (MCs). We now show that TMT reaches the external face of the plasma membrane when MCs are induced to degranulate. Analysis of purified recombinant TMT revealed that it is a two-chain neutral protease. Thus, TMT is the only MC protease identified so far which retains its 18-residue propeptide when proteolytically activated. The genes that encode TMT and tryptase βI reside on human chromosome 16p13.3. However, substrate specificity studies revealed that TMT and tryptase βI are functionally distinct even though they are ~50% identical. Although TMT is rapidly inactivated by the human plasma serpin $\alpha_1$-antitrypsin *in vitro*, administration of recombinant TMT (but not recombinant tryptase βI) into the trachea of mice leads to airway hyperresponsiveness (AHR) and increased expression of interleukin (IL) 13. T cells also increase their expression of IL-13 mRNA when exposed to TMT *in vitro*. TMT is therefore a novel exocytosed surface mediator that can stimulate those cell types that are in close proximity. TMT induces AHR in normal mice but not in transgenic mice that lack signal transducer and activator of transcription (STAT) 6 or the α-chain of the cytokine receptor that recognizes both IL-4 and IL-13. Based on these data, we conclude that TMT is an exocytosed MC neutral protease that induces AHR in lungs primarily by activating an IL-13/IL-4Rα/STAT6-dependent pathway.**

Mast cells (MCs)[1] play beneficial immunosurveillance and effector roles in the body, particularly during bacteria infections (1–5). Nevertheless, these effector cells also have been implicated in asthma and other pathologic conditions. The physiologic changes (*e.g.* vasodilation, edema, smooth muscle contraction, and leukocyte recruitment) that occur in humans and animals when their MCs are activated are caused by the release of different combinations of preformed and newly synthesized mediators. Neutral proteases are the major constituents of the secretory granules of MC, and granule proteases generally account for >25% of the total protein of a mature MC (6–8).

Nearly all human MCs contain at least one granule tryptase (9). The varied human tryptase genes reside at chromosome 16p13.3, and this locus represents the second largest cluster of protease genes in the human genome. Fourteen serine protease-like genes are present at the locus, including the genes that encode transmembrane tryptase (TMT; also known as tryptase γ), marapsin, eosinophil serine protease-1/testisin/PRSS21, and tryptases α, βI, βII, βIII, δ, and ϵ/PRSS22 (10–19). The corresponding tryptase locus in the mouse resides at chromosome 17A3.3-B1; it currently has four members (17, 20–22). No human MC has been found which expresses marapsin, eosinophil serine protease-1, or tryptase ϵ. Nevertheless, human MCs (including those in the lung) express TMT, and tryptases α, βI, βII, and βIII. Although human MCs probably express more tryptases than mouse MCs, interleukin (IL) 3-developed mouse MCs express the mouse ortholog of human TMT. Mouse TMT is 74% identical to human TMT (17). Human and mouse TMT differ from the other MC granule tryptases in that each contains a membrane-spanning domain at its C terminus (17).

Many substrates (*e.g.* high molecular weight kininogen, va-

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[S] The on-line version of this article (available at http://www.jbc.org) contains a table of the transcript analysis of Jurkat T cells before and after exposure to TMT.

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[1] The abbreviations used are: MC(s), mast cell(s); A1AT, α1-antitrypsin; Ab, antibody; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; Boc, *t*-butoxycarbonyl; Bz, benzoyl; DFP, diisopropyl fluorophosphate; EK, enterokinase; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; HBM-M, human bone marrow-derived mastocytosis; IL, interleukin; IL-R, interleukin receptor; KL, c-*kit* ligand; PMA, phorbol 12-myristate 13-acetate; *p*NA, *p*-nitroanilide; STAT, signal transducer and activator of transcription; Suc, succinyl; TBS, Tris-buffered saline; TMT, transmembrane tryptase/tryptase γ; TUNEL, terminal nucleotidyl transferase-mediated UTP nick end labeling; Z, benzyloxycarbonyl.

soactive intestinal peptide, urinary-type plasminogen activator, complement component C3, fibronectin, fibrinogen, procollagenase, proopiomelanocortin, proatrial natriuretic factor, and protease-activated receptor 2) can be cleaved *in vitro* by tryptase preparations from different human tissues. Nevertheless, because recombinant tryptases were not used in most of these earlier studies, it remains to be determined what are the physiologic substrates of the varied MC-derived neutral proteases. The pathologic roles of these tryptases in MC-mediated diseases such as asthma also have not been deduced. Tryptases have similar structural properties that hinder their separation in varied purification procedures. Allelic variants of these serine proteases also have been identified (18), as well as splice variants (23), that are predicted to encode proteases with altered substrate specificities. Even though the primary amino acid sequences of tryptase α and βII are 93% identical (10–12), it is now apparent that these two MC proteases are functionally distinct due, in part, to a single amino acid difference in one of the loops that forms the substrate binding cleft of each tryptase (24, 25).

To circumvent the substantial pitfalls encountered using complex tryptase preparations from human tissues, enzymatically active recombinant human tryptases α, βI, and βII have been generated recently using insect cell and *Pichia pastoris* expression systems (5, 24, 26, 27). These technological advances have begun to provide the amounts of pure enzyme needed to address the specificity, physiologic function, and potential pathologic roles of the different members of the chromosome 16p13.3 family of human serine proteases *in vivo*. For example, administration of small amounts of recombinant, mature human tryptase βI (but not protryptase βI or mature human tryptase α) into the trachea of MC-deficient $W/W^v$ mice confers protective immunity during a *Klebsiella pneumoniae* infection of the lung without inducing airway hyperresponsiveness (AHR) (5). This beneficial effect is mediated, in part, by the ability of tryptase βI to induce the selective extravasation of large numbers of neutrophils into the bacteria-infected lung.

Human asthma is a polygenic disorder (28, 29) that is influenced by varied environmental factors. MCs play important roles in this complex disorder. For example, MC activation enhances AHR to methacholine in varied mouse models of the disease (30). Of the multitude of factors thought to be involved in human asthma, overproduction of the pleiotropic cytokine IL-13 (31, 32) appears to contribute significantly to the development of the disease (33). Certain populations of activated MCs (34–36), basophils (37), and eosinophils (38) produce IL-13. However, it has been concluded that $CD3^+$ T cells are the primary source of this cytokine in the lungs of atopic asthma patients (39). Human lung MCs express TMT (17, 18), and it is well known that MCs physically interact with T cells (40, 41) and other cell types (42). Although it remains to be determined whether or not TMT is a functional protease, this newly identified granule constituent possesses a membrane-spanning domain at its C terminus analogous to the prohormone trypticlike convertase furin. It therefore has been speculated that TMT might be a furin/Kex2-like convertase that participates in the post-translational processing of the proteins that are stored in the secretory granules of MCs.

We now report that TMT reaches the external face of the plasma membrane of activated MCs where it can cleave and/or interact with proteins/peptides residing in the extracellular matrix and on the surface of those cells that MC contact. TMT, therefore, functions outside of activated MCs. Using recombinant material, we show that TMT is an enzymatically active protease that induces T cells to increase their expression of IL-13. Although TMT can be inactivated by human $\alpha_1$-antitrypsin (A1AT; also known as $\alpha_1$-proteinase inhibitor and serpin A1), this neutral protease is a potent inducer of AHR in mice because of its ability to activate an IL-13/IL-4Rα/STAT6-dependent pathway in the lung.

## EXPERIMENTAL PROCEDURES

*Generation of $TMT^+$ Human MCs/Basophils*—Umbilical cord blood was obtained from normal donors on the day of delivery of full term, newborn infants. Peripheral blood also was collected from patients with asthma. Approval for the study was obtained from the Institutional Ethics Committees at St. George Hospital and Brigham and Women's Hospital. Blood was collected in heparinized tubes (Greiner Labortechnik, Germany). The tubes were centrifuged at 800 × *g* for 10 min, and the leukocyte-enriched buffy coats were removed and resuspended in erythrocyte lysis buffer (Sigma) for 10 min at room temperature. The nonlysed cells were washed with Dulbecco's phosphate-buffered saline and resuspended in minimal Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 μg/ml penicillin-streptomycin. The resulting cell suspensions were seeded at a density of $10^6$ cells/ml and cultured in 50% (v/v) human bone marrow-derived mastocytosis (HBM-M) cell-conditioned medium supplemented with 50 ng/ml recombinant c-*kit* ligand (KL)/stem cell factor (Amgen Inc, Thousand Oaks, CA) in 25-$cm^2$ flasks for up to 3 weeks, as described previously (43, 44). The cells were cultured at 37 °C in a 5% $CO_2$ humidified atmosphere. Every 3–4 days, half of the conditioned medium was replaced by fresh medium. Human MCs also were generated by culturing cord blood progenitors from normal individuals for 6 weeks in medium supplemented with KL, IL-6, and IL-10, as described previously (45).

*Immunohistochemistry and Fluorescence-activated Cell Sorter (FACS) Analyses*—Slides containing cytocentrifugation preparations of cultured human MCs were placed sequentially in Carnoy's fixative, 0.3% $H_2O_2$ in methanol, and normal sheep serum diluted 1:5 in Tris-buffered saline (TBS) for 15, 10, and 10 min, respectively. The cells were washed and incubated for 2 h at 37 °C in TBS containing affinity-purified rabbit anti-TMT antibody (Ab) (~5 μg/ml in TBS) (17). They were then exposed to sheep anti-rabbit Ab conjugated to horseradish peroxidase (1.3 μg/ml) in TBS for 1 h at room temperature, followed by a freshly prepared 3,3′-diaminobenzidine solution. For double immunostaining, anti-TMT Ab-treated cells also were incubated for 2 h at 37 °C with 2 μg/ml of an alkaline phosphatase-conjugated mouse monoclonal Ab (designated here as anti-tryptase α/β Ab; Chemicon, Temecula, CA). This Ab recognizes all known human MC tryptases effectively except TMT. These cells were then placed for 20 min in a freshly prepared solution containing 0.2 mg/ml naphthol AS-MX phosphate, 0.1 mg/ml Fast Red TR, and levamisole in 0.1 M Tris-HCl (pH 8.2). In these immunohistochemical analyses, $TMT^+$/tryptase $\alpha/\beta^-$ cells stain brown, $TMT^-$/tryptase $\alpha/\beta^+$ cells stain pink, and $TMT^+$/tryptase $\alpha/\beta^+$ cells stain pink and brown depending on where the respective antigen resides.

KL/IL-6/IL-10-developed human MCs also were evaluated for their intracellular and surface expression of TMT by FACS analysis before and after a 15- or 30-min exposure to culture medium containing 0.5 μM calcium ionophore and 5 μg/ml phorbol 12-myristate 13-acetate (PMA) (Sigma). Calcium ionophore and PMA were used to induce degranulation of the cultured MCs rather than human IgE followed by rabbit anti-IgE Ab because the latter reagent cannot be used with rabbit anti-TMT Ab in the FACS analysis to demonstrate surface expression of the protease. Calcium ionophore and PMA also were used to optimize degranulation and to determine whether or not TMT is rapidly shed from the surface of an activated MC. In these analyses, 5% paraformaldehyde-fixed MCs were incubated (before and after a 1% saponin permeabilization step) on ice for 45 min with affinity-purified, rabbit anti-TMT Ab or an irrelevant rabbit Ab directed against a mouse MC protease in FACS buffer (Hanks' balanced saline solution containing 2% fetal calf serum and 0.1% normal human serum). The resulting cells were washed extensively with FACS buffer, incubated at 4 °C with fluorescein isothiocyanate-conjugated sheep anti-rabbit Ab (Jackson ImmnoResearch), and then analyzed using a Becton-Dickinson FACSort, as described previously for chemokine receptor expression (45).

*Generation and Biochemical Characterization of a Soluble Form of Recombinant Human TMT*—An enterokinase (EK)-susceptible, 5-residue peptide sequence was bioengineered in between the natural propeptide and the catalytic domain of human TMT, as described previously for other mouse and human tryptases (5, 19, 22, 24, 46, 47). In this way, recombinant pro-TMT could be proteolytically activated by EK (New England Biolabs, Beverly, MA) after its purification from the insect

TABLE I

*Effects of varied protease inhibitors on the enzymatic activity of recombinant TMT*

Using resorufin-labeled casein, the protease activity of trypsin and recombinant TMT was compared after an exhaustive exposure to pepstatin, bestatin ((2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl)-L-leucine hydrochloride, EDTA, phosphoranmidon, chymostatin, leupeptin, aprotinin, antipain, APMSF ((4-amidinophenyl)-methanesulfonyl fluoride hydrochloride monohydrate)), and Pefabloc (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride). Activities are expressed as percentages of the respective controls.

| Inhibitor | General specificity | Concentration | % Inhibition | |
|---|---|---|---|---|
| | | | TMT | Trypsin |
| | | *mM* | | |
| Pepstatin A | Aspartyl proteases | 0.10 | 0 | 0 |
| Bestatin | Aminopeptidases | 0.65 | 0 | 24 |
| EDTA | Metalloproteases | 4.00 | 0 | 5 |
| Phosphoramidon | Metalloproteases | 0.02 | 7 | 19 |
| Chymostatin | Serine and thiol proteases | 1.00 | 39 | 74 |
| Leupeptin | Serine proteases | 0.56 | 50 | 89 |
| Aprotinin (bovine) | Serine proteases | 0.003 | 74 | 95 |
| Antipain | Papain and trypsin | 0.74 | 78 | 89 |
| APMSF | Serine proteases | 0.20 | 90 | 61 |
| Pefabloc SC | Serine proteases | 6.00 | 94 | 100 |

cell-conditioned medium. Naturally occurring TMT contains a 40-mer, membrane-spanning domain at its C terminus (17). Protein modeling studies revealed that this C-terminal domain is not part of the 240-residue catalytic domain of the protease (17). As noted under "Results," TMT normally is a membrane-bound protease even after MCs are induced to degranulate. Recombinant TMT cannot be purified easily if it contains its C-terminal membrane-spanning domain. More important, no *in vivo* experiment can be carried out with the natural product because the protease would simply insert into the plasma membrane of any cell it touches. To overcome these technical limitations, residues 245–284 in human TMT were replaced by the 8-residue FLAG peptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys. The FLAG peptide was added because this short peptide does not appear to influence the substrate specificities of other tryptases. The epitope also enables the rapid purification of recombinant pro-TMT from the insect cell-conditioned medium using an anti-FLAG immunoaffinity column. Recombinant human tryptase βI was used as a control in the TMT experiments; it also has the FLAG peptide at its C terminus. Recombinant TMT was generated sterilely in High Five™ insect cells cultured in protein-free medium. Because all proteins in the conditioned medium are derived from the TMT-expressing insect cells in the cultures, the recombinant tryptase zymogen can be purified relatively easily. Another advantage of this expression system over a typical mammalian cell expression system is that one does not have to be concerned about biologically active factors (*e.g.* serpins that inactivate TMT) in the fetal calf serum routinely used in the latter culture systems. TMT was not generated in *Escherichia coli* because of the obvious concern of lipopolysaccharide contamination with this expression system, which would adversely impact the interpretation of the *in vivo* data.

High Five insect cells, infected with a low titer of baculovirus encoding pro-TMT (~ 8 million recombinant virus particles/6 million insect cells), were cultured in protein-free X-press medium for 6–7 days. Typically ~750 ml of conditioned medium was loaded at 4 °C onto a 1-ml column containing anti-FLAG M2 Ab (Sigma). After the column was washed with 250 ml of TBS (pH 7.0), 0.1 M glycine HCl buffer (pH 3.5) was added to elute the bound recombinant pro-TMT. Nine to ten 1-ml fractions were collected into tubes containing 20–40 μl of 1 M Tris-HCl (pH 8.0). Samples of the eluted fractions were analyzed by SDS-PAGE for the presence of Coomassie Blue$^+$ proteins and for immunoreactive TMT using either anti-FLAG Ab or anti-TMT Ab (17). Those column fractions that contained recombinant pro-TMT were pooled; the final protein concentration was estimated using the micro-BCA protein assay kit (Pierce). As assessed by the Limulus Amebocyte Lysate endotoxin assay (BioWhittaker, Walkersville, MD), the level of lipopolysaccharide was below detection (<0.06 enzyme units) in various 5-μg preparations of recombinant TMT used in this study.

Comparison of the primary amino acids of the zymogen forms of human TMT, tryptase α, tryptase βI, and tryptase βII revealed that pro-TMT has 10 Cys residues, whereas all other MC tryptases have only 8. The 2 extra Cys residues are located at positions −12 in TMT propeptide and +108 in its catalytic domain. Because these findings raised the possibility of an additional intramolecular disulfide bond linking the propeptide and catalytic domain, ~5 μg of recombinant pro-TMT was digested with 0.013 units of EK at 37 °C for 2 h in 5 mM $CaCl_2$ and 10 mM Tris-HCl (pH 5.5). Half of the sample was boiled in SDS-PAGE sample buffer containing β-mercaptoethanol; the other half was boiled in SDS-PAGE sample buffer lacking the reducing reagent. After electrophoresis, the resolved proteins were blotted onto a polyvinylidene difluoride membrane. Each protein blot was exposed to 15 ml of TBS containing 5% nonfat milk, 0.1% Tween 20, 0.5% goat serum, and 4 μg/ml mouse anti-FLAG Ab (Sigma) for 2 h at room temperature. The treated blot was washed three times with TBS containing 0.1% Tween 20. It was then incubated for 1 h at room temperature in 15 ml of TBS containing 5% nonfat milk, 0.1% Tween 20, 0.5% goat serum, and a 1,000-fold dilution of a stock solution of horseradish peroxidase-conjugated goat anti-mouse Ab (Bio-Rad). Immunoreactive proteins were visualized using a chemiluminescence kit (Geno Technologies, St. Louis, MO) and BioMax MR film (Eastman Kodak).

*Evaluation of the Substrate Specificity of Recombinant Human TMT, as well as Its Inactivation by Protease Inhibitors*—Diisopropyl fluorophosphate (DFP) is often used to radiolabel the catalytic triad Ser in enzymatically active serine proteases. Thus, recombinant pro-TMT and mature TMT (~15 μg) were suspended in 50 μl of 0.5 M Tris-HCl (pH 8.8) containing ~ 8 μCi of [$^3$H]DFP (4 Ci/nmol; 1 Ci = 37 gigabecquerels; Amersham Biosciences). The samples were incubated for 60 min at 37 °C. SDS-PAGE loading buffer was added, and each sample was boiled for 5 min before the [$^3$H]DFP-labeled proteins were subjected to SDS-PAGE analysis. The resulting gel was treated with En$^3$hance (PerkinElmer Life Sciences), dried, and exposed to x-ray film.

The substrate specificity of recombinant TMT was evaluated using the chromogenic substrates tosyl-Gly-Pro-Arg-*p*NA (Sigma), H-D-Leu-Thr-Arg-*p*NA, Boc-Leu-Gly-Arg-*p*NA, Bz-Phe-Val-Arg-*p*NA, H-Gly-Arg-*p*NA, Z-Arg-Arg-*p*NA, Suc-Ala-Ala-Pro-Arg-*p*NA, H-D-Val-Leu-Arg-*p*NA, and H-D-Ile-Phe-Lys-*p*NA (Bachem, King of Prussia, PA). Fifteen μl of pro-TMT (~7.5 μg) was suspended in 15 μl of EK activation buffer containing 5 mM $CaCl_2$, 0.013 unit of EK, and 10 mM Tris-HCl (pH 5.5). The resulting mixture was incubated at 37 °C for 2 h. After activation, 10 μl of 0.5 M Tris-HCl (pH 10.6) was added to raise the pH of the buffer. One microliter (~50 μg) of chromogenic substrate was added to each well containing mature TMT, pro-TMT, or the activation buffer that contains just EK. After a 3-h incubation at 37 °C, the enzymatic activity of the sample was measured as a change in $A_{405\ nm}$ using an ELISA plate reader (Molecular Devices, Sunnyvale, CA). Each enzymatic assay was carried out in triplicate. All mouse and human tryptases that have been examined to date have an optimal enzymatic activity at neutral pH. To determine the pH optimum of human TMT, ~7.5 μg of the mature recombinant protease was suspended in pH 5.5, 6.5, or 7.5 Tris-HCl buffers containing the chromogenic substrate H-D-Leu-Thr-Arg-*p*NA. The enzymatic activity of TMT was then measured at different time points.

Various nonphysiologic protease inhibitors (see Table I) from Roche Molecular Biochemicals were assessed for their ability to inhibit the cleavage of the protease-sensitive substrate, resorufin-labeled casein by TMT and trypsin. In each assay, ~7.5 μg of the protease was suspended in 100 μl of buffer containing 0.1 M Tris-HCl (pH 7.8), 10 mM $CaCl_2$, 200 μg of resorufin-labeled casein, and the protease inhibitor. After an exhaustive 18-h incubation at 37 °C, each reaction was stopped by the addition of 480 μl of 5% trichloroacetic acid followed by a 10-min centrifugation at >8,000 × *g*. A 400-μl sample of the resulting supernatant was mixed with 600 μl of 0.5 M Tris-HCl (pH 8.8), and the absorbance was measured at 574 nm. Because low molecular weight resorufin-labeled degradation products released from its native sub-

strate are not precipitated by the trichloroacetic acid step, the amount of proteolytic activity in the supernatant is directly proportional to the general activity present in the sample. Results were expressed as percent inhibition relative to control samples evaluated in the absence of a protease inhibitor.

The ability of purified human plasma A1AT (Sigma) and recombinant secretory leukocyte protease inhibitor (Cell Sciences, Norwood, MA) to inhibit TMT also were evaluated in more physiologic assays. For these studies, 1–8 μg of TMT or trypsin was preincubated with an ~5-fold molar excess of each protease inhibitor for 30 min. The ability of the treated serine protease to cleave H-D-Ile-Phe-Lys-*p*NA (Bachem) over a 1–5-h time period was then measured as determined above. The TMT·A1AT binary complex also was boiled for 5 min in SDS-PAGE buffer and subjected to electrophoresis to evaluate the affinity of the protease/serpin interaction. Trypsin was used as a positive control in this serpin/SDS-PAGE analysis.

*TMT Regulation of IL-13 Expression in T cells, and TMT-mediated Airway Responses in Normal and Transgenic Mice*—Sodium citrate-treated blood from normal individuals was subjected to a standard Ficoll-Paque lymphocyte isolation step. The T cells in the resulting buffy coat were obtained with anti-CD3 Ab-coated MicroBeads and MACS separation columns (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instruction. After a 3-h incubation in serum-free RPMI to allow the enriched T cells to recover from the trauma of the isolation procedure, ~$2 \times 10^6$ cells were placed in 1.5 ml of medium lacking or containing TMT. Twelve h later, total RNA was isolated from the two populations of cells with TRI Reagent (Molecular Research Center, Cincinnati, OH). cDNAs were prepared using a reverse transcription kit (Promega, Madison, WI). The oligonucleotides 5′-CTCCTCAATCCTCTCCTGTT-3′ and 5′-TTTCGCGAGGGACGGTTCAAC-3′ were then used to identify and amplify the IL-13 cDNAs. Each of the 33 cycles of the IL-13 PCR consisted of a 30-s denaturing step at 94 °C, a 30-s annealing step at 60 °C, and a 120-s extension step at 72 °C. In a similar manner, Jurkat T cells (line TIB-152/clone E6-1; American Type Culture Collection) were also evaluated to determine whether or not recombinant TMT is able to induce this cell line to increase its expression of the IL-13 transcript. In three of these experiments, the generated 452-bp products were subcloned and sequenced using standard methodologies to confirm that they corresponded to IL-13. For a positive control, the levels of the glyceraldehyde-3-phosphate dehydrogenase transcript were measured in comparable semiquantitative assays using a primer set supplied by Clontech (Palo Alto, CA). Each of the 30 cycles of the glyceraldehyde-3-phosphate dehydrogenase PCR consisted of a 30-s denaturing step at 94 °C, a 30-s annealing step at 65 °C, and a 120-s extension step at 72 °C to generate the relevant 983-bp product.

In one experiment, replicate flasks of $10^6$ Jurkat T cells were cultured for 1 h in serum-free medium (Opti-MEM). Ten μg/ml EK-activated TMT was added to first flask; a corresponding amount of EK was added to the other. After a 12-h incubation at 37 °C and 5% $CO_2$, ~15 μg of total RNA was isolated from both populations of cells. Comparative transcript profiling was then carried out at the GeneArray Technology Center (Brigham and Women's Hospital, Boston) using HG-U95A GeneChips (Affymetrix, Santa Clara, CA). Each GeneChip contains ~12,600 probe sets. The TUNEL assay was carried out as an additional control to ensure that TMT did not induce nonspecific damage to T cells. In this assay, Roche's *In Situ* Cell Death Detection Kit was used to identify apoptotic cells after Jurkat T cells were exposed to pro-TMT or mature TMT for 3 h.

Six- to seven-week old naïve male BALB/c, IL-4Rα-null (48), and STAT6-null (49) mice were used for functional studies. The latter transgenic mouse strains had been backcrossed at least 10 generations onto a BALB/c mouse genetic background. The animals were housed in a pathogen-free facility at the Australian National University, and all experimental procedures complied with the requirements of the Animal Care and Ethics Committee of the Australian National University. Mice were anesthetized with an intravenous injection of 100 ml of a Saffan solution (diluted 1:4 in distilled water) and then intubated with a 22-gauge catheter needle, through which vehicle alone or vehicle containing ~9 μg (~0.3 nmol) of recombinant human TMT or recombinant human tryptase βI (bound to heparin) was instilled into the trachea in a nonsurgical manner. This dose of TMT was selected because mice given comparable amounts of mouse MC protease-6 and -7, and the human tryptases α and βI do not develop AHR (5). AHR, mucus production, and tissue histochemistry were evaluated 6, 24, and 48 h after BALB/c mice were exposed to the tryptase or control solution. These same parameters were measured at the 24-h time point in the transgenic mice. The leukocytes in the lung sections and bronchoalveolar lavage (BAL) fluids were identified by morphological criteria and were quantitated as described previously (50, 51). Lung tissue representing the central (bronchi-bronchiole) and peripheral (alveoli) airways were fixed in 10% phosphate-buffered formalin, sectioned, and stained with Alcian blue-periodic acid-Schiff reagent to identify mucin-secreting cells. Replicate sections were stained with Carbol's Chromotrope-Hematoxylin to identify eosinophils.

Responsiveness to methacholine was assessed in conscious, unrestrained control mice, TMT-treated mice, and tryptase βI-treated mice by barometric plethymography, using an apparatus and software supplied by Buxco (Troy, NY). This system yields a dimensionless parameter known as enhanced pause (Phenh) that reflects changes in waveform of the pressure signal from the plethysmograph chamber, combined with a timing comparison of early and late expiration. Measurements were performed as described previously in other studies (52, 53). Briefly, mice were placed in the plethysmograph chamber and exposed to an aerosol of water (base-line readings) and then increasing concentrations of methacholine ranging from 3.1 to 25 mg/ml. The aerosol was generated with an ultrasonic nebulizer and drawn through the chamber for 2 min. The inlet was then closed and six Penh readings were taken over a 3-min time period. The results were averaged, and the values are reported as the percentage increase over base line.

Semiquantitative reverse transcription-PCRs were carried out as described previously for other cytokine transcripts (54) to evaluate the steady-state levels of the IL-13 and eotaxin-1 transcripts in the lungs of control and TMT-treated wild-type BALB/c mice, STAT6-null mice, and IL-4Rα null mice. Total RNA was isolated from the lungs of the treated mice. The oligonucleotides 5′-CTCCTCAATCCTCTCCTGTT-3′ and 5′-TTTCGCGAGGGACGGTTCAAC-3′ were used to identify and amplify the resulting IL-13 cDNAs (Invitrogen), whereas the oligonucleotides 5-TCCACCATGCAGAGCTCCACAG-3′ and 5′-CCCACATCTCCTTTCATGCCCC-3′ were used to identify and amplify the eotaxin-1 cDNAs. After 30–35 cycles, the generated products were separated by gel electrophoresis. DNA blots were prepared and probed with the IL-13-specific probe 5′-TCCAATTGCAATGCCATCTAC-3′ or the eotaxin-1-specific probe 5′-GGAACACAATGGGACGAGTTAGG-3′. The blots were then developed with the ECL detection system (Amersham Biosciences), as recommended by the manufacturer. The level of the hypoxanthine-guanine phosphoribosyltransferase transcript in each RNA sample also was determined to normalize RNA levels. The nucleotide sequences of the oligonucleotides used in this control assay have been described previously (55).

The level of a cytokine transcript in a tissue sometimes does not correlate with the level of its translated product. Thus, the amounts of IL-13 protein in the BAL fluids of control mice and TMT- and tryptase βI-treated mice were quantitated at the 24-h time point as described previously (53) using an anti-IL-13 Ab obtained from R & D Systems (Minneapolis, MN). The sensitivity of this IL-13 ELISA is ~0.5 ng/ml.

## RESULTS

*Expression of TMT in Cultured Human MCs/Basophils*—Cells isolated from the umbilical cord blood of normal individuals (Fig. 1, *a* and *c*) and from the peripheral blood of asthma patients (Fig. 1*b*) were cultured in the presence of HBM-M cell-conditioned medium and KL. Although TMT immunoreactive cells were not detected in the former cultures at day 0, many $TMT^+$ cells were detected in the day 7 cultures. The number of these cells gradually declined over the next 2 weeks of culture. Double immunostaining with anti-TMT Ab and anti-tryptase α/β Ab demonstrated that ~45% of the $tryptase^+$ umbilical cord cells at day 7 contained both TMT and tryptase α/β immunoreactivity, whereas 17% contained just TMT immunoreactivity. Although <5% of the cells in the starting population of cells from the peripheral blood of the asthma patients were metachromatic MCs and/or basophils, ~90% of the tryptase $\alpha/\beta^+$ cells in these patients additionally expressed TMT (Fig. 1*b*). The number of $TMT^+$ MCs in these cultures gradually declined even though >20% of the $tryptase^+$ cells in the 3-week cultures still expressed TMT. These data confirm earlier data (17) that indicated that TMT is not coordinately expressed with the other tryptases in its family.

In contrast to the MCs/basophils developed for 3 weeks with HBM-M cell-conditioned medium, nearly all of the cells in the cultures developed 6 weeks with KL, IL-6, and IL-10 expressed TMT (Fig. 1*d*). Based on previous immunogold location studies

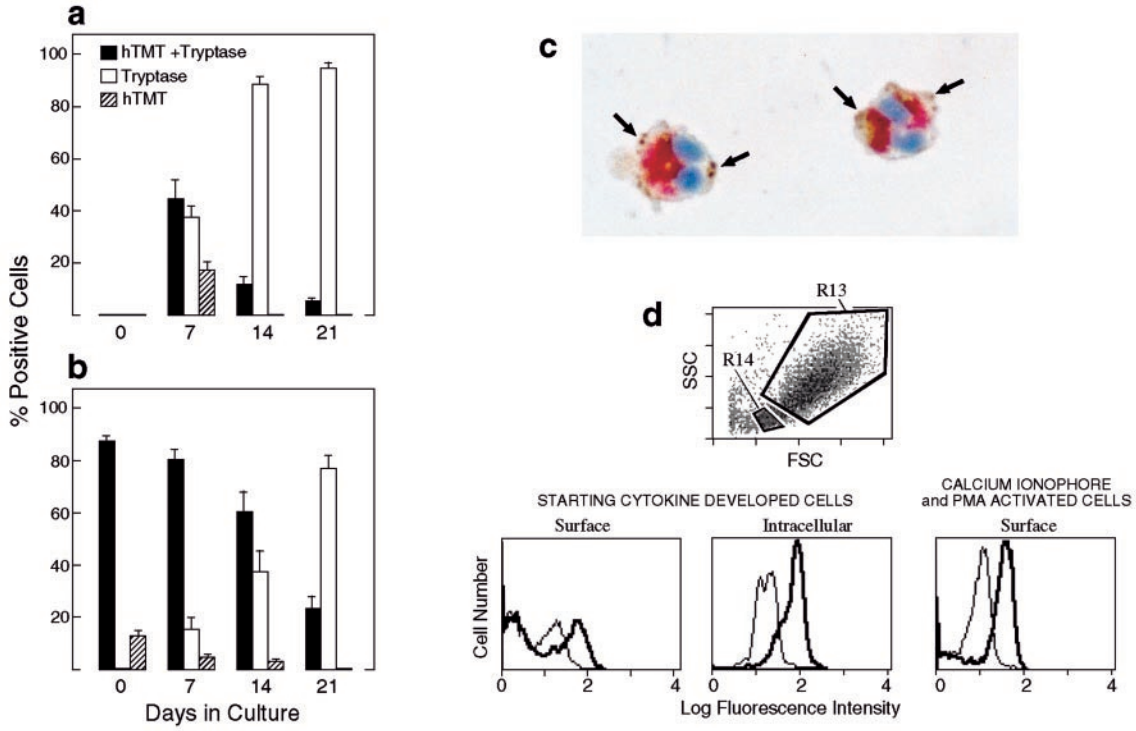



FIG. 1. **Generation of tryptase$^+$ human MCs.** MCs were developed by culturing the progenitors in umbilical cord blood of normal individuals (*a*, *c*, and *d*) or peripheral blood of asthma patients (*b*) in medium containing KL and HBM-M cell-conditioned medium (*a–c*) or in medium containing KL, IL-6, and IL-10 (*d*). At the indicated time points, aliquots of the cells in the first type of culture were cytocentrifuged and stained with anti-TMT Ab followed by anti-tryptase $\alpha/\beta$ Ab. Results are expressed in *a* and *b* as the percentage of immunoreactive cells in each preparation which are TMT$^+$/tryptase $\alpha/\beta^+$ (*solid bars*), TMT$^-$/tryptase $\alpha/\beta^+$ (*open bars*), or TMT$^+$/tryptase $\alpha/\beta^-$ (*hatched bars*) relative to cells that express at least one tryptase. Fewer than 1 and 5% of the starting cells in *a* and *b* contain immunoreactive tryptase, respectively. However, ~20% of the resulting cells in the KL/HBM-M cultures expressed at least one tryptase after 3 weeks of culture. Shown in *c* is an *in vitro* differentiated, TMT$^+$/tryptase $\alpha/\beta^+$ MC. Shown in *d* are the FACS analyses of MCs derived by culturing cord blood progenitors in the presence of KL, IL-6, and IL-10. Two cell populations are present in this type of culture which differ in their side and forward scatter because of differences in their granularity and size, respectively. We reported previously that the MC-committed progenitors residing in the R14 quadrant of the FACS analysis give rise to the more mature MCs in the R13 quadrant (45). The gated cells in the R13 quadrant were evaluated for their intracellular and surface expression of TMT before and after a 30-min exposure to calcium ionophore and PMA. Cells were stained with affinity-purified, rabbit anti-TMT Ab (*bold tracings*) or with an irrelevant rabbit Ab (*light tracings*). Essentially all of the cells in the 6-week KL/IL-6/IL-10 cultures expressed TMT.

(17), TMT resides predominantly in the secretory granules of nonactivated tissue MCs. Nevertheless, it is well known that exposure of MCs to KL often results in continuous but low grade degranulation. Because very few contaminating cells were detected in the KL/IL-6/IL-10 cultures, this *in vitro* derived MC/basophil population of cells was more suitable for evaluating surface expression of TMT than the cell population developed using KL and HBM-M cell-conditioned medium. Some of the MCs/basophils in the KL/IL-6/IL-10 cultures constitutively expressed TMT on the external face of their plasma membranes (Fig. 1*d*). However, essentially all of the cells in these cultures expressed TMT on their surfaces when they were induced to undergo more extensive degranulation. The fact that surface-bound TMT is recognized by an ~150-kDa Ab indicates that TMT is not sterically prevented from interacting with proteins in the extracellular matrix or on the surface of adjacent cell types. The data therefore implicate an extracellular function for TMT. Because human MCs/basophils exposed to calcium ionophore and PMA for 30 min actually expressed more TMT on their surfaces than cells exposed to the degranulating agents for only 15 min (data not shown), exocytosed TMT is not rapidly shed from the surface (at least in these *in vitro* conditions).

*Expression, Purification, and Biochemical Characterization of Recombinant Human TMT*—To facilitate the biochemical and functional characterization of human TMT, a recombinant pseudozymogen form of this tryptase was generated using a modification of the insect cell expression system we developed previously to generate other recombinant tryptases. Pro-TMT (Fig. 2*A*) was secreted into the conditioned medium of High Five insect cells, and the recombinant protein could be purified to homogeneity using an immunoaffinity column (Fig. 2, *B* and *C*). Recombinant pro-TMT became enzymatically active after EK treatment. However, as assessed by SDS-PAGE, the mature protease did not become smaller in size unless the activated protease was exposed to the reducing agent $\beta$-mercaptoethanol (Fig. 3*A*). Amino acid sequence analysis of the four tryptases known to be expressed in human MCs revealed that Cys$^{-12}$ and Cys$^{108}$ in TMT are not found in tryptases $\alpha$, $\beta$I, or $\beta$II. Thus, we concluded that these two Cys residues probably form the intramolecular disulfide bond that links the short 18-mer propeptide and the catalytic main chain of TMT (Fig. 3*B*).

*Substrate Specificity of Recombinant Human TMT, and Evaluation of Its Susceptibility to Protease Inhibitors*—Mature TMT, but not pro-TMT, was readily radiolabeled by [$^3$H]DFP (Fig. 4*a*). Thus, mature TMT is an enzymatically active protease even when its cleaved propeptide remains covalently attached to the catalytic domain. Proteolytic cleavage of the chromogenic substrate H-D-Leu-Thr-Arg-*p*NA by recombinant TMT was dose- (data not shown) and kinetic- (Fig. 4*b*) dependent. Similar to tryptase $\beta$I, the enzymatic activity of recombinant TMT was influenced greatly by the pH of the buffer. Optimal enzymatic activity was obtained at pH values above 7. The three-dimensional model of human TMT suggested that this MC protease probably possesses a substrate specificity more restricted than that of pancreatic trypsin. Consistent with this

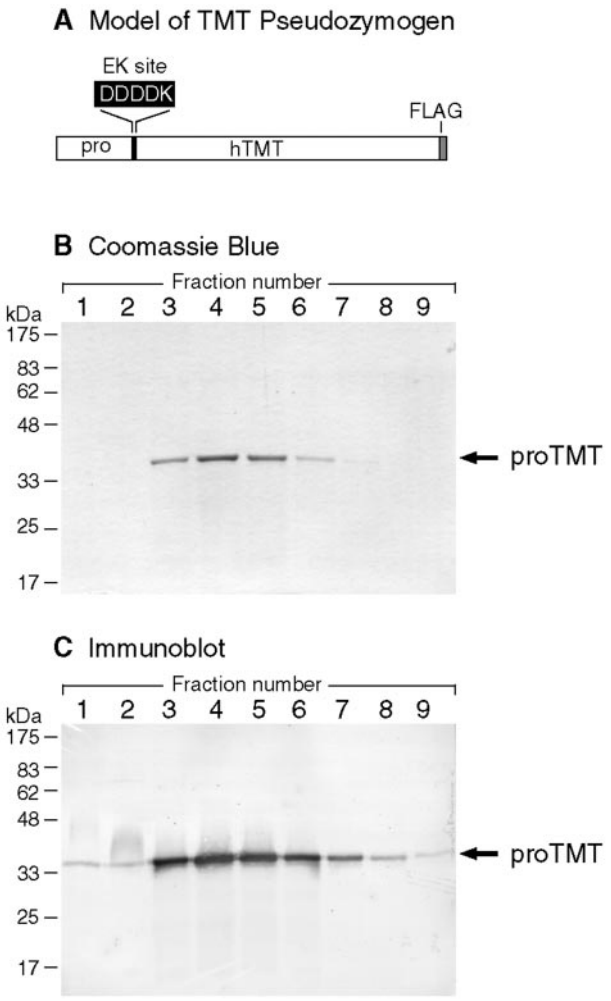



FIG. 2. **Generation of recombinant pro-TMT.** *A*, schematic diagram depicting the pseudozymogen form of human TMT expressed in High Five insect cells. *B–C*, insect cells were cultured in sterile protein-free medium, and the resulting 6-day conditioned medium was applied to an immunoaffinity column. After the column was washed extensively, it was exposed to a pH 3.5 glycine buffer to elute bound pro-TMT. The resulting eluate fractions were subjected to SDS-PAGE. One gel was stained with Coomassie Blue to evaluate the purity of the preparation (*B*). A protein blot, prepared from the duplicate gel, was stained with anti-TMT Ab (*C*). *Arrows* on the *right* indicate pro-TMT. Molecular mass standards are indicated on the *left*.

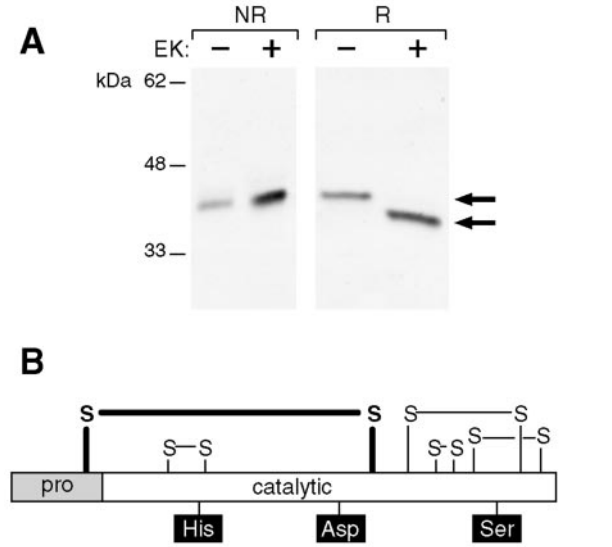



FIG. 3. **Identification of a novel intramolecular disulfide bond that covalently links the propeptide and the catalytic domain of human TMT.** *A*, recombinant pro-TMT before (−) and after (+) EK activation was subjected to SDS-PAGE analysis under nonreducing (*NR*) or reducing (*R*) conditions. The resulting protein blots were probed with mouse anti-FLAG M2 Ab. *Arrows* on the *right* indicate activated TMT with and without a covalently attached propeptide. Molecular mass markers are shown on the *left*. *B*, schematic diagram depicting the five intramolecular disulfide bonds in human TMT. Shown in *bold* is the $Cys^{-12}$-$Cys^{108}$ bond in TMT which is not present in human tryptase α, βI, βII, or βIII. The locations of the catalytic triad amino acids are also depicted.

prediction, mature TMT cleaved some, but not all, of the seven trypsin-susceptible chromogenic substrates we examined. The most susceptible substrates tested were H-D-Ile-Phe-Lys-*p*NA, H-D-Leu-Thr-pNA, and tosyl-Gly-Pro-Arg-*p*NA (Fig. 4*c*). TMT was only marginally inhibited by those reagents that selectively inactivate varied aspartyl proteases, amino peptidases, and metalloproteases even when the tested protease inhibitor was present for 18 h (Table I). Although TMT was slowly inactivated by secretory leukocyte protease inhibitor, TMT was quite susceptible to the Sigma preparation of A1AT (Fig. 5). Exposure of human TMT to human A1AT for only 30 min was sufficient for total inactivation. TMT also formed a binary complex with A1AT which could tolerate boiling in SDS-PAGE buffer.

*IL-13 Expression in TMT-treated T cells, and AHR in TMT-treated Mice*—Exposure of peripheral blood T cells or Jurkat T cells to lipopolysaccharide-free recombinant TMT resulted in increased expression of hundreds of transcripts (see GeneChip data in Supplemental material), including the one that encodes IL-13 (Fig. 6). IL-13 is a central mediator in asthma. Because pulmonary MCs express TMT, the biologic consequences of the exocytosis of TMT from activated MCs was evaluated next. Administration of ~0.3 nmol of recombinant TMT into the airways of mice caused an AHR effect 24 (Fig. 7, *middle panel*) to 48 h (data not shown) later, as determined by barometric plethysmography when the animals were subsequently given methacholine. AHR was not obtained 6 h after TMT exposure (Fig. 7, *top panel*). Thus, the tryptase induces its biologic response in the lung in an indirect manner. As found earlier (5), comparable amounts of recombinant human tryptase βI were unable to induce a significant AHR response even if this tryptase was given to replicate mice bound to heparin to increase its bioactivity and stability (Fig. 7, *bottom panel*). Because TMT-treated T cells increase their expression of IL-13 mRNA, we next looked for the expression of this cytokine in the lungs of TMT-treated mice. The levels of immunoreactive IL-13 protein in BAL fluids of BALB/c mice were 8.7 ± 0.6 ng/ml (mean ± S.E., $n = 3$) 24 h after these animals received TMT. In contrast, the levels of IL-13 protein in the BAL fluids of tryptase βI-treated mice were below detection. At the mRNA level, TMT increased the expression of the IL-13 transcript in the lungs of BALB/c mice 2–6-fold at the 24-h time point (Fig. 8).

To evaluate whether or not the biologic effects of TMT on airway function were caused primarily by increased IL-13 levels in the lung, transgenic mice that are unable to express IL-4Rα or STAT6 were examined next. The TMT-mediated AHR seen in wild-type mice was not obtained in STAT6- and IL-4Rα-null mice (data not shown) even though the IL-13 mRNA levels were increased in both transgenic mouse strains after exposure to TMT (Fig. 8). One of the biologic consequences of activation of the IL-13/IL-4Rα/STAT6 pathway in the lung is increased transcription of the eotaxin-1/SCYA11 gene. As noted in Fig. 8, the steady-state levels of the eotaxin-1 transcript were increased dramatically in TMT-treated BALB/c mice but not in STAT6- and IL-4Rα-null mice. Despite the increase in the levels of the eotaxin-1 transcript in BALB/c mice that had been given TMT 24 h earlier, large numbers of

eosinophils did not extravasate into the lungs even though the number of neutrophils increased (data not shown).

## DISCUSSION

TMT is a major granule constituent of numerous populations of human and mouse MCs, including those that reside in the lung (17, 18). However, unlike other human and mouse MC tryptases, TMT possesses a membrane-spanning domain at its C terminus which causes its cellular retention when MCs are induced to release the contents of their secretory granules (Fig. 1). TMT is therefore a potential novel mediator the MC can use to activate nearby cells. In terms of the asthma relevance of this finding, measuring the levels of immunoreactive TMT in the BAL fluid of a patient undergoing an acute asthmatic attack might give no insight about the potential importance of TMT in this lung disorder.

To begin to address the extracellular role of TMT in the lung, a pseudozymogen form of pro-TMT was generated in insect cells which could be activated after its purification from the conditioned medium (Fig. 2). The removal of the membrane-spanning domain was a technological advance because it allowed us to carry out numerous *in vivo* and *in vitro* studies without concern about the nonspecific insertion of the recombinant protease into the plasma membranes of TMT-treated cells and tissues. More important, it allowed us to evaluate the biologic effects of this protease in the absence of IL-13 and the other mediators that are also released from activated MCs.

Human TMT is initially translated as an ~35-kDa zymogen that consists of 321 amino acids (17). The initial 19 residues likely represent the signal peptide of the protein. Thus, removal of these hydrophobic residues in the endoplasmic reticulum results in a zymogen that possesses an 18-mer propeptide and a 284-mer catalytic domain. The overall amino acid sequence of the propeptide domain of TMT does not resemble that of any other member of its family. Based on its crystallographic structure (56), the mature domain of human tryptase βII contains eight Cys residues that form four intramolecular disulfide bonds. Because the corresponding Cys residues are present in the mature domains of human and mouse TMT, the same four

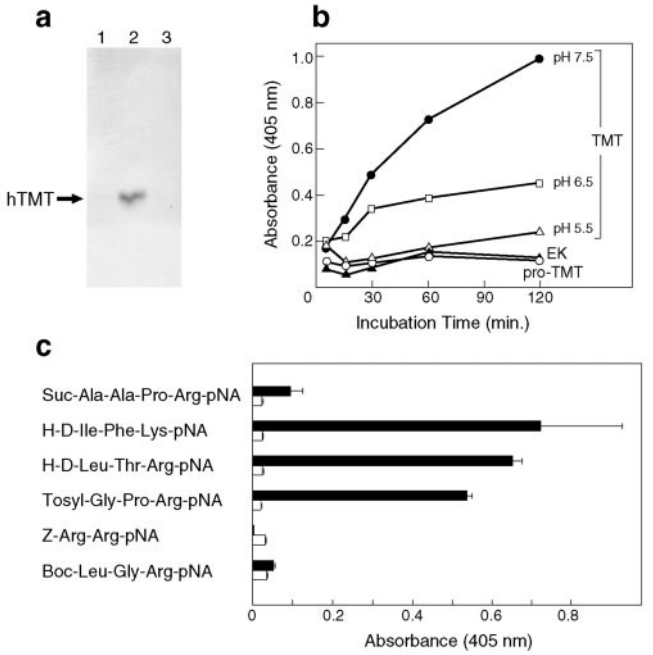



FIG. 4. **Evaluation of the substrate specificity of recombinant human TMT.** *a*, to determine whether the substrate binding cleft of human TMT is able to form properly even when the propeptide remains covalently attached to the catalytic domain, pro- (*lane 3*) and mature (*lane 2*) TMT were incubated with [$^3$H]DFP for 1 h at room temperature. The resulting products were subjected to SDS-PAGE analysis. The gel was treated with En$^3$hance, dried, and exposed to x-ray film. As a control, activation buffer containing identical amounts of EK was incubated with [$^3$H]DFP (*lane 1*). Although EK also will bind [$^3$H]DFP, a prominent radiolabeled protein is not detected in *lane 1* because of the low amount of EK in the activation buffer. *b*, pro-TMT (○), EK activation buffer (▲), and EK-activated TMT (△, □, ●) were evaluated for their ability to cleave the chromogenic substrate, H-D-Leu-Thr-Arg-*p*NA at pH 5.5 (△), pH 6.5 (□), and pH 7.5 (●). At the indicated time points, the amount of cleaved substrate was measured as a change in absorbance at 405 nm using an ELISA plate reader. Each enzymatic assay was done in duplicate. The results represent the average of two different experiments. *c*, the ability of pro-TMT (*open bars*) and mature TMT (*solid bars*) to cleave six trypsin-susceptible chromogenic substrates was evaluated. After a 3-h incubation, the amount of proteolysis of each substrate was measured. Each enzymatic assay was done in triplicate. The results represent the mean ± S.D. of three assays for each substrate.

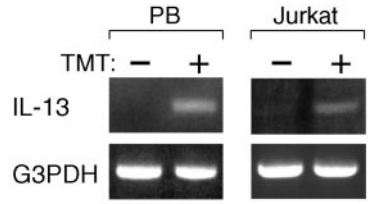
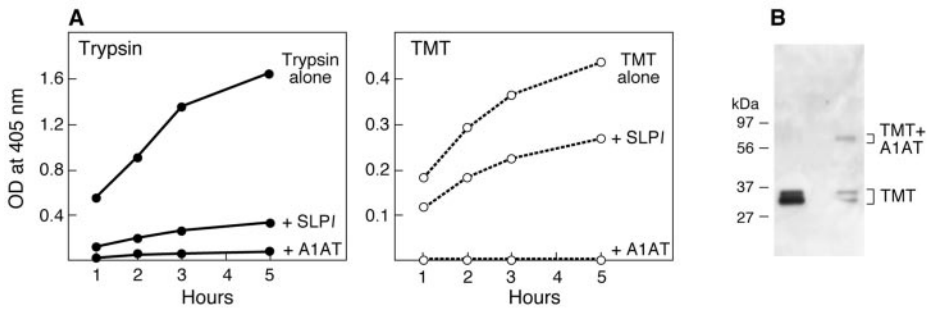



FIG. 6. **IL-13 mRNA levels in control and TMT-treated T-cells.** Jurkat T cells (*right lanes*) and CD3$^+$ T cells isolated from the peripheral blood of a normal individual (*PB*; *left lanes*) were cultured for 12 h in medium lacking (−) or containing (+) recombinant TMT. A semi-quantitative reverse transcription-PCR approach was then used to measure the steady-state levels of the IL-13 transcript in the four populations of T cells. Glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*)-specific primers were used to document equivalent amounts of intact mRNA in both cell populations. 30 and 33 cycles were used in the glyceraldehyde-3-phosphate dehydrogenase and IL-13 assays, respectively. Similar findings were obtained in two other experiments using peripheral blood T cells. Nucleotide sequence analyses confirmed that the generated 452-bp products correspond to the IL-13 transcript. TMT-treated T cells continued to exclude trypan blue. Moreover, as assessed by the TUNEL assay, virtually no apoptotic cells were found in the TMT-treated Jurkat T cell cultures.

FIG. 5. **Susceptibility of human TMT to A1AT and secretory leukocyte protease inhibitor.** *A*, trypsin and TMT were preincubated for 30 min in buffer lacking any protease inhibitor or buffer containing an ~5-fold molar excess of either secretory leukocyte protease inhibitor (*SLPI*) or A1AT. The ability of the treated protease to cleave the colorimetric substrate H-D-Ile-Phe-Lys was then evaluated. *B*, recombinant TMT was incubated for 30 min in buffer lacking (*left lane*) or containing (*right lane*) A1AT purified from human plasma. As noted, the TMT/A1AT complex that is formed can tolerate boiling in SDS buffer and electrophoresis.



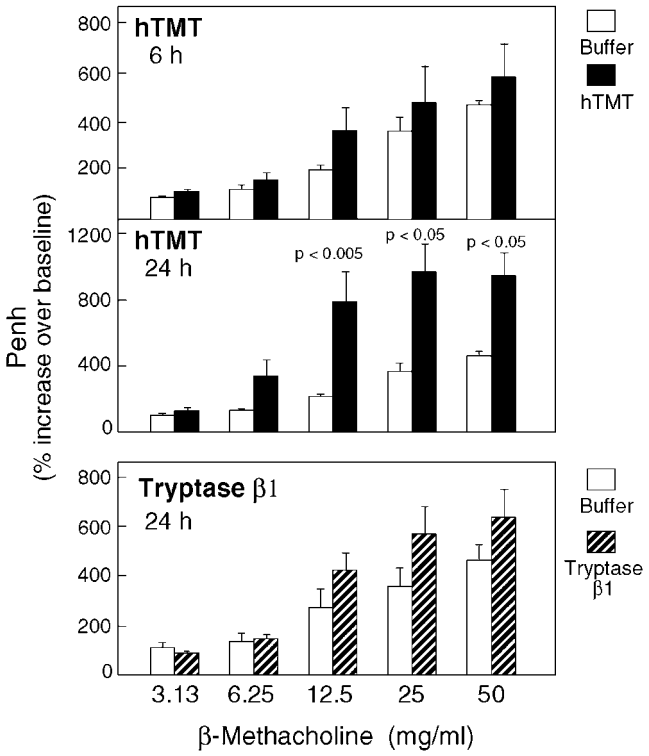



FIG. 7. **Induction of AHR using purified, recombinant human TMT.** Vehicle (*open bars*) or vehicle containing human TMT (*solid bars*) or tryptase βI (*hatched bars*) were placed in the trachea of separate mice. Six and 24 h later, airway reactivity to the indicated amounts of methacholine was determined by barometric plethysmography. Data represent the percent increase in Penh over base-line reactivity in the absence of cholinergic stimuli (mean ± S.E., four mice/group). TMT-treated mice also exhibited increased reactivity to methacholine 48 h after they had been exposed to the tryptase (data not shown). Thus, TMT exerts its adverse effects in the lung for at least 2 days.

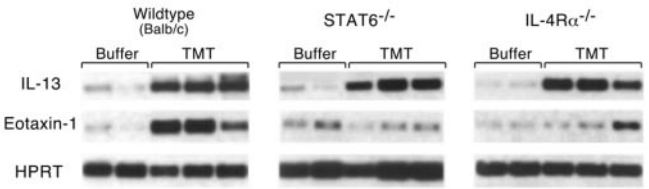



FIG. 8. **IL-13 and eotaxin-1 mRNA expression.** The steady-state levels of the IL-13, eotaxin-1, and hypoxanthine-guanine phosphoribosyltransferase transcripts were evaluated in the lungs of five wild-type BALB/c mice, five STAT6-null mice, and five IL-4Rα-null mice 24 h after the mice were exposed to buffer ($n = 2$) or TMT ($n = 3$).

intramolecular disulfide bonds (namely, $Cys^{26}$-$Cys^{42}$, $Cys^{122}$-$Cys^{191}$, $Cys^{155}$-$Cys^{173}$, and $Cys^{181}$-$Cys^{209}$) are presumed to be present in mature TMT (Fig. 3). However, human pro-TMT differs from the tryptase βI zymogen in that it has two additional Cys residues at positions −12 and +108. These two additional Cys residues also are present in mouse TMT. SDS-PAGE analysis of the activated product revealed that mature human TMT is a two-chain protease (at least when expressed in insect cells) presumably because of the formation of the $Cys^{-12}$-$Cys^{108}$ disulfide bond. Although TMT is the only MC protease identified so far which retains its propeptide when proteolytically activated, a similar situation occurs for the related airway epithelium protease human tryptase ε (19) and for many coagulation proteases. For example, the N-terminal kringle domain of plasminogen also remains bound to the C-terminal catalytic portion of the protease via a similar disulfide bond when this zymogen is proteolytically converted into plasmin.

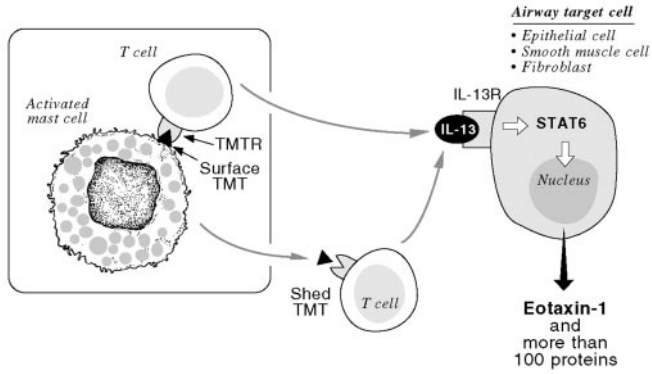



FIG. 9. **Schematic model of the TMT-mediated activation of IL-13/IL-4Rα/STAT6-dependent pathways in the lung.** TMT normally resides predominantly in the secretory granules of resting MCs. However, unlike the other preformed granule mediators, exocytosed TMT (▲) is retained at the cell plasma membrane for an extended period of time because of its C-terminal, membrane-spanning domain. The possibility has not been ruled out that some TMT is slowly shed from the MC plasma membrane via a metalloproteinase- or exosome-dependent pathway to activate distant cells in the lung. However, exocytosed TMT probably is used preferentially to activate those cell types that MCs physically contact. When MCs interact with resident T cells in the lung, surface-bound TMT induces these immune cells to increase their expression of IL-13. Newly generated IL-13 then binds to the IL-4Rα/IL-13Rα1 heterodimer on the surfaces of bronchial epithelial cells, endothelial cells, and fibroblasts. This cytokine/cytokine receptor interaction leads to movement of activated STAT6 into the nucleus where it induces the transcription of hundreds of downstream genes, including the gene that encodes eotaxin-1. It is possible that TMT induces eosinophils to produce IL-13 in a similar manner. In a more complex scenario, TMT could induce bronchial epithelial cells, endothelial cells, smooth muscle cells, and/or fibroblasts to express an intermediary factor that then induces T cells and/or eosinophils to increase their expression of IL-13.

The reason for the propeptide remaining bound to the catalytic domain of TMT remains to be determined. TMT might retain its propeptide to prevent its rapid inactivation by one or more of the other proteases stored in the MC secretory granules. Nevertheless, as occurs with plasmin, the covalent attachment of the cleaved propeptide does not appear to suppress the biological activity of TMT *in vitro* (Figs. 4 and 5) or *in vivo* (Figs. 6 and 7).

Human TMT and tryptase βI are ~50% identical, and both MC proteases are enzymatically active at neutral pH (Fig. 4). However, six of the seven loops that form the substrate binding clefts of TMT (17) and tryptase βI (12) are distinct. As expected based on these structural differences, recombinant TMT possesses a substrate specificity different from that of recombinant tryptase βI (Fig. 4). Recombinant human tryptases α and βI cannot cleave H-D-Leu-Thr-Arg-*p*NA effectively, whereas this substrate is readily cleaved by recombinant human TMT. Based on these and other data, it is now apparent that a primordial serine protease gene at chromosome 16p13.3 duplicated repeatedly during evolution to give rise to multiple tryptic-like proteases in human MCs that possess different substrate specificities. The fact that human TMT and tryptase βI are functionally distinct proteases even though their amino acid sequences are ~50% identical highlights the importance of using recombinant TMT in varied *in vitro* and *in vivo* studies.

In its mature state, TMT is rapidly inhibited by DFP (Fig. 4*a*) and by A1AT (Fig. 5). The ability of A1AT to inhibit TMT quickly was a surprise because it had been concluded that MC tryptases are not inhibited by this serpin. At least 35 human serpins have been cloned (57). Because recombinant serpins are just beginning to be generated, we cannot rule out the possibility that TMT is regulated in the lung by multiple protease inhibitors. Human pulmonary macrophages normally

produce minuscule amounts of A1AT relative to hepatocytes. However, the levels of A1AT in the BAL fluids of our BALB/c mice before and after TMT treatment were not measured. It also remains to be determined whether mouse A1AT can inactivate human TMT. Nevertheless, the exquisite susceptibility of human TMT to human A1AT is noteworthy in that low levels of this serpin in the circulation is a major risk factor in the development of asthma and emphysema (58). MC degranulation results in edema and the influx of A1AT and other plasma proteins into tissues. It has been concluded that the primary beneficial role of A1AT in the lung is the inactivation of neutrophil elastase. Our data now suggest that plasma-derived A1AT also plays an important role in dampening TMT-mediated events in the lung during MC-mediated inflammatory reactions. The finding that TMT is somewhat susceptible to secretory leukocyte protease inhibitor (Fig. 5) is also noteworthy in that the aerosol delivery of secretory leukocyte protease inhibitor into the lungs is able to reduce the early and late phases of bronchoconstriction in a sheep bronchoprovocation model (59).

The derivation of recombinant TMT (Figs. 2–4) allowed us to deduce one of the biological consequences that occur when TMT is released from the secretory granules of an activated MC. MCs often contact T cells (40, 41). Although T cells are the major source of IL-13 in the lungs of asthma patients (39), it is not known what endogenous factors in the lung induce T cells to increase their expression of this key cytokine. As noted in Fig. 6, exposure of Jurkat and peripheral blood T cells to recombinant TMT results in increased expression of the IL-13 transcript. The mouse ortholog of human TMT (17) resides at the ~12 cM position on mouse chromosome 17 near a site that is associated with methacholine-mediated AHR in two mouse gene linkage studies (60, 61). Whether or not TMT could induce an AHR effect in a living mouse was therefore investigated. Despite its susceptibility to human A1AT (Fig. 5), a one-time exposure to 0.3 nmol of recombinant TMT was sufficient to induce AHR in normal BALB/c mice (Fig. 7). The levels of IL-13 protein were markedly increased in the BAL fluids of these animals. Moreover, a comparable effect was not obtained in STAT6- and IL-4Rα-null mice. Thus, it appears that TMT exerts its adverse effect in the lung in this animal model primarily by inducing the expression of IL-13 (Fig. 8).

IL-13 is a central mediator in allergic asthma (31–33). Transcript profiling data revealed that the expression of eotaxin-1 and hundreds of other genes are dramatically altered in IL-13-treated bronchial epithelial cells, smooth muscle cells, and fibroblasts *in vitro* (62, 63). The levels of IL-13 mRNA and protein are elevated in the lungs of asthma patients (39, 64, 65), and gene-linkage studies have mapped an asthma susceptibility locus to human chromosome 5 where the IL-13 gene resides (66). IL-13R-Fc inactivates IL-13 (but not the related cytokine IL-4), and allergic mice given this bioengineered soluble receptor exhibit decreased AHR, eosinophil recruitment, and mucus production (67, 68). Naïve A/J mice also develop AHR within 72 h after recombinant IL-13 is injected into their airways (68). Adoptive transfer of ovalbumin-specific T cells from normal mice, but not IL-13-null mice, into naïve nonsensitized mice that are subsequently exposed to ovalbumin results in AHR, enhanced mucus production, high levels of eotaxin-1, and a transient increase in the number of eosinophils in the lung (69). The chronic production of large amounts of IL-13 in the lungs of transgenic mice results in AHR, airway fibrosis, eosinophil extravasation, and increased expression of eotaxin-1 and varied metalloproteinases and cathepsins (70, 71). Although we obtained no evidence of increased fibrosis and eosinophil extravasation in our TMT-treated mice, the mice used in our experiments were only exposed to TMT once, and AHR was measured within 48 h of TMT treatment. Because of our findings of increased eotaxin-1 mRNA levels in BALB/c mice 24 h after exposure to TMT, we suspect that increased numbers of eosinophils would be seen in the lungs of TMT-treated mice if a later time point had been examined or if animals had been exposed to the tryptase multiple times. Nevertheless, our TMT findings support our previous data (72) and that of others which showed that AHR can be obtained in mice in the absence of an eosinophilia.

The functional IL-13R is a heterodimer composed of the IL-4Rα and IL-13Rα1 chains (73–75). Although IL-13 and IL-4 are only ~25% identical, IL-4 often exhibits IL-13-like responses *in vivo* and *in vitro* because IL-4 can recognize the IL-4Rα/IL-13Rα1 heterodimer. Signaling through this surface receptor complex leads to activation of STAT6 and subsequent downstream events (69, 76–78). The level of IL-4Rα mRNA is increased in the lungs of asthma patients (79) and genetic variants of IL-4Rα also are associated with the development of atopic asthma (80, 81). Finally, blockade of IL-4Rα/IL-13Rα1-dependent signaling in mouse models of experimental asthma abrogates AHR and mucus production in allergic lungs. Transcript profiling revealed that TMT induces the expression of hundreds of genes in T cells (see Supplemental material). Nevertheless, IL-4 and IL-13 are the only known ligands for IL-4Rα. Signal transduction events mediated by this cytokine receptor are dependent on STAT6. TMT induces AHR in normal BALB/c mice but not in IL-4Rα- or STAT6-null mice. Thus, TMT induces AHR primarily by activating an IL-13/IL-4Rα/STAT6-dependent pathway in the lungs (Fig. 9). The accumulated data suggest that MC-derived TMT induces resident T cells (and possibly eosinophils) in the lung to increase their expression of IL-13 by a manner that remains to be determined. The resulting IL-13 binds to the α-chain of the cytokine receptor that recognizes both IL-4 and IL-13 residing on the surface of those cell types that control AHR in the lung. This cytokine/cytokine receptor interaction results in downstream signaling events (*e.g.* increased eotaxin-1 expression (Fig. 8)) that are exquisitely dependent on STAT6. Activated MCs release 60–100-nm exosomes (42), and these small membrane-containing vesicles can activate T cells (82). Some MC populations transiently express varied metalloproteinases (83). Thus, it is also possible that TMT is slowly shed from the plasma membrane of an activated MC in the lung by a metalloproteinase- or exosome-dependent pathway so that a portion of the exocytosed tryptase can exert its biological effects at more distant sites. Whether or not this occurs *in vivo*, TMT is a novel type of granule mediator the MC uses to induce signal-transduction events in those cell types that it physically contacts.

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