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δ Tryptase Is Expressed in Multiple Human Tissues, and a Recombinant Form Has Proteolytic Activity[1]

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Tryptases are neutral serine proteases selectively expressed in mast cells and have been implicated in the development of a number of inflammatory diseases including asthma. It has recently been established that the number of genes encoding human mast cell tryptases is much larger than originally believed, but it is not clear how many of these genes are expressed. A recent report suggested that the transcript for at least one of these genes, originally named mMCP-7-like tryptase, is not expressed. To further address this question, we screened tissue-specific RNA samples by RT-PCR, using primers designed to match the putative exonic sequence of this gene. We successfully generated and cloned the correctly sized RT-PCR product from mRNA isolated from the human mast cell-I cell line. Two distinct clones were identified whose nucleotide sequence matched the published sequence of the mMCP-7-like I and mMCP-7-like II genes. Transcripts were detected in a wide variety of human tissues including lung, heart, stomach, spleen, skin, and colon. A polyclonal antipeptide Ab that specifically recognizes the translated product of this transcript was used to demonstrate its expression in mast cells that reside in the colon, lung, and inflamed synovium. A recombinant form of this protein expressed in bacterial cells was able to cleave a synthetic trypsin-sensitive substrate, D-Ile-Phe-Lys pNA. These results suggest that the range of functional tryptases is larger than previously recognized. For simplicity, we suggest that the gene, transcripts, and corresponding protein product be named δ tryptase. ***The Journal of Immunology*****, 2002, 169: 5145–5152.**

Mast cells are highly granulated, tissue-resident, effector cells of the immune system. They may be activated via their high-affinity IgE receptors or by a number of alternate mechanisms (reviewed in Ref. 1). Following activation, they secrete a variety of preformed mediators including histamine, proteoglycans, and a range of serine proteases that are active at neutral pH (2, 3). Two major families of these proteases have been identified: chymases and tryptases, and they represent the major protein component of the mast cell.

A role for mast cell tryptases has been proposed in the development of a number of inflammatory diseases, including rheumatoid arthritis and asthma. Despite the underlying causes of these diseases not being fully understood, it is known that the number of mast cells is increased in the airways of patients with asthma (4) and in the synovial tissue of patients with rheumatoid arthritis (5–7). Although many of the products expressed by mast cells are capable of contributing to an inflammatory phenotype (4), it is becoming increasingly apparent that especially in asthma, tryptase is one of the most important (8–10).

A major impediment to determining the role of tryptases has been the confusion over how many different functional human tryptases are expressed. In 1989, Miller and coworkers (11) cloned the first cDNA that encoded a human mast cell tryptase. In 1990, the same group cloned a second cDNA (12), naming it β tryptase, and retrospectively naming the original cDNA α tryptase. In the same year, Vanderslice and colleagues (13) cloned three distinct cDNAs from a skin-derived library, skin tryptases I, II, and III. Skin tryptase II cDNA was recognized to be identical with β tryptase, and all skin tryptase cDNAs appeared to be more closely related to β tryptase than to α tryptase. Therefore, these are now referred to as βI tryptase, βII tryptase, and βIII tryptase (14, 15).

It was not known how many of these cDNAs were the product of distinct gene loci and how many were allelic variants. Recent progress in the sequencing of the human genome has shed light on this question. In 1990, Vanderslice (13) reported the cloning of the first human tryptase gene, which encoded skin tryptase I (βI tryptase); and in 1999 and 2000, researchers from the same group cloned a number of tryptase genes that resided on two overlapping bacterial artificial chromosome clones that mapped to 16p13.3 (14, 15). These included the gene encoding βI tryptase and its allelic partner αII tryptase (itself presumably an allelic variant of the original α tryptase), the allelic genes encoding βII and βIII tryptase, two allelic variants of a transmembrane tryptase called γ tryptase, and two allelic variants of another tryptase originally named "mouse mast cell protease (mMCP)[3]-7-like" (14, 15). The mMCP-7-like tryptase was so named due to homology between its fifth exon and the murine tryptase mMCP-7 (14, 16). Of these, cDNAs have been cloned for all loci except for that encoding mMCP-7-like tryptase. Wong et al. (17) have recently described the cloning of a more distantly related member, ε tryptase, which is ~40% similar to the αβ tryptases.

Recently, it has been reported that the mMCP-7-like gene is not transcribed and is thus likely to be a pseudogene (18). In this study, we describe the cloning of the cDNA for this gene, the expression of its mRNA and protein in multiple tissues, and report that a

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[3] Abbreviations used in this paper: mMCP, mouse mast cell protease; HMC-1, human mast cell-I cell line; RTQ, real-time quantitative; EK, enterokinase; *p*NA, *p*-nitroanilide.

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recombinant form expressed in bacterial cells is proteolytically active. To simplify the nomenclature of human mast cell tryptases, we suggest that this gene and its protein product be named δ tryptase.

Materials and Methods

Sources of RNA

Human mast cell-I cell line (HMC-1) cells (5×10^6, a kind gift from Dr. J. H. Butterfield, Mayo Clinic, Rochester, MN) were lysed in 1 ml of TRI REAGENT (Sigma-Aldrich, Sydney, Australia), and 0.2 ml of chloroform added. Following centrifugation, the aqueous phase was transferred to a fresh tube and 0.5 ml of isopropanol was added. The RNA pellet was collected by centrifugation, washed with 75% ethanol, dissolved in dH_2O, and stored at −80°C until required.

Total RNA from adult lung, heart, stomach, spleen, skin, colon, fetal heart and fetal lung, and poly(A^+) RNA isolated from human lung were obtained from commercial sources (Invitrogen, Carlsbad, CA).

Preparation of cDNA

First strand cDNAs were generated using the cDNA Cycle kit (Invitrogen) from total RNA isolated from the HMC-1 cell line and from poly(A^+) RNA isolated from human lung. A total of 1.5 μg of HMC-1 total RNA (or 300 ng of lung poly(A^+) mRNA) and 1 μl of oligo(dT) primer were heated at 65°C for 10 min to remove secondary structure. Reverse transcription was performed for 1 h at 42°C in a solution containing 1 μl of RNase inhibitor, 4 μl of 5× reverse transcriptase buffer, 1 μl of 100 mM dNTPs, 1 μl of 80 mM sodium pyrophosphate, and 0.5 μl avian myeloblastosis virus reverse transcriptase. The reaction was terminated by incubating the mixture at 95°C for 2 min, and was placed on ice immediately.

PCR amplification and cloning of cDNAs

PCR amplification of first strand cDNA was performed within 2 h of the reverse transcription reaction. Initially a nested PCR approach was used to amplify cDNAs, using primers designed according to the sequence of a gene that we isolated independently and named δ tryptase (data not shown), and according to the published sequence of the mMCP-7-like genes (GenBank accession nos. AF099147 and AF098327; Ref. 14). Two sets of primers (F1 = 5′-CCC GTC CTG GCG AGC CCG-3′/R1 = 5′-CAG TGA CCC AGG TGG ACA C-3′ and F2 = 5′-AGT GGC CAG GAT GCT GAG C-3′/R2 = 5′-TTT GGA CAG GAG GGG CTG GCT-3′) were used to amplify the initial product, and a single nested primer pair (NF1 = 5′-GAG CAA GTG GCC CTG GCA-3′/NR1 = 5′-GGA CAT AGT GGT GGA TCC AG-3′, see Fig. 2) was used on the resulting template. In later experiments, a single primer pair (F3 = 5′-TGC AGC AAA CGG GCA TTG TTG-3′ and R3 = 5′-AAA GCT GTG GCC CGT ATG GAG-3′) was used to amplify δ tryptase cDNAs.

The PCR were conducted with 2.5 U of AmpliTaq Gold (PerkinElmer, Branchburg, NJ) and 2 μl of the reverse transcriptase reaction mixture. The total reaction volume was 50 μl with a final concentration of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 0.1 μM of the appropriate 5′ and 3′ primers. After an initial incubation for 5 min at 94°C, samples were subjected to 35 cycles of PCR (45 s at 95°C, 60 s at 55°C, and 60 s at 72°C). This was followed by a final extension step of 72°C for 10 min. PCR products (10 μl) were visualized on a 1% agarose gel. Appropriately sized products were excised from the gel, purified with QIAquick gel extraction kit (Qiagen, Chatsworth, CA) and ligated into the plasmid vector pCR 2.1-TOPO (Invitrogen). The ligation mixture was then used to transform TOP10 cells (Invitrogen). The transformation mixture was plated onto LB/agar plates containing ampicillin (50 μg/ml), and coated with X-gal to enable blue/white color selection. Plasmids containing the appropriate sized inserts were screened for the presence of tryptase cDNAs by PCR using the primer set NF1/NR1. Plasmid DNA was then purified from clones identified in this manner using a commercial kit (Qiagen). Nucleotide sequencing was performed either in-house using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI377 PRISM DNA Sequencer (PE Applied Biosystems, Foster City, CA), or at a core facility (SUPAMAC, Sydney, Australia).

In vivo expression of δ tryptase transcripts

Initially semiquantitative RT-PCR (with primers F3/R3) was performed to screen a broad range of total RNA samples isolated from lung, heart, stomach, spleen, skin, and colon. The expression of δ tryptase mRNA was then quantified using a real-time quantitative (RTQ) RT-PCR approach performed on a AB7700 Sequence Detection system (PE Applied Biosystems). Reverse transcription was performed using a commercial kit (PerkinElmer). Briefly, 1 μg of total RNA purified from human lung, heart, spleen, stomach, colon, and the HMC-1 cell line were reverse transcribed according to the manufacturer's instructions. In control experiments, reverse transcriptase was omitted from the reaction mixture to control for possible contamination of the sample with genomic template DNA. Total reaction volume was 50 μl.

Oligonucleotide primers (forward primer DF1 = GGC CAC AGC TTT CAA ATC GT, reverse primer DR1 = GCA GTT AGG TGC CAT TCA CCT T) and a TaqMan probe DTP1 (6 FAM-CCT GCC AGG GTG ACT CCG GAG GG) were designed using the PrimerExpress software (PE Applied Biosystems) to specifically detect reverse transcribed δ tryptase mRNA, and not the mRNA of other tryptases (see Fig. 2). Coamplification of genomic DNA was avoided by locating the forward and reverse primers in separate exons and designing the probe so that it straddled the exon 5/exon 6 boundary.

Optimal concentrations and conditions for amplification were determined using the plasmid containing the δ tryptase cDNA as template. The specificity for δ tryptase was determined by comparing PCR amplification of δ tryptase, αII tryptase, or βI tryptase cDNA templates. Cycling conditions were 50°C for 2 min, 95°C for 10 min, then 40–45 cycles of 95°C for 15 s, 60°C for 60 s.

For determination of mRNA levels, 6 μl of the appropriate reverse transcriptase reaction mixture was added to 12.5 μl of PCR master mix, 2.5 μl of TaqMan probe (2.5 μM), 1 μl each of the forward and reverse primers (18 μM each), and 2 μl of dH_20 to give a total reaction mixture of 25 μl.

Relative quantitation of δ tryptase mRNA expression in various tissues was determined by comparing the sample threshold cycle number to a standard curve constructed with serial log dilutions (10^{-1} –10^{-8} ng) of a plasmid containing the δ tryptase cDNA. Relative copy number was determined using an algorithm (1 ng of plasmid = 2.01342×10^8 copies) and then expressed per microgram of total RNA.

For each RNA sample, δ tryptase expression was then normalized for β-actin expression. The relative quantity of δ tryptase mRNA was determined using commercially available TaqMan probe and primers (PE Applied Biosystems), and a standard curve constructed by serial dilution of a plasmid containing the β-actin cDNA. The SD for the resulting δ tryptase: β-actin ratio was determined using the equation: coefficient of variation $(CV)_{ratio} = \sqrt{(CV_{\delta\ tryptase}{}^2 + CV_{\beta\text{-}actin}{}^2)}$ where CV = SD/X (SD/mean). Standards were tested in duplicate and samples in triplicate.

Generation of polyclonal anti-peptide Abs to δ tryptase

New Zealand white rabbits (Institute of Medical and Veterinary Science, Gilles Plains, Australia) were immunized with a δ tryptase-specific peptide that possessed an amino terminal cysteine and the δ tryptase residues Y^{162}HTGLHTGHSFQIVRDD^{178} conjugated to diphtheria toxin (Mimotopes, Melbourne, Australia). The peptide sequence, located in the region translated from exon 5, has only ~50% identity to the αβ tryptases (see Fig. 3). A search of protein databases detected no other protein that shared this epitope. Anti-δ tryptase Abs were affinity purified from antisera using the peptide Y162-D178 conjugated to thiopropyl Sepharose. The specificity of the δ tryptase Ab was confirmed by Western blot. Purified recombinant δ tryptase (~0.5 μg) and rβII tryptase (~1 μg; Promega, Madison, WI) were separated on a 10% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After blocking for 2 h at room temperature with 5% skim milk powder/TBS/0.1% Tween 20, membranes were incubated with affinity purified δ tryptase anti-peptide Ab (1 μg/ml in TBS/0.1% Tween 20). Bound primary Ab was detected using a goat anti-rabbit HRP-conjugated second Ab (Bio-Rad, Hercules, CA) diluted 1/10,000 (2 h at room temperature), followed by exposure to an HRP-chemiluminescence substrate for 5 min (Renaissance Enhanced Luminol Reagent; NEN, Boston, MA). The resulting bands were visualized by exposure to Biomax ML photographic film (Kodak, Rochester, NY). Replicate blots were probed as described above with normal rabbit IgG (1 μg/ml), and with the mouse monoclonal anti-tryptase Ab AA1 diluted 1/200 (DAKO, Glostrup, Denmark) followed by goat anti-mouse HRP-conjugate.

Immunohistochemistry

Immunohistochemistry was performed on 4-μm serial sections cut from formalin-fixed and paraffin-embedded samples of human lung, colon, stomach, heart, spleen, and rheumatoid synovium. Sections were deparaffinized, dehydrated, and rinsed in tap water. Ag retrieval was performed by incubating the sections with proteinase K (25 μg/ml in 0.1 M Tris (pH 7), 50 mM EDTA) at 37°C for 30 min. Sections were then rinsed with TBS and blocked with 20% normal goat serum/TBS at room temperature for 20 min. Sections were incubated with primary Ab diluted in TBS/2% BSA (δ

tryptase = 4 μg/ml overnight at 4°C, normal rabbit IgG = 4 μg/ml overnight at 4°C, AA1 anti-tryptase Ab = 1/50 dilution for 1 h at room temperature). Sections were then washed four times for 5 min in TBS, and then incubated at room temperature for 30 min with the appropriate biotinylated secondary Ab diluted 1/200 in TBS/2% BSA:goat anti-rabbit for δ tryptase and normal rabbit IgG, and goat anti-mouse for AA1 tryptase Ab. Sections were washed four times for 5 min in TBS, incubated with avidin-conjugated alkaline phosphatase (Vector Laboratories, Burlingame, CA) for 30 min at room temperature, and then washed four times for 5 min in TBS. The sections were incubated in the dark for ~15 min with alkaline phosphatase substrate (Vector Red; Vector Laboratories), which gives a red reaction product in the presence of alkaline phosphatase. All incubations were performed in a humidified chamber. Sections were rinsed in tap water, counterstained with hematoxylin for 30 s, rinsed in tap water, and coverslipped with CrystalMount (Biomeda, Foster City, CA). Stained sections were examined using an Olympus BX-60 microscope (Olympus, Melville, NY) and images captured using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Generation of recombinant δ tryptase

When compared with the αβ human tryptases, mature δ tryptase has a 40-aa C-terminal truncation. To determine whether it is a functional protease, recombinant δ tryptase was expressed in bacterial cells and tested for the ability to cleave a panel of trypsin-sensitive substrates.

The recombinant fusion protein included an N-terminal His-patch thioredoxin region (to increase translation efficiency and solubility), an enterokinase (EK) recognition site (to allow activation of the proenzyme), the mature δ tryptase sequence, and C-terminal V5 and 6× His tags (to aid detection and purification). As the δ tryptase cDNA (GenBank accession no. AF206664) used to generate the expression construct did not include the sequence coding for the beginning of the mature tryptase, the forward primer (5′-CAC CAT GAT TGT TGG GGG GCA GGA GGC CCC CAG GAG CAA GTG GCC CTG G-3′) was designed to include this region. A reverse primer (5′-GGT GCC ATT CAC CTT GCA-3′) was designed immediately 5′ of the stop codon. The resulting PCR fragment was directionally cloned into the pET102D-TOPO vector (Invitrogen), sequenced in both directions, and the construct used to transform BL21 DE3 cells. Following the addition of isopropyl-β-thiogalactopyranoside (0.5 mM final concentration), the bacterial cells were incubated for 6 h at 37°C while being agitated vigorously. The cells were pelleted by centrifugation and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole (pH 8.0)). The lysate was centrifuged to remove cellular debris, and the His-tagged recombinant protein purified from the supernatant using a Ni-NTA column.

To enable refolding of the protein, it was first denatured in 6 M guanidine hydrochloride buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 5 mM DTT, 6 M GuHCl (pH 8.0)) and introduced slowly (3 ml/h) into refolding buffer (50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 10 mM CHAPS, 2 mM DTT) while stirring gently. The refolded protein was repurified using a Ni-NTA column and dialysed with 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 2 mM $CaCl_2$ overnight.

Using the same approach, a recombinant form of αII tryptase was generated based on the cDNA we have cloned (GenBank accession number AF 206665), and that matched the predicted exonic sequence of the αII gene reported by Pallaoro et al. (14).

The recombinant enzymes were activated proteolytically by incubating the refolded purified protein with recombinant EK (Novagen, Madison, WI) for 16 h at 20°C in a buffer containing 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 2 mM $CaCl_2$. Following activation, EK was removed from the reaction mixture using an EK cleavage capture kit (Novagen).

The enzymatic activity of recombinant δ tryptase was evaluated by testing its ability to cleave a panel of three trypsin-susceptible *p*-nitroanilide (*p*NA) chromogenic substrates: *N*-benzoyl-Pro-Phe-Arg-*p*NA, D-Ile-Phe-Lys *p*NA, and *N*-p-Tosyl-Gly-Pro-Lys 4-*p*NA (Sigma-Adrich), and was compared with that of rαII tryptase and commercially available native human lung tryptase (ICN, Costa Mesa, CA) and recombinant human lung βII tryptase (Promega). Pro-δ tryptase (i.e., before removal of the EK-susceptible activation peptide), recombinant EK alone, and buffer alone acted as negative controls. Approximately equal amounts (~2 μg) of the enzymes were incubated with each substrate (10 μg) at 37°C for 2 h in 100 mM HEPES (pH 7.5) 10% glycerol (total reaction volume = 50 μl), and then analyzed by HPLC using a reverse-phase column (4.6 × 50 mm RP18 Xterra; Waters, Bedford, MA). In this initial investigation, no exogenous heparin was added. Substrate cleavage was determined by the detection of new peaks representing the separated peptide and nitroanilide moieties. The amount of substrate cleaved was estimated by measuring the area under the HPLC peak that corresponded to the liberated peptide moiety using Delta T Scan software version 2.04 (Delta T Devices, Cambridge, U.K.). The retention time of the liberated nitroanilide was constant and was experimentally determined to be 2.67 min.

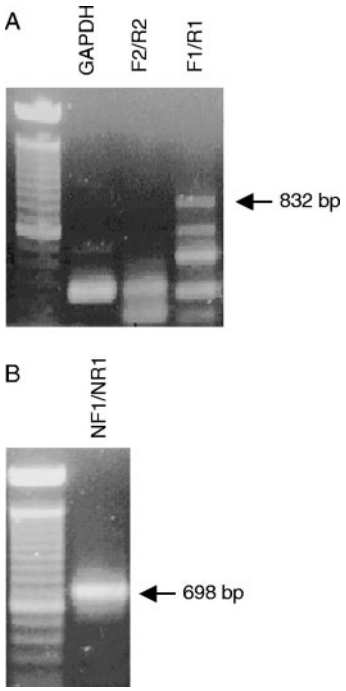

FIGURE 1. RT-PCR amplification of the transcript for δ tryptase. *A*, RT-PCR of total RNA isolated from the HMC-1 cell line using two primer pair combinations, F1/R1 and F2/R2. GAPDH was amplified as a control. The correct sized band (832 bp) for δ tryptase was only generated using the F1/R1 primer pair. *B*, PCR amplification using the purified 832-bp band from *A* as the DNA template, and the nested primer set NF1/NR1 to generate the expected 698-bp product.

Three-dimensional protein modeling

Three-dimensional structure of the novel tryptase was modeled from the reported 3.0 Å x-ray structure of human βII tryptase (Protein Data Bank identifier 1AOL; Ref. 19).

Results

Cloning of the δ tryptase cDNA

PCR amplification of the first-strand cDNA template from the HMC-1 cell line generated multiple bands, but only reactions using the F1/R1 primer pair resulted in amplification of the expected 832-bp product (Fig. 1*A*). The correctly sized band was excised and used as a PCR template with the nested primer pair NF1/NR2. The expected 698-bp PCR product was generated (Fig. 1*B*), excised, and cloned into the pCR2.1 vector. Sequencing of 10 clones revealed the presence of two distinct cDNAs that we have named δI tryptase and δII tryptase (GenBank accession nos. AY055427 and AF206664, respectively[4]). The δI tryptase cDNA sequence matched that predicted from the published partial sequence of the mMCP-7-like I gene, and the δII tryptase cDNA matched the published exonic sequence of the mMCP-7-like II gene (14). The cDNA and putative amino acid sequence of δII tryptase is shown in Fig. 2. The cDNA sequence of δI tryptase (sequence not shown) was identical with that of δII except for two nucleotide differences; G216 (δII cDNA) to A (δI cDNA) (nucleotide numbering starts from the translation initiation codon), and G226 (δII cDNA) to A

[4] The nucleotide sequences reported in this paper have been deposited in GenBank database with accession nos. AY055427, AF206664, and AF206665.

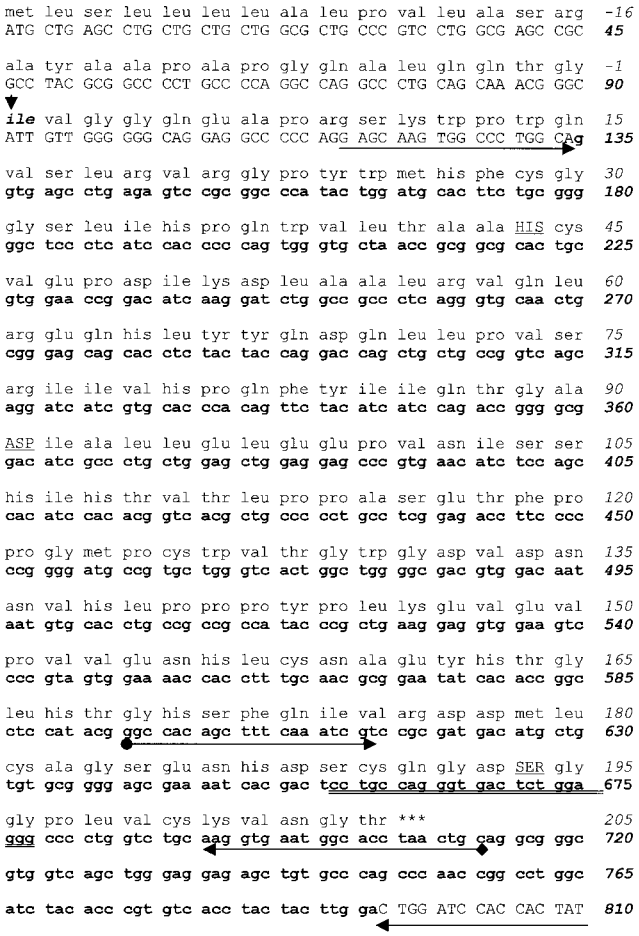

FIGURE 2. The cDNA and putative amino acid sequences of δII tryptase (GenBank accession no. AF206664). The δII cDNA sequence matched the putative exon sequence of the mMCP-7-like II gene. The δI cDNA (GenBank accession no. AY055427) is not shown but matched the exonic sequence of the partial mMCP-7-like I gene (14). Consistent with the published gene sequences, there were two nucleotide differences between the two cDNA sequences; G216 (δII cDNA) to A (δI cDNA) (nucleotide numbering starts from the translation initiation codon), and G226 (δII cDNA) to A (δI cDNA). Only the second of these results in an amino substitution (Val in δII to Met in δI). Actual nucleotide sequence of cloned RT-PCR product is shown in bold lowercase lettering. The location of the forward (NF1) and reverse (NR1) primers are indicated by arrows. The first amino acid of the mature enzyme is italicized and bold. The three members of the catalytic triad, His, Asp, and Ser, are in capitals and underlined. Nucleotide numbering begins from the translation initiation codon (Met). Amino acid numbering begins from the first residue of the mature enzyme (Ile). The position of the forward (right arrow with circle) and reverse (left arrow with diamond) primers, and the TaqMan probe (heavy double underlines) for RTQ RT-PCR are indicated.

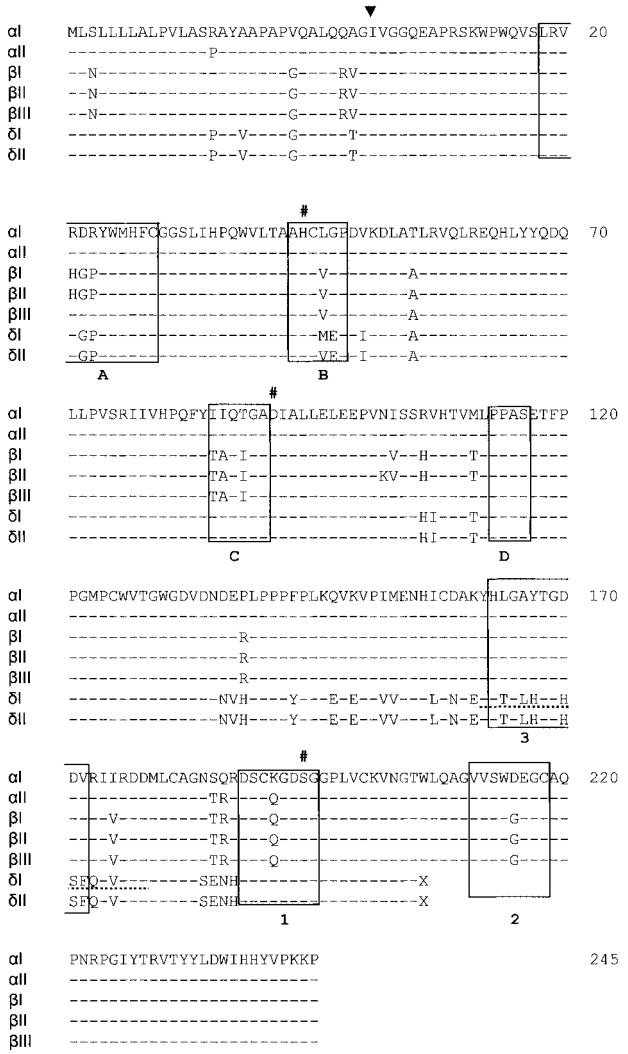

FIGURE 3. Amino acid sequences of δI tryptase and δII tryptase compared with that of tryptases αII, βI, βII, and βIII. A dash (—) indicates the presence of an identical amino acid. Numbering begins at the first residue of the mature enzyme, which is indicated by an arrow (▼). The seven loops comprising the substrate binding cleft as described by Huang (20) are boxed and labeled A, B, C, D, 1, 2, and 3. The H, D, and S of the catalytic triad are marked with a hash (#). The premature termination codons of the δ tryptases are marked with an X. The peptide sequence used as the immunogen for anti δ tryptase is underlined (·····).

(δI cDNA). Of the nucleotide differences described above, only the second, G226 (δII) to A (δI), results in an altered amino acid residue in the putative protein products (Val in δII and Met in δI).

Our results confirm that the primary transcript is spliced as predicted by Pallaoro et al. (14). The consequence of this is that compared with other tryptases, the δ tryptases possess a premature translation termination codon (T706AA) at the beginning of exon 6 which would result in the translation of a mature enzyme that is 40 aa shorter than the αβ tryptases (Fig. 3). Despite this truncation, the serine protease catalytic triad His^{44}, Asp^{91}, and Ser^{194} (initial Met numbered as position 1), which is an absolute requirement for proteolytic activity in these enzymes, remains intact. A further consequence of this truncation is the complete loss of the residues that comprise loop 2, one of seven that form the substrate-binding cleft in the αβ tryptases (19, 20).

A search of the expressed sequence tag database found no sequence with high similarity to any of the cDNAs cloned in the present study.

In vivo expression

Standard RT-PCR analyses revealed that the δ tryptase genes are transcribed in a wide range of tissues. Correctly sized ethidium bromide-staining bands were visible after 30 cycles when amplified from RNA isolated from the HMC-1 cell line, lung, heart, stomach, spleen, skin, and colon, as well as in fetal lung and heart (data not shown). Sequencing of RT-PCR products amplified from HMC-1, lung, and fetal lung confirmed their identity (data not shown). The lack of contaminating sequence indicated that no other tryptase transcripts were being coamplified.

Using an RTQ RT-PCR approach, the relative abundance of δ tryptase and β-actin mRNA in a range of human tissue was determined using log-linear regressions derived from standard curves (representative regressions, δ tryptase: $y = -3.0918x + 12.996$, $R^2 = 0.9516$; β-actin: $y = -4.0552x + 11.089$, $R^2 = 0.9776$, where y = nanograms of cDNA and x = threshold cycle number). When normalized for β-actin, δ tryptase was most abundant in the colon and lung, less abundant in the heart and stomach, and just detectable in the spleen (Fig. 4). Significant amounts of δ tryptase mRNA was also detected in the HMC-1 cell line. No amplification was apparent in the no template control (Fig. 4) when primers were omitted from the PCR, when other tryptase cDNAs were used as template in PCR, or when reverse transcriptase was omitted from the reverse transcription reactions (data not shown).

In vivo expression of δ tryptase protein

Western blot analyses indicated that the δ tryptase Ab recognized recombinant δ tryptase (see below for details) as a single band of <30 kDa, but not rβII tryptase (Fig. 5*A*). Conversely, the AA1 Ab detected rβII tryptase as a major band of >30 kDa, but did not recognize recombinant δ tryptase (Fig. 5*B*).

Immunohistochemical analyses revealed that the δ tryptase protein is expressed in a range of human tissue including colon (Fig. 6, *A*, *B*, and *E*), lung, heart, and synovial tissue (data not shown). No positive-staining cells were observed in any tissue when the primary Ab was omitted, or when normal nonimmune rabbit Ig was used as the primary Ab (Fig. 6, *C* and *F*). Many of the positive-staining cells possessed the morphological characteristics of mast cells. Staining of serial sections of colon tissue revealed the presence of cells that were positive for both δ tryptase (Fig. 6*E*) and for the αβ tryptases (i.e., with the AA1 Ab; Fig. 6*D*), indicating that some mast cells express δ tryptase. It was noticeable in the colon tissue that the vast majority of δ tryptase-positive cells were in the mucosa, specifically in the lamina propria between the crypts. δ Tryptase-positive cells were virtually absent from the submucosa and muscle layers.

Generation of active recombinant δ tryptase

Recombinant δ tryptase was expressed in bacterial cells, purified on a metal chelating column, refolded, and the mature form of the enzyme generated by EK cleavage. The mature form of the enzyme was recognized by an anti-peptide Ab as a single band of <30 kDa (Fig. 5*A*). Recombinant δ tryptase, rαII tryptase, and commercially available rβII tryptase and native lung tryptase were tested for the ability to cleave a panel of three trypsin-susceptible *p*NA chromogenic substrates. rβII tryptase was able, with different efficiencies, to cleave all three substrates (Fig. 7*B*; *N*-*p*-Tosyl-Gly-Pro-Lys > *N*-benzoyl-Pro-Phe-Arg-*p*NA = D-Ile-Phe-Lys *p*NA; only data for D-Ile-Phe-Lys *p*NA is shown for all enzymes). Native lung tryptase was able to cleave two of the substrates (Fig. 7*C*; *N*-*p*-Tosyl-Gly-Pro-Lys > D-Ile-Phe-Lys *p*NA), while rαII tryptase was able to cleave all three substrates equally, but less efficiently than rβII tryptase (Fig. 7*D*). Recombinant δ tryptase, while ineffective against *N*-benzoyl-Pro-Phe-Arg-*p*NA or *N*-*p*-Tosyl-Gly-Pro-Lys 4-nitroanilide, was able to cleave D-Ile-Phe-Lys *p*NA (Fig. 7*E*). No substrate cleavage was detected in the presence of buffer alone (Fig. 7*A*), prorecombinant δ tryptase (i.e., not activated by EK cleavage, Fig. 7*F*), and EK alone (data not shown). The amount of substrate cleaved by each enzyme was determined by estimating the area under the peaks that represent the liberated peptide portion of the cleaved substrate. The amount of D-Ile-Phe-Lys *p*NA substrate cleaved by recombinant δ tryptase was ~12% as much as that cleaved by rβII tryptase.

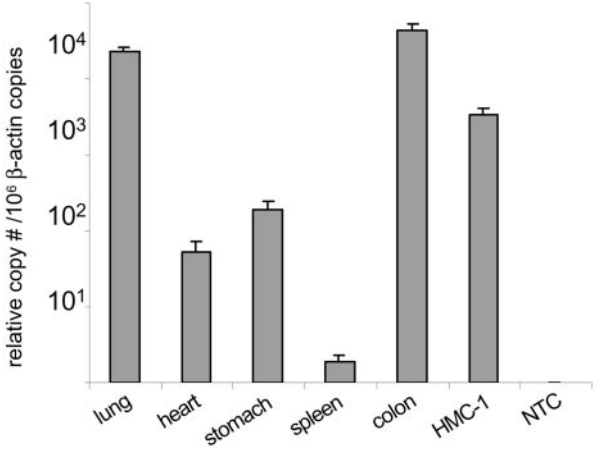

FIGURE 4. δ Tryptase is transcribed in various tissues. The relative abundance of δ tryptase mRNA (normalized with respect to β-actin mRNA) in a range of human tissues was determined using RTQ RT-PCR. The data represent the mean (± SD) from a single experiment. All samples were tested in triplicate and have been tested in at least two independent experiments, except for colon RNA, which was tested once. NTC, no template control.

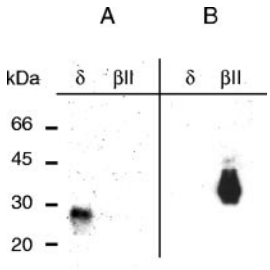

FIGURE 5. Affinity-purified anti-δ tryptase Ab recognizes recombinant δ tryptase but not rβII tryptase. Approximately 0.5 μg of purified recombinant δ tryptase and 1 μg of rβII tryptase (Promega) were separated on a 10% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride membrane, blocked, and incubated with affinity-purified δ tryptase anti-peptide Ab (1 μg/ml) (*A*). Replicate blots were probed with normal rabbit IgG (1 μg/ml; data not shown), and with the mouse monoclonal anti-tryptase Ab AA1 (1/200) (*B*).

Three-dimensional protein modeling

Fig. 8 is a three-dimensional model of βII tryptase showing the position of the terminal 40 aa that appear in the α and β human tryptases, but that are deleted in δ tryptase due to the presence of a premature termination codon in the transcript.

The deletion of 40 C-terminal amino acids is expected to have a significant effect on the structure and function of δ tryptase. Although the catalytic triad is likely to remain intact, the floor of the S1 substrate-binding site may be dramatically affected. At a minimum, truncation is likely to result in significantly altered substrate specificity. Although the tetramerization interface is not as severely affected, there are likely to be disturbances to monomer interactions, which may have an additional influence upon substrate specificity in the light of recently proposed "substrate sharing" by adjacent monomers (21). Due to the relatively large size of the deletion and the subtlety of monomer interactions, we have not attempted to build a (potentially misleading) three-dimensional structure of the mutant. Protein expression, substrate specificity studies, and quaternary structure determination are required to form well-grounded conclusions on the structure-function effects.

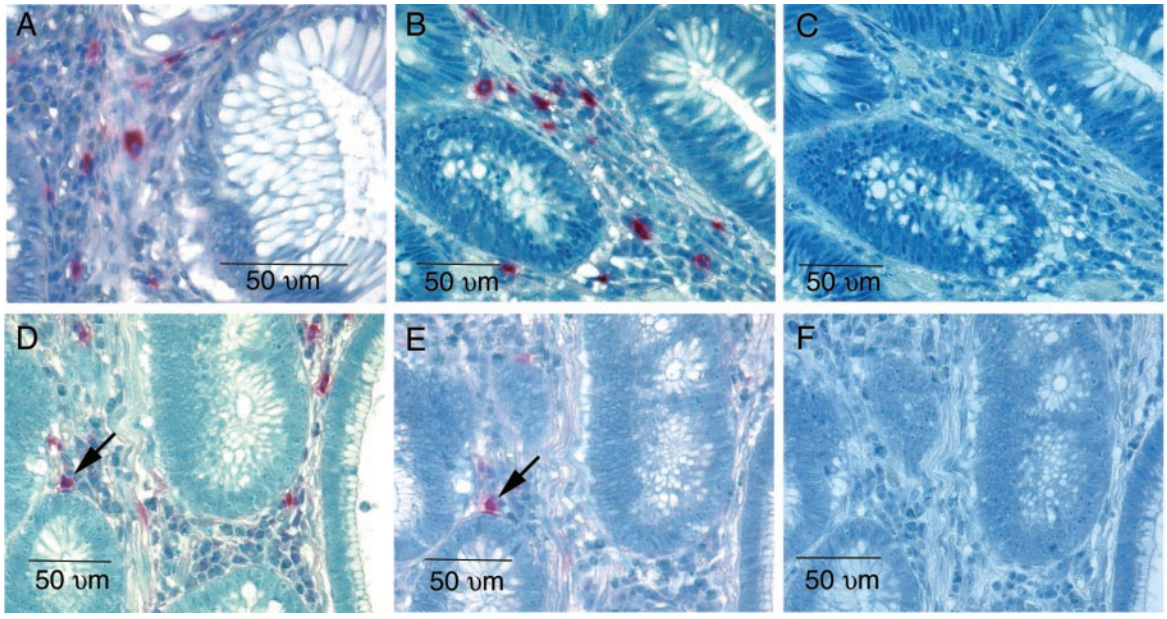

FIGURE 6. δ Tryptase protein is expressed in human colon tissue. Sections of human colon were stained immunohistochemically using anti-δ tryptase Ig (*A*, *B*, and *E*), the monoclonal anti-tryptase Ab AA1 (*D*), and normal rabbit IgG (*C* and *F*). *B–F*, Serial sections. *D* and *E*, Arrows indicate the location of a cell that is stained with both anti-δ tryptase and the AA1 Ab.

Discussion

The cDNAs first reported in this study, designated δI tryptase and δII tryptase, match the predicted exonic sequences of the genes encoding alleles I and II of the so-called mMCP-7-like human tryptase gene (14). In that report, no evidence was presented regarding the expression of this gene, and indeed the authors reported that a search of existing human expressed sequence tag databases was negative. Recently, Min and coworkers (18) cloned a third allele of this gene, but failed to detect transcripts from any of the three known alleles by RT-PCR. In this study, we report the cloning of cDNAs transcribed from this gene, the expression of the mRNA and protein in multiple tissue sites, and that a recombinant

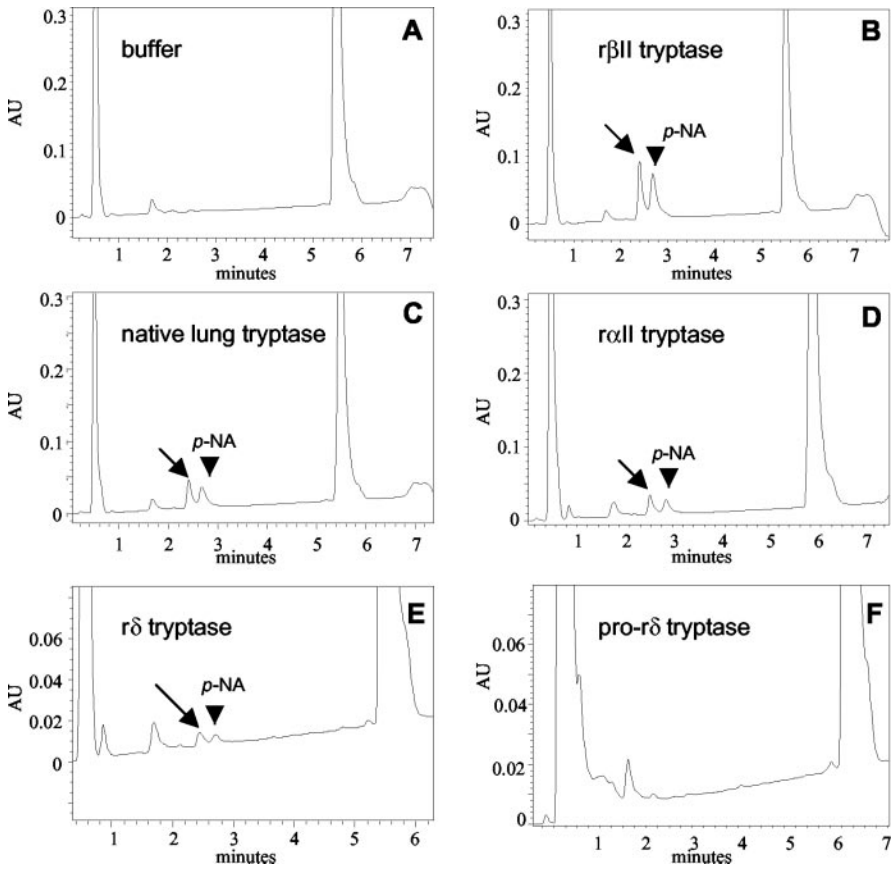

FIGURE 7. Recombinant δ tryptase cleaves a trypsin-sensitive substrate. Reverse-phase HPLC of D-Ile-Phe-Lys *p*NA cleavage products when digested with *A*, buffer alone; *B*, rβII tryptase; *C*, native lung tryptase; *D*, rαII tryptase; *E*, recombinant δ tryptase; or *F*, pro-recombinant δ tryptase. Aproximately 2 μg of each enzyme was used to digest 10 μg of substrate in a 50-μl reaction. Liberated pNA (▼) and peptide (↓) moieties are indicated. The large peak at the *right* of each panel (5.6 min) represents undigested substrate. Samples were tested in duplicate and representative chromatograms are shown.

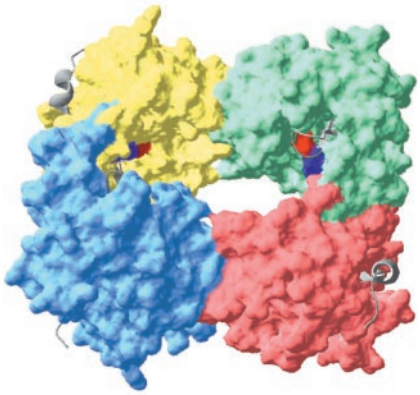

FIGURE 8. Three-dimensional model of βII tryptase showing the position of the terminal 40 aa that appear in the α and β human tryptases, but that are deleted in δ tryptase. The figure is a surface representation showing the tryptase tetramer ring with a ribbon (gray) representation of the 40 aa C-terminal deletion in each monomer. Monomers A, B, C, and D are depicted as yellow, green, pink, and blue, respectively. The catalytic Ser and His residues are colored red and purple, respectively.

form is proteolytically active. For simplicity, we suggest that the gene and its products be renamed δ tryptase.

We initially isolated the δ tryptase cDNAs from HMC-1 cells and confirmed that the splicing pattern matched that of the other related tryptases, and as predicted by Pallaoro and coworkers (14). Although the putative mature enzyme is 40 aa shorter than the well-described αβ family of human tryptases, the catalytic triad, essential for proteolytic activity, remains intact. Using δ tryptase-specific PCR primer-pairs, we demonstrated the in vivo expression of this transcript in a wide variety of tissues, including lung, heart, stomach, spleen, skin, and colon. RTQ RT-PCR analyses revealed that the δ tryptase transcript, when corrected for β-actin expression, is most abundant in the lung and colon but is also expressed in the heart and stomach. This is the first evidence that this gene is expressed at the transcriptional level. Detection of the δ tryptase transcript in the HMC-1 cell line indicated that its expression may be primarily restricted to mast cells in a manner similar to that described for other tryptases. One consequence of these findings is that the presence of δ tryptase transcripts has to be accommodated when designing primers and probes to detect the expression of the αβ family of tryptases. The exceptional degree of homology between δ tryptase and the αβ tryptases (in all regions except exon 5) means that there is a high probability of cross-reactivity, unless the primers/probes are designed to specifically exclude detection of δ tryptase.

Given the unique truncated structure of the putative enzyme, it was not clear whether δ tryptase would be translated in vivo. Our immunohistochemical demonstration that δ tryptase is indeed translated in vivo expands the number of structurally different serine proteases present in the chromosome 16p13.3 complex. Interestingly, the number of δ tryptase-positive cells in a particular tissue was generally consistent with the level of the δ tryptase mRNA detected using RTQ RT-PCR, despite the mRNA and tissue samples being derived from different individuals.

At least some of the cells expressing δ tryptase also stained positive with the AA1 Ab, an Ab generally recognized to be immunohistochemically specific for mast cells. This, together with the finding that δ tryptase mRNA is expressed by HMC-1 cells, indicates that mast cells represent at least one cellular source of δ tryptase. Not all δ tryptase-positive cells were AA1 positive, and not all AA1-positive cells were δ tryptase-positive. This suggests that δ tryptase may be expressed by other cells in addition to mast cells, and/or that different mast cell phenotypic subsets may exist characterized by discordant expression of δ tryptase and the AA1-staining αβ tryptases.

A close examination of the colon sections indicated that the vast majority of δ tryptase-positive cells were in the mucosal compartment, with only one or two individual δ tryptase-positive cells located in the submucosa or muscle layers. In contrast, AA1-positive cells were common throughout. This suggests that, at least in the colon, δ tryptase may be useful as marker of a new mast cell phenotype that is more common in the mucosa. The δ tryptase Ab we have developed will allow us to explore human mast cell phenotypic heterogeneity, in much the same manner as mMCPs are used to phenotype mouse mast cell populations (22).

Perhaps the most interesting finding of our study is that a recombinant form of the enzyme was able to cleave a synthetic trypsin-susceptible substrate, D-Ile-Phe-Lys-*p*NA, albeit apparently less efficiently than rβII tryptase. The substrate binding cleft of tryptases comprises seven major loops in the polypeptide chain named A, B, C, D, 3, 1, and 2 in order from the N terminus (Ref. 20; see Fig. 3). The loss of the terminal 40 aa means that loop 2 (residues 211–218 in the αβ tryptases) is not present in δ tryptase. This loop forms part of the S1, S2, and S3 sites of the enzyme, and even single amino acid changes within this loop alter the substrate specificity of tryptases (20) and granzyme B (23). This is consistent with our finding that recombinant δ tryptase and rβII tryptase differed in their ability to cleave a panel of three substrates. It is not certain whether the lower efficiency of δ tryptase in cleaving D-Ile-Phe-Lys *p*NA is due to it being a less effective proteolytic enzyme than the αβ tryptases, or whether the substrates chosen for this study were suboptimal. Future studies may identify substrates that δ tryptase hydrolyzes more efficiently, or may reveal that it is an inherently flawed proteolytic enzyme. We are presently attempting to identify the extended substrate specificity of this enzyme, and the identification of suitable substrates will allow us to investigate this.

The results of two recent investigations may have important implications for understanding questions concerning the possible function of δ tryptase. First, Huang et al. (24) reported that two mouse tryptases, mMCP-6 and mMCP-7, could form enzymatically active heterotypic tetramers. Second, Harris and coworkers (21) suggested that residues on neighboring tryptase monomers acted together within the tetramer framework to form parts of the extended substrate recognition site. Taken together, these results suggest that even if its proteolytic efficiency is low, δ tryptase may modulate the activity of other tryptases by forming heterotypic tetramers.

The expression of δ tryptase may have clinical relevance as mast cell proteases have been implicated in the development of certain lung diseases, especially asthma. Chymase is a potent secretagogue of airway mucous glands (25), while tryptases are known to cleave vasoactive intestinal peptide, the only known bronchodilator apart from NO which occurs in the airway (26). In addition, tryptases cause smooth muscle hyperreactivity (9), while tryptase inhibitors have been shown to inhibit allergen-induced airway hyperreactivity and markers of inflammation in vivo (8). Mast cell tryptase is also a potent mitogen for airway smooth muscle cells (27), probably via proteinase activated receptor-2 (28, 29), and may be associated with the smooth muscle hypertrophy seen in the airway wall of asthmatic subjects. There is evidence that it may induce IL-8 secretion from airway epithelial cells, promoting airway inflammation (30). Determining the exact role of the different tryptases, including δ tryptase, remains a challenge. However, our

results indicate that the range of structurally and functionally different human tryptases expressed may be wider than previously believed.

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