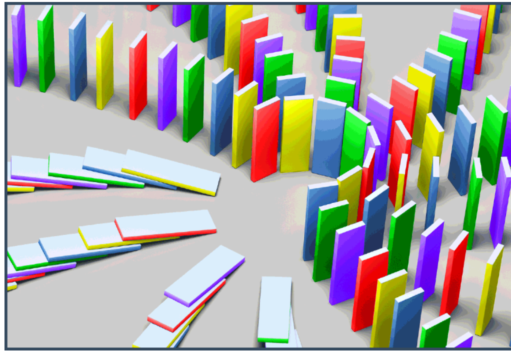

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Lawrence B. Schwartz; ... et. al

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Tryptase Precursors Are Preferentially and Spontaneously Released, Whereas Mature Tryptase Is Retained by HMC-1 Cells, Mono-Mac-6 Cells, and Human Skin-Derived Mast Cells[1]

Lawrence B. Schwartz,[2] Hae-Ki Min, Shunlin Ren, Han-Zhang Xia, Jiang Hu, Wei Zhao, George Moxley, and Yoshihiro Fukuoka

Tryptase (α and β) levels in serum are used to assess mast cell involvement in human disease. Using cultured cells, the current study examines the hypothesis that protryptase(s) are spontaneously secreted by mast cells at rest, whereas mature tryptase(s) are stored in secretory granules until their release by activated cells. HMC-1 cells have only β-tryptase genes and the corresponding mRNA. Mono-Mac-6 cells have both α- and β-tryptase genes but preferentially express α-tryptase. Mono-Mac-6 cells spontaneously secrete most of their tryptase, which consists of α-protryptase, whereas mature tryptase is retained inside these cells. HMC-1 cells also spontaneously secrete most of their tryptase, identified as β-protryptase, and retain mature tryptase. Skin-derived mast cells retain most of their tryptase, which is mature, and spontaneously secrete protryptase(s). Total tryptase levels in plasma are detectable but no different in healthy subjects with and without the gene for α-tryptase, consistent with pro forms of both α- and β-tryptase being spontaneously secreted. Thus, protryptase(s) are spontaneously secreted by resting mast cells, whereas mature tryptase is retained by mast cells until they are activated to degranulate. ***The Journal of Immunology,* 2003, 170: 5667–5673.**

Tryptase (EC 3.4.21.59) is the most abundant protein product produced by human mast cells and is derived principally from two genes on chromosome 16p13.3, α-tryptase and β-tryptase (1). There are two major forms of α-tryptase (αI and αII) and three major forms of β-tryptase (βI, βII, and βIII) (2). Also on chromosome 16p13.3 are the δ-tryptases, initially named mouse mast cell protease-7-like tryptases (3). The product of this gene has close homology to $\alpha\beta$-tryptases over exons 1–4, but exon 5 is more closely related to mouse mast cell protease-7. Although it is expressed by mast cells and possibly other cell types, a seemingly premature stop codon terminates translation 40 aa earlier than both α- and β-tryptases (4). The product of the β-tryptase gene(s) is autoprocessed in vitro from β-protryptase to β-pro'tryptase at acidic pH, optimally in the presence of heparin proteoglycan, and then to β-tryptase by a dipeptidase, thought to be dipeptidyl peptidase I in humans (5). Tryptase in murine mast cells uses a different dipeptidase (6). Mature β-tryptase is stored in secretory granules as an enzymatically active tetramer in a complex with heparin proteoglycan until the cells are activated to degranulate and release the protease:proteoglycan complex. In contrast, α-protryptase (and δ-tryptases) may not undergo autoprocessing from protryptase to pro'tryptase, because a tryptase-resistant Q^{-3} rather than a tryptase-sensitive R^{-3} is present in the -3 position of the propeptide (3, 5). Indeed, when mature α-tryptase is produced in vitro, although it forms a tetramer, the protein appears to be enzymatically inactive (7–9).

The major form of tryptase found in normal blood fails to bind to the G5 mAb, which recognizes recombinant human (rh)[3] β-tryptase but not rhα-protryptase (5, 10). Thus, an immunoassay using this mAb measures mature β-tryptase. Although β-tryptase levels are undetectable in normal serum (<1 ng/ml), they are elevated in the blood of most cases of systemic anaphylaxis with hemodynamic compromise, particularly when the precipitating agent is administered parenterally. In such cases, the magnitude of mast cell degranulation appears to be the primary determinant of clinical severity (11–13). In contrast, based on mAbs that recognize both α-protryptase and β-tryptase, a total tryptase immunoassay was developed that detected levels of tryptase in baseline serum from essentially all individuals (mean $\pm$ SD, 4.9 $\pm$ 2.3 ng/ml) (14). So-called total tryptase levels are elevated in subjects with systemic mastocytosis and reflect the total body burden of mast cells (10).

Several issues relating to these mature and total tryptase assays must be clarified. Approximately 25% of individuals lack a gene for α-tryptase (15, 16). Whether such a genetic difference affects the level of tryptase in the blood has not been determined. Also, the possibility that α-protryptase might be processed to mature α-tryptase in vivo has not been excluded; thus, the ability of tryptase immunoassays to detect this putative form of the protein also should be determined. Finally, the hypothesis that mature forms of tryptase are preferentially stored in secretory granules, whereas immature forms of tryptase are preferentially selected for spontaneous secretion, has not been directly examined.

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[3] Abbreviations used in this paper: rh, recombinant human; Mac-6, Mono-Mac-6.

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The current study examines retained and spontaneously secreted tryptase in two human cell lines, HMC-1 cells derived from a mast cell leukemia (17) and Mono-Mac-6 (Mac-6) cells derived from a monoblastic leukemia (18). Only tryptase mRNA encoding β-tryptase was previously found in HMC-1 cells (19, 20), whereas only tryptase mRNA encoding α-tryptase was found in Mac-6 cells (21). In contrast, most human lung-derived mast cells, skin-derived mast cells, and mast cells derived from fetal liver progenitors in vitro contain both α-tryptase and β-tryptase mRNA (20, 22). HMC-1 cells contain ~50 ng of tryptase/10^6 cells (23), whereas human lung-derived (12 μg/10^6 cells) and skin-derived (35 μg/10^6 cells) mast cells contain substantially greater amounts (24). The current study concludes that precursor forms of both α- and β-tryptases are preferentially secreted spontaneously. Thus, serum levels of precursor forms of tryptase in health subjects reflect the total body burden of mast cells, regardless of whether the subject is α-tryptase deficient, whereas serum levels of mature tryptase reflect mast cell activation.

Materials and Methods

Materials

Tosyl-Gly-Pro-Lys-*p*-nitroanilide, HEPES, MES, porcine heparin glycosaminoglycan (1,200–20,000 Da), dextran sulfate (500 kDa), 3-(cyclohexylamino)-1-propanesulfonic acid (Sigma-Aldrich, St. Louis, MO); *Hin*dIII, *Xho*I, *Sac* II, *Eco*RI, 100-bp DNA ladder, dNTP, genomic DNA purification kit, PCR size markers, DNase, oligodeoxythymidylate primers, and *Taq* DNA polymerase with the PCR reagents (Promega, Madison, WI); SYBR Green I (FMC Bioproducts, Rockland, ME); Moloney murine leukemia virus-derived reverse transcriptase (Life Technologies, Gaithersburg, MD); XL-1-Blue-competent cells, *Pfu* DNA polymerase, and the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA); and RNA isolation kit (Qiagen, Valencia, CA) were obtained as indicated. Tryptase from human lung and rhβII-tryptase (both tetramers with subunits of 29 to 35 kDa by SDS-PAGE) were prepared and purified to homogeneity by immunoaffinity chromatography using the B2 mAb, and heparin-agarose chromatography as described (5, 25). rhαI-protryptase (a monomer of ~30 kDa by SDS-PAGE) also was prepared and purified as described (26). Purified preparations of tryptase were stored at −70°C in 20% (v/v) glycerol, 0.01 M MES (pH 6.5), 0.8 M NaCl. IgG1κ mAb B12, which recognizes a conformational epitope present on tryptase, was prepared as before (14, 25). mAb B12 Affi-Gel was prepared by coupling B12 with activated Affi-Gel (Bio-Rad Laboratories, Hercules, CA). mAbs G4 (IgG1κ) and G5 (IgG2bκ), which recognize linear epitopes on all forms of tryptase and on β-tryptase, respectively, were prepared as described (27–29). An HMC-1 mast cell leukemia cell line was provided by Dr. G. Gleich and Dr. J. Butterfield (Mayo Clinic, Rochester, MN) (17). The Mac-6 myelomonocytic leukemia cell line was provided by Dr. H. W. Ziegler-Heitbrock (University of Munich, Munich, Germany) (18). Primer synthesis and DNA sequencing reactions were performed by the Virginia Commonwealth University Nucleic Acids Core Laboratory (Richmond, VA). All studies using human serum, cells, and tissues were approved by the human studies Institutional Review Board of Virginia Commonwealth University (Richmond, VA). Tryptase ELISAs with the B12 mAb used for capture, and either biotin-G5 (mature tryptase) or biotin-G4 (total tryptase) used with streptavidin-alkaline phosphatase for detection was performed as described (10).

Preparation of mature α-tryptase

αI-Tryptase cDNA (26) in the pBluescript II SK vector (Stratagene) was mutated as recommended by the manufacturer (QuickChange Site-Directed Mutagenesis Kit, QCM) with slight modifications (30) to yield $Q^{-3}R$ by changing the −3 codon from CAA to CGA. First, two independent extension reactions were performed with *Pfu* DNA polymerase, one containing the sense primer, 5′- AGCGAGTGGGCATCGTTGGGGGTCAGG-3′ and the other containing the antisense primer, 5′-CCTGACCCCCAACGAT GCCCACTCGCT-3′. The underlined nucleotides show the mutation. Extension reactions were initiated by preheating the reaction mixtures to 95°C for 5 min, followed by five cycles at 95°C for 30 s, 58°C for 1 min, and 68°C for 8 min. Second, equal portions of the two reactions were mixed together along with an additional 1 U of *Pfu* DNA polymerase and the same sense and antisense primers and heated at 95°C for 5 min, followed by 22 cycles at 95°C for 30 s, 58°C for 1 min, and 72°C for 12 min and a final extension reaction at 72°C for 15 min. 10 U of *Dpn*I were mixed into the final PCR mixture and incubated at 37°C for 2 h to destroy parental plasmid before transformation of XL-1-Blue cells was performed, which in turn were selected with ampicillin. After confirmation of the mutated sequence, $Q^{-3}R$α-tryptase was excised by *Not*I and *Hin*dIII double digestion and ligated into the pFastBac1 transfer vector (Invitrogen, Carlsbad, CA). pFastBac1, which contained $Q^{-3}R$β-tryptase cDNA, was used to transform DH10Bac *Escherichia coli*-competent cells, which contained helper plasmid and bacmid DNA. Transformed cells with the transfer plasmid integrated into the bacmid by homologous recombination were selected on LB agar plates containing kanamycin (50 μg/ml), gentamicin (7 μg/ml), tetracycline (10 μg/ml), Bluo-gal (100 μg/ml; Invitrogen), and isopropyl β-D-thiogalactoside (40 μg/ml). The inserted $Q^{-3}R$α-tryptase cDNA sequence was confirmed using both vector- and tryptase-specific primers. Sf9 insect cells were then transfected with recombinant bacmid DNA mixed with CellFECTIN reagent (Invitrogen). Recombinant baculovirus was collected from the cell culture medium, produced in high titer, and used to express recombinant $Q^{-3}R$ β-tryptase. Sf9 cells were infected at a multiplicity of infection of 0.1 and cultured at 2×10^6 cells/ml of Sf-900II serum-free medium. *trans*-Epoxysuccinylleucylamido(4-guanidino)butane (1 μM; Sigma-Aldrich) was added 2 days after transfection, and medium was typically harvested on day 4. $Q^{-3}R$α-protryptase was purified from the medium (1–1.5 mg/L) by B2-agarose chromatography (26). Before immunoaffinity chromatography, the medium was adjusted to 1 M NaCl and 10 mM MES, pH 6.5. The B2 column, once loaded, was washed with several column volumes of 10 mM MES buffer, pH 6.5, containing 1 M NaCl and then 10 mM MES buffer, pH 6.5, containing 0.2 M NaCl. Elution occurred with 10 mM diethanolamine buffer, pH 10, containing 0.2 M NaCl, 50% ethylene glycol in 2-ml fractions. Eluted fractions were immediately neutralized with 1 M MES buffer, pH 6.5 (0.1 fraction volume). Tryptase was measured by the total tryptase ELISA.

To generate mature α-tryptase, the eluted $Q^{-3}R$α-protryptase (10–30 μg/ml) was combined with dextran sulfate (100 μg/ml) and BSA (0.5 mg/ml) and dialyzed at 4°C overnight against 1 liter of 0.01 M MES buffer, pH 6.0, containing 0.15 M NaCl. Dialyzed $Q^{-3}R$α-protryptase was incubated for 3 h at 37°C with purified human lung-derived tryptase (1–3% of $Q^{-3}R$α-protryptase concentration), dipeptidylpeptidase I (10 μg/ml), and DTT (0.1 mM) in 10 mM MES buffer, pH 6.0, containing 0.15 M NaCl. Controls included omitting purified human lung-derived tryptase, omitting purified human lung-derived tryptase and dipeptidylpeptidase I, or omitting $Q^{-3}R$α-protryptase. With all incubation components included, nearly all of the $Q^{-3}R$α-protryptase was converted from a monomer to a tetramer by Superose 12 gel filtration performed in 10 mM MES buffer, pH 6.5, containing 1 M NaCl. In the absence of either lung-derived tryptase or dipeptidylpeptidase I, only monomer was detected. The N-terminal amino acid sequence of the processed tryptase was determined as described below.

Determination of spontaneous release of tryptase

HMC-1 cells were maintained in Iscove's medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin D, 10% (v/v) heat-inactivated FCS, and 0.01% α-thioglycerol. Mac-6 cells were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin D, and 10% (v/v) heat-inactivated FCS. Both cell types were cultured at 37°C in an incubator maintained at 5% CO_2. To examine the time course of spontaneous tryptase secretion, cells were washed three times and suspended in fresh medium at 0.5×10^6 cells/ml at time 0. Cells and medium were harvested at different times as indicated. Cells were separated from medium by centrifugation at 2000 rpm for 10 min at 4°C in a model PR6 centrifuge (IEC, Needham Heights, MA) using a swinging bucket rotor. Solubilized tryptase was obtained from the cell pellets by sonicating cells suspended at 10^7 cells/ml 10 mM MES, pH 6.0, containing 1 M NaCl at 4°C and centrifugation at 10,000 × *g* for 30 min at 4°C. Cell lysate supernatants and culture media were then analyzed as described.

Skin-derived mast cells were obtained as described (31). Briefly, cells were dispersed from fresh surgical skin using collagenase and hyaluronidase, partially purified by Percoll density-dependent sedimentation, and placed into culture in serum-free AIM-V medium containing 100 ng/ml rh stem cell factor (a gift from Amgen, Thousand Oaks, CA). Mast cells were studied after 6 wk of culture, by which time they were >99% pure and >97% viable. Media and cells were collected 6 days after addition of fresh medium. Tryptase levels in cells, after extraction of the protein by sonication in 10 mM MES, pH 6.5, containing 1 M NaCl as above, and in the medium were measured by the mature and total tryptase ELISAs.

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Purification of tryptase from cell media

Cells and cell media were harvested 4 days after seeding the cells at 0.5 × 10^6 cells/ml. Tryptase in cell lysates (~10^9 cells) was solubilized as above and incubated with B12-Affi-Gel beads overnight at 4°C. The beads were washed with 10 volumes of 10 mM MES, pH 6.0, containing 1.0 M NaCl, 10 volumes of 10 mM diethanolamine, pH 10, containing 0.2 M NaCl and 50% ethylene glycol, and 10 volume of 10 mM Tris, pH 8.5, containing 0.2 M NaCl. Tryptase was then eluted with 8 M urea. Cell medium (5 liters) was adjusted to contain 1 M NaCl and incubated with B12-Affi-Gel beads overnight at 4°C. Washing and elution were as for the cell lysate tryptase. Each sample of eluted tryptase was dialyzed against 1.0 M NaCl and then against 0.12 M NaCl in 10 mM MES, pH 6.0. Dialyzed tryptase was subjected to heparin-agarose chromatography, being loaded in dialysis buffer and eluted with a salt gradient in 10 mM MES, pH 6.0, from 0.1 to 2.0 M NaCl. Protein was measured by the bicinchoninic acid-modified Lowry assay (32); tryptase was measured by total and mature tryptase ELISAs.

Amino acid sequencing

Tryptase preparations from HMC-1 and Mac-6 cell lysates and culture medium, purified as above, were further subjected to SDS-PAGE in 12% polyacrylamide gels under reducing conditions, blotted onto PVDF membranes (0.2 mm) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer, pH 11, containing 10% methanol at 50 V for 0.5–1 h, stained with 0.1% Coomassie Brilliant Blue R250 in 40% methanol, 1% acetic acid, destained with 50% methanol, 10% acetic acid, washed with water, and dried. The tryptase band was excised and subjected to N-terminal amino acid sequencing by the Protein Structure Laboratory (University of California, Davis, CA) using an Applied Biosystems 477A sequencer.

RT-PCR and genomic DNA analysis of tryptases in the HMC-1 and Mac-6 cells

Genomic DNA from each cell line was purified using a commercial genomic DNA purification kit, whereas total RNA from each cell line was extracted using a commercial RNA isolation kit, in each case according to the manufacturer's instructions. Also, to remove genomic DNA from preparations of total RNA the samples were treated with RNase-free DNase. To assess the presence of tryptase in genomic DNA, PCR were performed in a total volume of 50 μl containing 1× reaction buffer with 50 ng of genomic DNA, 200 mM dNTP, and 20 pmol of each primer, including α-tryptase-specific and β-tryptase-specific pairs (see below). To directly assess the presence of α-tryptase and β-tryptase mRNA, cDNA was synthesized from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase and oligodeoxythymidylate primers and subjected to PCR as above. α-Tryptase-specific primers (sense: 5′-TGCAGCAA GCGGGTATCGT-3′; antisense: 5′-AGTCTGGATGATGTAGAACTGT-3′) and β-tryptase-specific primers (sense: 5′-TGCAGCGAGTGGG CATCGT-3′; and antisense: 5′-GATCTGGGCGGTGTAGAACTGT-3′) were used for PCR. In each case, the sense and antisense primers were located in exons 3 and 4, respectively, and amplified 386-bp regions from genomic DNA and 278-bp regions from cDNA. The optimal $MgCl_2$ concentration for PCR was 0.5 mM. Hot start reactions (95°C for 5 min) were initiated with 1 U *Taq* DNA polymerase followed by 35 cycles of amplification in a volume of 50 μl. Each cycle consisted of 1 min at 94°C, 1 min at 62°C and 1 min at 72°C; the final extension was performed at 72°C for 10 min. Reaction conditions were as above except that a portion (2 of 20 μl) of the cDNA was used as template. PCR products were analyzed by electrophoresis in a 6% polyacrylamide gel and visualized by staining with SYBR Green I (1/10,000 dilution).

Analysis of the relationship between the presence and absence of the gene for α-tryptase and serum tryptase levels

Plasma and blood cells were derived from EDTA-anticoagulated venous blood collected from 109 healthy subjects (56 male and 53 female). Plasma was stored at −70°C, and genomic DNA was purified from peripheral blood leukocytes using a salting-out method (PureGene; Gentra, Minneapolis, MN) (33). Samples of DNA were assessed for the presence of the α-tryptase gene by PCR with α-tryptase-specific primers as described above. Purified genomic DNA (50 ng) was subjected to PCR as above except that 1.5 U of *Taq* DNA polymerase were included and 35 cycles were used. The PCR product was subjected to electrophoresis in 1.5% agarose gels and visualized with ethidium bromide. Plasma was subjected to the total tryptase ELISA.

Results

Detection of recombinant forms of α- and β-tryptases by ELISA

Mature and precursor forms of recombinant α- and β-tryptases were prepared and analyzed by two ELISAs. Both mature recombinant α-tryptase and β-tryptase were tetrameric by gel filtration and had the predicted N-terminal amino acid sequences of IVGG. Whereas recombinant β-tryptase exhibited a specific activity with 0.1 mM tosyl-Gly-Pro-Lys-*p*-nitroanilide of ~25 U/mg, that of recombinant α-tryptase was ≤ 0.2 U/mg. Each ELISA used the B12 mAb for capture. One used biotin-conjugated G5 mAb for detection; the other used biotin-conjugated G4 mAb. As shown in Fig. 1*A,* the dose-response curves with the biotin-G4/B12 combination were no different among the recombinant and lung-derived forms of tryptase. In contrast, the biotin-G5/B12 combination failed to detect α-protryptase, β-protryptase, and β-pro'tryptase but showed similar dose-response curves for mature recombinant β- and α-tryptases and lung-derived tryptase. Thus, in the context of mast cell α- and β-tryptases, the biotin-G4/B12 sandwich serves as a total tryptase assay; the biotin-G5/B12 sandwich serves as an assay for mature tryptases.

Spontaneously secreted and retained levels of tryptase in cultures of HMC-1 and Mac-6 cell lines and of skin-derived mast cells

Total and mature tryptase levels were measured in the medium and in cell lysates collected at different times from cultures of HMC-1

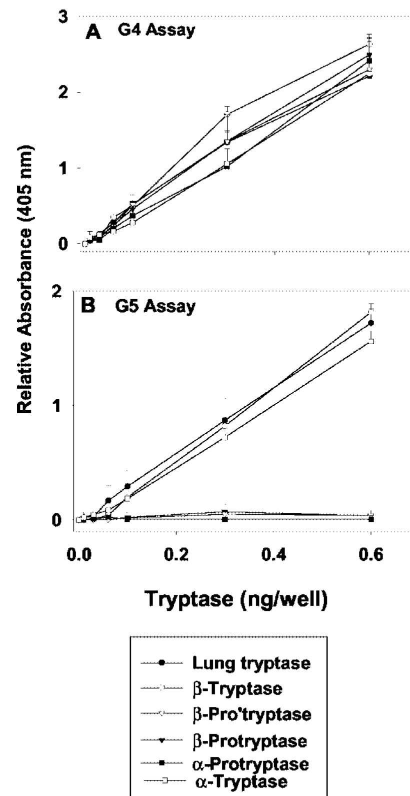

FIGURE 1. Detection of mature and precursor forms of α-tryptase and β-tryptase by two ELISAs. Purified human lung-derived tryptase, rhα-protryptase (native sequence), rhα-tryptase (derived from $Q^{-3}R$α-protryptase), rhβ-protryptase, rhβ-pro'tryptase, and rhβ-tryptase molecules, at concentrations from 0.01 to 0.6 ng/well, were measured as described in *Materials and Methods* using biotin-G4 (*A*) or biotin-G5 (*B*) for detection. The absolute amounts of each form of tryptase were determined by direct measurement of purified protein (bicinchoninic assay) and confirmed by comparing the optical density of the Coomassie blue-stained band after SDS-PAGE of each form of tryptase to that of human lung-derived tryptase.

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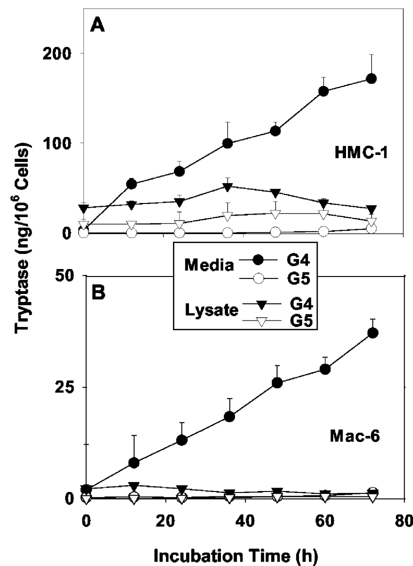

FIGURE 2. Spontaneous secretion of tryptase by HMC-1 and Mac-6 cells. Cells in culture were washed and suspended in fresh medium at 0.5×10^6 cells/ml. Samples were harvested from three wells at each time point, separated into cells and medium, and assayed for tryptase with the two immunoassays as described. The data were normalized by the amount of tryptase divided by cell number (nanograms per 10^6 cells). *A,* HMC-1 cell line; *B,* Mac-6 cell line. Tryptases in medium and in the cell lysates are expressed as nanograms or micrograms per 10^6 cells or cell equivalents. Mean ± SD values are shown at each time point ($n = 3$).

and Mac-6 cells. As shown in Fig. 2, total tryptase levels in the medium progressively increased during the 3-day experiment for both HMC-1 and Mac-6 cells, whereas mature tryptase levels remained low throughout the culture (Table I). For HMC-1 cells, the total tryptase level detected in the medium after 3 days of culture (172 ± 27 ng/10^6 cell equivalents) was higher than the tryptase level detected inside the cells (36 ± 5 ng/10^6 cells). However, 42% of the tryptase that was retained in the cell was mature by ELISA, compared with only 3% of the spontaneously secreted tryptase. For Mac-6 cells the total tryptase level detected in the medium after 3 days of culture (37 ± 3 ng/10^6 cell equivalents) was considerably higher than that detected inside the cells (1.3 ± 0.3 ng/10^6 cells). Again, the portion of retained tryptase that by ELISA was mature (23%) was higher than for the spontaneously secreted tryptase (4%).

Cultured skin-derived mast cells were examined 6 days after addition of fresh medium (Table II). For these primary cultures of nontransformed mast cells, the total tryptase level measured in the medium (5.2 ± 1.3 μg/10^6 cell equivalents) was lower than that retained by the cells (23 ± 3 μg/10^6 cells). Levels of tryptase associated with these skin-derived mast cells were substantially higher than for both leukemic cell types, being more comparable with freshly isolated skin-derived mast cells (35 μg/10^6 cells) (24). Like the leukemic cells, only a small portion of spontaneously secreted tryptase was mature (6%). In contrast, nearly all of the tryptase retained by the skin-derived mast cells was mature (94%).

Identification of mature and precursor forms of tryptase

To obtain more precise molecular data as to the types of tryptase either retained or secreted by HMC-1 and Mac-6 cells, tryptase from these sources was purified by sequential immunoaffinity and heparin-agarose chromatography and subjected to N-terminal amino acid sequencing. Whereas medium-derived tryptase eluted from heparin-agarose near 0.2 M NaCl, cell lysate-derived tryptase eluted at ~0.8 M NaCl, suggesting different physicochemical forms of this protein. Thus, cell lysate-derived tryptase elutes from heparin at an ionic strength comparable with that of enzymatically active, human lung-derived tryptase, whereas tryptase in the medium elutes at a salt concentration comparable with those of precursor forms of the protein (26).

After purification, both media and cell lysate tryptase preparations produced a single diffuse Coomassie blue-stained band after SDS-PAGE (data not shown). The protein in each of these bands was subjected to up to 12 cycles of N-terminal amino acid sequencing. Fig. 3 summarizes the results. The single sequence obtained for HMC-1 medium tryptase was consistent with β-pro-tryptase, indicating this was the predominant form that was spontaneously released. In contrast, the two N-terminal sequences for tryptase in the Mac-6 culture medium indicated that α-pro-tryptase was the predominant form that was spontaneously secreted and that partial digestion of the propeptide had occurred, perhaps by an N-terminal peptidase. In contrast, tryptase in the cell lysates of both cell types yielded the mature sequence, consistent with the ELISA and chromatography data indicating that mature forms of tryptase predominate inside the cells.

Tryptase genes and their expression in HMC-1 and Mac-6 cells

Previous reports indicated that HMC-1 cells express predominantly βI-tryptase mRNA (19, 20) and Mac-6 cells express predominantly α-tryptase mRNA (21). In the current study, PCR and RT-PCR were used to examine genomic DNA and mRNA for α-tryptase and β-tryptase. Primers specific for α-tryptase and β-tryptase were designed. As shown in Fig. 4, amplification by PCR with α-tryptase-specific primers yielded a 278-bp product with an α-tryptase cDNA template but no product with a β-tryptase cDNA template (*lanes 6* and *7,* respectively). Likewise, β-tryptase-specific primers produced a 278-bp product with a β-tryptase cDNA template and no product with an α-tryptase cDNA template (*lanes 11* and *10,* respectively). Whereas genomic DNA from both HMC-1 and Mac-6 cells yielded a 386-bp product reflecting a β-tryptase gene (*lanes 3* and *4,* respectively), only Mac-6 genomic DNA yielded an α-tryptase product (*lane 2*), indicating HMC-1 cells were deficient for the α-tryptase gene (*lane 1*). RT-PCR using α-tryptase primers for PCR

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Table I. *Retained and secreted mature and total tryptase levels in HMC-1 and Mac-6 cells*[a]

Mast Cell Source	Medium Tryptase: Mature (ng/10^6 cell equivalents)	Medium Tryptase: Total (ng/10^6 cell equivalents)	Medium Tryptase: % Mature	Cell Lysate Tryptase: Mature (ng/10^6 cells)	Cell Lysate Tryptase: Total (ng/10^6 cells)	Cell Lysate Tryptase: % Mature
HMC-1	4.7 ± 0.6	172 ± 27	3	15 ± 9	36 ± 5	42
Mac-6	1.3 ± 0.3	37 ± 3	4	0.3 ± 0.2	1.3 ± 0.3	23

[a] Cells and media were collected 3 days after a medium change for HMC-1 cells and Mac-6 cells, respectively. Mean ± SD concentration values, normalized to 10^6 cells, are shown. Percent mature values were calculated by multiplying the ratio of mature to total tryptase values by 100.

Table II. *Retained and secreted mature and total tryptase levels in skin-derived mast cells*[a]

Mast Cell Source	Medium Tryptase: Mature (μg/10^6 cell equivalents)	Medium Tryptase: Total (μg/10^6 cells equivalents)	Medium Tryptase: % Mature	Cell Lysate Tryptase: Mature (μg/10^6 cells)	Cell Lysate Tryptase: Total (ng/10^6 cells)	Cell Lysate Tryptase: % Mature
Skin	0.3 ± 0.1	5.2 ± 1.3	6	21 ± 3	23 ± 3	94

[a] Cells and media were collected 6 days after a medium change for skin-derived mast cells. Mean ± SD concentration values, normalized to 10^6 cells, are shown. Percent mature values were calculated by multiplying the ratio of mature to total tryptase values by 100.

showed that α-tryptase mRNA was present in Mac-6 cells (*lane 9*), but not in HMC-1 cells (*lane 8*). In contrast, RT-PCR using β-specific primers for PCR showed that β-tryptase mRNA was present in both HMC-1 cells (*lane 12*) and Mac-6 cells (*lane 13*). However, there appeared to be far less β-tryptase product from Mac-6 cells than from HMC-1 cells, suggesting a corresponding paucity of β-tryptase mRNA in Mac-6 cells.

Plasma tryptase levels in subjects with and without the gene for α-tryptase

Samples of genomic DNA from the peripheral blood leukocytes of 109 healthy subjects were assessed for the presence or absence of the α-tryptase gene by PCR. Tryptase gene haplotypes may bear either one α and one β gene, or, alternatively, two β genes; with our method, a sample typing positive for the α-tryptase gene may represent either a heterozygote ($\alpha\beta$:$\beta\beta$) or a homozygote ($\alpha\beta$:$\alpha\beta$). Representative PCR results are shown for nine of these subjects in Fig. 5*A,* where the results reflect the presence of an α-tryptase gene in six of the nine samples. Overall, 28 of the 109 specimens (26%) lacked the α-tryptase gene. All 109 DNA samples contained β-tryptase gene(s) (data not shown). Total tryptase levels also were measured in corresponding samples of plasma, as illustrated in Fig. 5B. The median (25th percentile, 75th percentile) plasma tryptase value among α-tryptase$^+$ subjects, 5.2 (4.1, 7.5) ng/ml was slightly but not significantly ($p = 0.11$, Mann-Whitney rank sum test) higher than that of α-tryptase$^-$ subjects, 4.7 (3.4, 6.3) ng/ml. The range also was higher among α-tryptase$^+$ (1.1–19 ng/ml) than α-tryptase$^-$ (1.8–8.4 ng/ml) subjects. Total tryptase levels in male subjects (4.7 (3.5, 6.8) ng/ml) were slightly lower than in female subjects (5.6 (4.3, 7.5) ng/ml) ($p = 0.02$). Regression analyses using log-transformed tryptase values and correcting for the sex difference confirmed no significant effect of the α-tryptase gene on total tryptase levels. Thus, the median total plasma tryptase level of healthy subjects lacking the α-tryptase gene was not significantly different from the median for those having at least one α-tryptase gene.

Cell Lysate

HMC-1 **Ile^{+1}-Val-Gly-Gly-Gln-Glu ~ α/β-tryptase**

MAC-6 **Ile^{+1}-Val-Gly-Gly-Gln-Glu ~ α/β-tryptase**

Culture Media

HMC-1 **Ala^{-12}-Pro-Ala-Pro-<u>Gly</u>-Gln-Ala-Leu-Gln-<u>Arg^{-3}</u>-<u>Val</u>-Gly ~ β-protryptase**

MAC-6 **Ala^{-10}-Pro-<u>Val</u>-Gln-Ala-Leu-Gln-<u>Gln^{-3}</u>-<u>Ala</u>-Gly ~ α-protryptase**

<u>Val^{-8}</u>-Gln-Ala-Leu-Gln-<u>Gln^{-3}</u>-<u>Ala</u>-Gly ~ α-protryptase

FIGURE 3. N-Terminal amino acid sequence of retained and spontaneously secreted tryptase from HMC-1 and Mac-6 cells. In each case, the first N-terminal 12-aa positions were assigned. α-Tryptase and β-tryptase can be distinguished from one another by the propeptide amino acids at positions −8, −3, and −2, namely, Val, Gln, and Ala for α-tryptase and Gly, Arg, and Val for β-tryptase. Mature tryptase could not be subclassified by the obtainable sequence.

Discussion

A major finding of the current study is that precursors of both α-tryptase and β-tryptase are spontaneously released, while fully processed β-tryptase is retained by the cells, principally in secretory granules. This finding applies to both tryptase-expressing leukemic cell lines (HMC-1 and Mac-6) and primary cultures of skin-derived mast cells. The consensus 12-aa α-protryptase was dominant in tryptase purified from the medium of Mac-6 cultures, whereas the β-protryptase sequence was dominant in tryptase purified from the culture medium of HMC-1 cells. This is the first time the β-protryptase molecule has been directly observed in a mast cell system and supports this consensus pro sequence as being the actual pro sequence. In contrast to medium-derived tryptase, cell lysate-derived tryptase exhibited a sequence consistent with the mature portion of either β-tryptase or α-tryptase. Because prior work showed that recombinant human β-protryptase could be autoprocessed next to R^{-3} to β-pro'tryptase, and then to β-tryptase by dipeptidyl peptidase I, whereas α-protryptase did not undergo autoprocessing (5), we presume that most if not all of the mature tryptase detected in the HMC-1 cells, Mac-6, cells and skin-derived mast cells is mature β-tryptase. This is certainly the case in HMC-1 cells, which lack the α-tryptase gene. However, for Mac-6 cells, which express small amounts of β-tryptase mRNA along with larger amounts of α-tryptase mRNA, the gene of origin for the mature tryptase has not been directly determined. In the case of skin- and lung-derived mast cells, it is likely that most, if not all, of the mature tryptase is β-tryptase. As reported previously (7–9, 34) and confirmed in the current study, mature α-tryptase, like mature β-tryptase, forms a tetramer, but has little, if any, enzymatic activity. Because tryptase purified from skin- and lung-derived mast cells has a specific enzymatic activity nearly identical with that of mature recombinant β-tryptase, little if any mature α-tryptase is likely present.

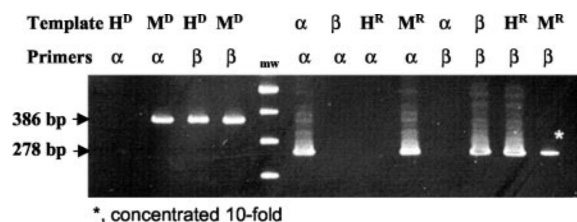

FIGURE 4. Analysis of α-tryptase and β-tryptase in genomic DNA and mRNA of HMC-1 and Mac-6 cells. Samples of genomic DNA from HMC-1 (H^D) and Mac-6 (M^D) cells were amplified by PCR with α-tryptase-specific and β-tryptase-specific primers. Samples of RNA (H^R and M^R) were subjected to RT-PCR using the same primer pairs. The size marker lane is noted by mw. The β-tryptase RT-PCR product (50 μl) from Mac-6 cells was purified and concentrated before loading, whereas 5 μl of each of the other reaction mixtures were applied to the gel. DNA was visualized with SYBR Green I.

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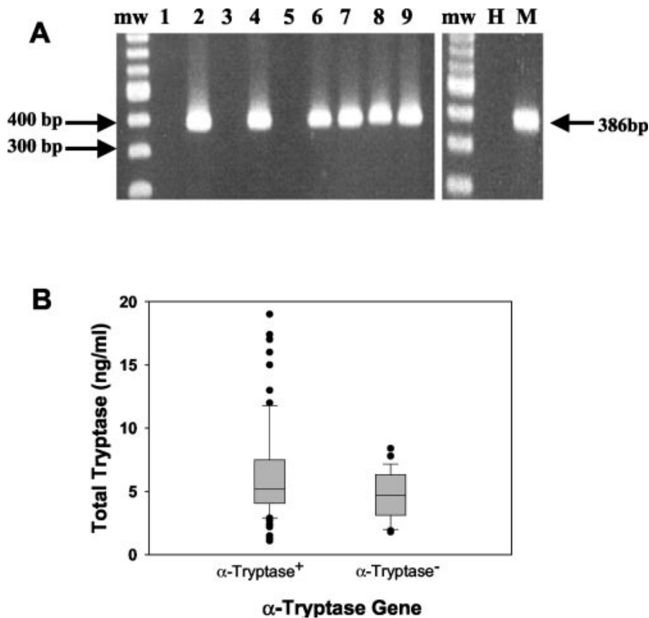

FIGURE 5. Plasma tryptase levels in subjects with and without the gene for α-tryptase. *A,* Analysis of genomic DNA for α-tryptase. Genomic DNA was extracted from peripheral blood leukocytes and amplified by PCR using α-tryptase-specific primers as described. *Lanes 1–9,* Representative PCR results from 9 of the 109 subjects in the study group; H, HMC-1 cells; M, Mac-6 cells. A 100-bp DNA ladder was used to estimate molecular mass (mw). Arrows point to the 300- and 400-bp markers, and to the 386-bp PCR product. *B,* Plasma total tryptase levels. Box and whisker plots show median (horizontal bar within gray boxes), 25th and 75th percentiles (box ends), 10th and 90th percentiles (whiskers), and outliers (●).

Whether either of the immunoassays used in the current study detects δ-tryptase was not directly addressed. However, like α-protryptase, δ-protryptase has a Q^{-3}, possibly making autoprocessing inefficient. Further, in Western blots with extracts of various types of mast cells, a low mw band reflecting the 40 amino acid deficit of δ-tryptase was not apparent (our unpublished results). Thus, δ-tryptase may be present in relatively low amounts compared with α- and β- tryptases or may not be recognized by the mAb(s) used for Western blotting. Nevertheless, δ-tryptase remains to be precisely quantified.

Why protryptase molecules are spontaneously secreted by mast cells is unclear. Failure to adopt a particular conformation needed for targeting the protein to secretory granules is one possibility. A region within the pro portion that actively targets the protein to a constitutive secretory pathway is another possibility. A third consideration is that the protein is targeted to the secretory granules through its high affinity interaction with heparin proteoglycan, which for β-tryptase occurs after the protein has been processed to the mature, tetrameric enzyme. β-Protryptase binds to heparin with lower affinity and is optimally processed to the mature tetramer when bound to heparin. If production of β-protryptase in excess of cofactor heparin occurred, it would most likely lead to inefficient processing of the excess portion of the enzyme, and spontaneous secretion of the unprocessed protein. Whether Mac-6 cells can express small amounts of heparin proteoglycan or use distinct processing enzymes and stabilizing cofactors must be considered.

Although α-tryptase and β-tryptase mRNA are found together in preparations of lung-derived and skin-derived mast cells (20) and increase in parallel during the development of mast cells from fetal liver progenitors in vitro (22), the current study supports previous observations in Mac-6 cells that indicate that differential expression of these genes is possible (19, 21). Mac-6 cells express predominantly α-tryptase and can now be said to also contain small amounts of β-tryptase mRNA. Other cell lines, U-937 monocytic leukemia cells and the KU812 basophil leukemia cells, express predominantly β-tryptase mRNA, whereas normal basophils from peripheral blood of two individuals expressed predominantly α-tryptase mRNA (20, 21, 35). Normal peripheral blood monocyte preparations of 85% purity that were T cell depleted reportedly contain trace amounts of tryptase mRNA (21), but basophils possibly present in this preparation should be considered as a potential source of the tryptase mRNA detected. Factors regulating the differential expression of tryptase genes remain to be determined.

A finding of practical importance to clinicians is that the ELISA using the G5 mAb for detection preferentially detects mature tryptase, regardless as to whether the product originates from the α-tryptase or β-tryptase genes. This is likely even though all subtypes of these gene products were not examined. For example, the primary sequence of βIII-tryptase is entirely represented in α- and the other β-tryptases. Thus, the total and mature tryptase assays should recognize the precursor and mature forms of this tryptase variant. Because mature tryptase is stored in secretory granules and released during degranulation, measurement of this form of tryptase in biological fluids should reflect the magnitude of mast cell activation, as has been postulated previously (10, 11, 36). In contrast, the ELISA using the G4 mAb for detection detects both pro and mature forms of α-tryptase and β-tryptase, i.e., the total tryptase level. Because the tryptase in normal blood is measurable by the total but not mature tryptase assay, it is likely that protryptase(s) is the major form in normal blood. Although it was previously postulated that α-protryptase accounted for the protryptase in normal blood (10), this cannot be the case in subjects who lack a gene for α-tryptase. The current study shows that both α-protryptase and β-protryptase are spontaneously secreted by tryptase-producing cells in culture. The total tryptase level, in a subject without symptoms or signs of anaphylaxis, is likely to reflect mast cell number. Indeed, the total tryptase level is elevated ($>$20 ng/ml) in people with systemic mastocytosis during nonacute periods (10, 37, 38). However, total tryptase levels also rise during systemic anaphylaxis (14). The current study shows the difference in median total tryptase levels in the plasma of subjects with (5.2 ng/ml) and without (4.7 ng/ml) the α-tryptase gene is small and is not statistically significant. If each haploid genome contains either one α-tryptase and one β-tryptase gene, or two β-tryptase genes, as postulated (16), then subjects with four β-tryptase genes in their diploid genomes produce almost the same levels of total plasma tryptase as do the combined groups with either two β-tryptase and two α-tryptase, or three β-tryptase and one α-tryptase genes. However, whether a difference might have been appreciated if the latter two groups had been identified and separately analyzed was not directly assessed by the current study. Also, the subtypes of α- and β-tryptases were not assessed, leaving open the possibility of variations in tryptase levels that correspond to specific subtypes.

Subjects with systemic mastocytosis have elevated levels of total tryptase ($>$20 ng/ml) and little if any mature tryptase in their plasma or serum during nonacute periods, presumably reflecting the increased burden of mast cells in tissues such as liver, spleen, and bone marrow. Some subjects with acute myelocytic leukemia and myelodysplastic syndromes also have markedly elevated levels of total tryptase (39, 40). Based on the immunoassay characteristics of the current study, most of the plasma tryptase detected in such patients is likely to be precursor forms of α- and β-tryptases; however, direct N-terminal sequencing of tryptase purified

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from such sources would provide the best evidence for this inference. Unfortunately, plasma tryptase purified from two mastocytosis subjects had blocked N termini (our unpublished data). Whether the absence or presence of α-tryptase in the genomes of individuals with these disorders affects total tryptase levels to a greater extent than for healthy subjects remains to be studied. Also, it is possible that the coordinated expression of β-protryptase and heparin proteoglycan in mastocytosis mast cells is awry and leads to the spontaneous secretion of a higher portion of the tryptase produced by such cells. The HMC-1 cells used in the current study secrete a far greater portion of their β-tryptase compared with cultured skin-derived mast cells, even though the latter cells produce substantially more tryptase.

In conclusion, the current study supports the previous hypothesis that levels of mature tryptase in biological fluids serve as an indicator for mast cell activation, because this form is selectively stored in mast cell secretory granules. In contrast, levels of the precursors of mature tryptase reflect mast cell numbers, because they are spontaneously secreted by mast cells. Further, whether or not the four putative tryptase genes in the normal diploid chromosome lack α-tryptase does not influence the circulating level of tryptase precursors in healthy subjects.

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