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Characterization of Human \( \gamma \)-Tryptases, Novel Members of the Chromosome 16p Mast Cell Tryptase and Prostasin Gene Families


Previously, this laboratory identified clusters of \( \alpha \)-, \( \beta \)-, and mast cell protease-7-like tryptase genes on human chromosome 16p13.3. The present work characterizes adjacent genes encoding novel serine proteases, termed \( \gamma \)-tryptases, and generates a refined map of the multityptase locus. Each \( \gamma \) gene lies between an \( \alpha \)I\( \alpha \) channel gene (\( \text{CACNA1H} \)) and a \( \beta \)I- or \( \beta \)II-tryptase gene and is \(~30\) kb from polymorphic minisatellite MS205. The tryptase locus also contains at least four tryptase-like pseudogenes, including mastin, a gene expressed in dogs but not in humans. Genomic DNA blotting results suggest that \( \gamma \)- and \( \gamma \)II-tryptases are alleles at the same site. \( \beta \)II- and \( \beta \)III-tryptases appear to be alleles at a neighboring site, and \( \alpha \)II- and \( \beta \)I-tryptases appear to be alleles at a third site. \( \gamma \)-Tryptases are transcribed in lung, intestine, and in several other tissues and in a mast cell line (HMC-1) that also expresses \( \gamma \)-tryptase protein. Immunohistochemical analysis suggests that \( \gamma \)-tryptase is expressed by airway mast cells. \( \gamma \)-Tryptase catalytic domains are \( \sim48\% \) identical with those of known mast cell tryptases and possess mouse homologues. We predict that \( \gamma \)-tryptases are glycosylated oligomers with tryptic substrate specificity and a distinct mode of activation. A feature not found in described tryptases is a C-terminal hydrophobic domain, which may be a membrane anchor.

Although the catalytic domains contain tryptase-like features, the hydrophobic segment and intron-exon organization are more closely related to another recently described protease, prostasin. In summary, this work describes \( \gamma \)-tryptases, which are novel members of chromosome 16p mast cell/prostasin gene families. Their unique features suggest possibly novel functions.

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Tryptases are expressed by most human mast cells and are abundant, comprising as much as 25% of cell protein (1). They are trypin-like proteases secreted as active, heparin-bound oligomers that resist inactivation by plasma antiproteases (2). Extracellular targets include neuropeptides, procoagulant proteins, urokinase,stromelysin, and proteinase-activated receptor-2 (3–7).

Several studies suggest roles for tryptases in allergic airway disease. Lavage tryptase levels rise in asthmatics following endobronchial allergen challenge (8). In dog and human bronchi, tryptases increase histamine-induced bronchoconstriction (9, 10) and also may promote constriction by degrading vasoactive intestinal peptide (4). By stimulating airway smooth muscle cell (11) and fibroblast growth (12, 13), as well as chemotaxis and collagen synthesis (14, 15), they may contribute to muscle hypertrophy and subepithelial fibrosis in asthma. The hypothesis that tryptases worsen asthma is buttressed by reports that tryptase inhibitors block allergic bronchoconstriction and eosinophilic inflammation in sheep (16) and, in the first human trials of tryptase inhibitors, reduce asthmatic responses to inhaled allergen in asthmatics (17).

Known human tryptases divide into two groups, \( \alpha \) and \( \beta \). \( \alpha \)-Tryptase is the major circulating isofrom and may be the main type expressed by basophils (18, 19). \( \beta \)-Tryptases appear to be the major type stored in secretory granules and are the major form isolated from lung extracts and mast cells purified from lung and skin (19). Four different human cDNAs (\( \alpha \), \( \beta \), \( \beta \)III, and \( \beta \)III) have been isolated from lung and skin mRNA (20–22). \( \beta \)-Tryptases are 98–99% identical in amino acid sequence. \( \alpha \)-Tryptases are less closely related (\( \alpha \)I is 91% identical with \( \beta \)I). Our laboratory reported recently that \( \alpha \)II, \( \beta \)I, \( \beta \)III, and \( \beta \)III genes are clustered on chromosome 16p13.3 along with genes encoding novel tryptases, the fifth exon of which is related to the mouse tryptase, mouse mast cell protease (mMCP)-7 (23). The intron-exon organization of tryptase genes is unique, suggesting that tryptases are a distinct and perhaps ancient branch of the trypsin clan of serine peptidases.

In mice, tryptases mMCP-6 and -7 have been identified (24, 25). Structurally, human \( \alpha \beta \)-tryptases are more closely related to each other than to mMCP-6 and -7, which are much more different from each other after the point when known mouse and human tryptases shared a common ancestor (23, 26). In dogs, our laboratory characterized a mast cell protease termed mastin, a relative of tryptase sufficiently different from known tryptases that it forms a separate branch of the tree (27–29). No expressed human or mouse homologue has been identified.

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3 Abbreviations used in this paper: mMCP, mouse mast cell protease; EST, expressed sequence tag; HTGS, high throughput genome sequence; BAC, bacterial artificial chromosome.
Here we demonstrate expression of novel human tryptases, termed γ because they are distinct from α, β, and other human tryptases. We also present a refined map of α/β genes and identify a gene encoding a human homologue of dog mastin.

Materials and Methods

Database screening

Human β-tryptase cDNA (22) and gene sequence (23) and Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov) algorithms were used to query expressed sequence tag (EST) and high throughput genome sequence (HTGS) in GenBank. Predicted novel human cDNAs were used to query GenBank's mouse EST database to identify mouse homologues.

Cell culture

Cells from the human mast cell line HMC-1 (5C6 subclone, kindly provided by Dr. Beate M. Henz) were cultured as described (30). HMC-1 cells express active β-tryptase but not α-tryptase (19, 30–32).

Amplification and cloning of cDNAs

cDNA predicted from EST and genomic sequence was used to design PCR primer pairs, which were then used to screen cDNAs prepared from a spectrum of human tissues for expression of γ-tryptases. Sequencing of amplimers allowed us to prove the identity of the PCR-derived bands, to confirm exon sequences predicted from genomic and EST DNA, and to confirm intron-exon splice site predictions. Amplified fragments of γ-tryptase cDNA from human lung and from HMC-1 5C6 cells were subcloned into pCR2.1 (Invitrogen, Carlsbad, CA) for further sequencing and for generation of specific probes to use in blotting studies. Bands containing amplimers from colon, small intestine and testis were sequenced directly.

DNA and protein sequence comparisons

DNA sequencing was conducted by University of California at San Francisco's Biomolecular Resource Center using standard dyeoxy techniques with 20% dehydrated ethanol washes. Multiple sequence alignments, matrix analyses, and dendrograms were generated using GeneWorks software (Oxford Molecular, Campbell, CA).

Chromosomal localization and screening of bacterial artificial chromosomes (BACs)

BAC clones 324 and 48 previously were identified and localized to chromosome 16p13.3 as described (23). In the current study, γ-tryptase genes were detected in these BACs by PCR of gene-specific fragments and by DNA blotting using the general approaches we described previously in connection with these BACs (23). BAC fragments generated by digestion with HindIII were subcloned. Genes were mapped to specific sites in BAC λ subclones by blotting of electrophoresed DNA restriction digestes and by sequencing of PCR amplimers, BAC ends, and portions of selected λ subclones.

mRNA and DNA blotting

mRNA extracted and purified from cultured HMC-1 5C6 cells was electrophoresed in agarose and blotted to nylon membranes. These blots, along with blots of mRNA from a range of human tissues (Invitrogen), were hybridized with radiolabeled cDNA probes corresponding to protein-coding exons. BAC and full human genomic DNA were digested with restriction endonucleases, size-fractionated in agarose, then blotted, baked, and prehybridized as described (23). The resulting blots were probed with a radiolabeled 1100-bp fragment of the BAC 48 γ-tryptase gene bracketing the internal NorI site, or with labeled subclones of this fragment lying on each side of the NorI site, then subjected to autoradiography.

Ab generation, immunoblotting, and immunohistochemistry

Polyclonal Abs recognizing human γ-tryptase were raised in chickens immunized with a keyhole limpet hemocyanin-conjugated synthetic peptide (CRRHYDPGPPGSILQPP) corresponding to residues 192–206 of human prepro-γ-tryptase. Conjugations and immunizations were conducted by AnaSpec (San Jose, CA). In preparation for immunoblotting, HMC-1 5C6 cell proteins were extracted into detergent solution (4% SDS in 0.2 M DTT, 20% glycerol, 0.125 M Tris, pH 6.8). Extracts were electrophoresed in 12.5% SDS-polyacrylamide gels and electroblotted to polyvinylidene difluoride membranes, which were preincubated with 0.3% Tween-20 in 50 mM Tris (pH 7.2) and 0.5 M NaCl, then hybridized with 1:1000 dilutions of chicken antiserum for 1 h in the same buffer. Bound Abs were detected with goat anti-chicken IgG–alkaline phosphatase (1:5000) and Fast Red TR/Naphthol AS-MX Phosphate (Sigma, St. Louis, MO). Control blots were incubated with preimmune chicken serum or with secondary Ab.

For immunohistochemical analysis using the same anti-γ-tryptase antiserum, samples of large airways were obtained (with approval of the University of California at San Francisco Committee on Human Research) from patients undergoing lung transplantation for cystic fibrosis. Five-micrometer airway cryosections placed onto glass slides were equilibrated in 0.3% H2O2 and 90% methanol for 10 min. washed with PBS, then incubated for 15 min with blocking solution (PBS containing 5% dehydrated milk, 3% nonimmune goat serum, 0.1% Triton X-100, and 1% glycerine) at 18°C. Blocking solution was removed and tissues probed either with a 1:100 dilution of chicken nonimmune serum or anti-γ-tryptase antiserum overnight at 4°C. Tissues were then washed in PBS plus 0.05% Tween-20, incubated with 1:200 dilution of alkaline phosphatase-conjugated goat anti-chicken IgG Ab (Promega, Madison, WI) for 10 min at 18°C, then washed again. Bound alkaline phosphatase was detected using the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Zymed Laboratories, South San Francisco, CA).

Molecular modeling

We constructed a homology-model of the γ-tryptase catalytic domain with assistance of an automated protein modeling tool and server (Swiss PDB Viewer and Swiss-Model, respectively) (33). The pro- and C-terminal hydrophobic sequences were excluded from the model. X-ray diffraction-derived coordinates of human β-tryptase (1AOL) (34) served as template for the model, which was optimized by idealizing bond geometry and removing unfavorable nonbonded contacts.

Results and Discussion

Identification of γ-tryptase cDNAs and prediction of amino acid sequence

cDNAs encoding parts of human γ-tryptase were obtained initially by screening EST databases using known tryptases as query sequences. Because these sequences are partial and disagree in certain areas of overlap, PCR primers based on EST sequence were used to amplify more complete cDNAs from human lung cDNA. The full amino acid sequence of 321 amino acids deduced from a combination of PCR-derived lung cDNA and EST sequence is identical with that predicted for gene-derived γ-tryptase in Fig. 1, which compares gene-derived prepro-γ-tryptase primary sequences with those of related serine proteases. The γ and γI structures aligned in Fig. 1 represent amino acid sequences predicted from separate BAC genes (see below). The predicted m.w. of prepro-γ-tryptase is 33,689 without glycosylation or other post-translational modification. Based on database searches and computer-assisted multiple sequence alignments and phylogenetic comparisons, the γ-tryptase catalytic domain amino acid sequence is most similar to that of known mast cell tryptases, exhibiting 47% identity to βII-tryptase. Some structural features, e.g., LPPPY (residues 173–177), are particularly tryptase-like. Other features are common to all active serine proteases with tryptic specificity, such as “catalytic triad” residues His78 (His57, by standard chymotrypsinogen numbering), Asp102 (Asp192, standard numbering), and Ser195 (Ser55, standard numbering), and also residue Asp216 (Asp187, standard numbering), which is the prime determinant of specificity for Arg or Lys on the N-terminal side of the scissile bond of peptide substrates. These features, along with the clustering of γ-tryptase genes with known tryptase genes and evidence of mast cell expression, are the basis of labeling these enzymes tryptases. However, the catalytic domains are related almost as closely to the recently described proteases prostasin (35) and testisin (36). Indeed, when full presequence and gene structures (see below) are compared, γ-tryptase appears to be more similar to prostasin and testisin than to α/β-tryptases. Features shared with prostasin and testisin include a propeptide ending in Arg and a predicted C-terminal membrane anchor, neither of which are
present in α/β-tryp-, and a gene organization more similar to that of prostanin than of previously described tryptases (see below).

Termination of the putative propeptide in a basic residue suggests that pro-γ-tryp- is activated directly from its catalytically inactive zymogen form by a tryptic protease, which will sever the propeptide from the catalytic domain, allowing it to adopt an enzymatically active conformation. This mode of activation would differ from that of human β-tryp-, which lack a terminal propeptide basic residue and are activated in a two-step process involving tryptase and dipeptidyl peptidase I (37). Mature, active γ-tryp- resembles known tryptases, may be a two-chain protein, with part of the propeptide remaining covalently attached via a disulfide linkage involving Cys26 (Cys 1 in chymotrypsinogen) by analogy to the propeptide-catalytic domain linkage in activated chymotrypsin (38). Prostasin and testisin also contain this predicted linkage. It is possible that the γ-tryp- propeptide will be removed through the action of exopeptidases such as dipeptidyl peptidase I, which is highly expressed in mast cell secretory granules (39). Based on a consensus site at Asn85, mature γ-tryp- may be N-glycosylated. The mature protein is likely to be an active protease with tryptic specificity, based on conservation of critical triad residues and of specificity-determining Asp216 (Asp 189, by chymotrypsinogen numbering), as noted above. Due to conservation of the LPPPY sequence found to form noncovalent contacts between subunits in the βII-tryptase tetramer (34), γ-tryp- may form oligomers of catalytically active units. However, γ-tryp- lacks the pair of Tyr residues that also are predicted to promote LPPPY-mediated oligomerization. Therefore, it is not yet possible to predict with certainty whether γ-tryp- will oligomerize, like all heretofore-characterized mast cell tryptases and close relatives, such as mastin (29).

γ-Tryp- I and II contain a C-terminal extension with a highly hydrophobic segment long enough to form a single-pass transmembrane helix, as shown by the hydropathy plot in Fig. 2. This predicts that the catalytic domain will be C-terminally membrane anchored. Within the endoplasmic reticulum, Golgi, and secretory granules, the γ-tryp- catalytic domain should lie in the lumen. If γ-tryp- traffics to the cell surface, then the catalytic domain will lie on the extracellular surface. The hydropathy analysis predicts a small cytoplasmic tail of uncertain significance.

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**FIGURE 1.** Alignment and phylogenetic comparison of γ-tryp- with related serine proteases. A, Computer-aligned preprosequences of αI-II-tryptase, β-tryptase, γ-tryp-, testisin, and prostasin. Chymotrypsin is also included to show the positions of key residues numbered according to the sequence of chymotrypsinogen. Residues that are identical in all seven proteases are enclosed in boxes. B, A dendrogram developed from the same sequences (catalytic domains only) exchanging chymotrypsin for trypsin, which is more closely related to tryptases in substrate specificity. The length of each branch is proportional to the percentage of sequence mismatches between pairs or groups of sequences. The results suggest that the catalytic domains of γ-tryp- are more closely related to α/β-tryp- than to testisin and prostasin. C, Alignment of γII-tryp- amino acid sequence with the partial sequence of a putative mouse homologue deduced from ESTs. The high degree of correspondence between the two sequences suggests that mice possess one or more homologues of human γ-tryp-.

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10 residues on the N-terminal side of the hydrophobic tail embody features typical of GPI-anchored proteins (40). Possibly, the transmembrane sequence in mature \( \gamma \)-tryptase is severed in the endoplasmic reticulum and replaced with a GPI membrane anchor. If this is the case, \( \gamma \)-tryptase could be released in a soluble form by GPI-specific phospholipases. The predicted m.w. of the unglycosylated \( \gamma \)-tryptase catalytic domain plus C-terminal extension is 30,063. Without the C-terminal extension, the predicted m.w. is 25,000. The deduced amino acid sequences of \( \gamma \)-tryptases I and II are 98–99% identical with that of recently described “transmembrane tryptase” (41).

Using our human \( \gamma \)-tryptase cDNA, we identified mouse ESTs (e.g., AI466869 and AA266560) collectively encoding 158 residues of contiguous sequence in the catalytic domain of a related mouse enzyme. Based on an amino acid identity of \( \sim \)74% compared with human \( \gamma \)-tryptase in regions of overlap, the mouse products are likely to be orthologs of human \( \gamma \)-tryptases. Whether they are products of more than one gene remains to be determined. This level of identity is similar to that between human \( \gamma \)-tryptases and the mouse tryptases mMCP-6 and -7 (23). Among features conserved between mouse and human enzymes are catalytic triad residues (His and Asp but not Ser are covered by the partial mouse sequence), the consensus N-glycosylation site, and five of six cysteines.

**Tissue expression of \( \gamma \)-tryptase mRNA**

As shown by the results of PCR in Fig. 3, we identify transcription of human \( \gamma \)-tryptase in multiple tissues, including lung and small intestine. However, several tissues screened using the same conditions yield no signal or faint signals, suggesting tissue-specific differences in levels of \( \gamma \)-tryptase transcription. Tissues expressing no detectable \( \gamma \)-tryptase mRNA include peripheral blood leukocytes and thymus, a finding that suggests that \( \gamma \)-tryptases are not highly expressed in circulating immune cells. In contrast, \( \gamma \)-tryptase mRNA is abundant in the 5C6 subclone of HMC-1 cells, which also express active \( \beta \)-tryptase (19, 30–32). Sequencing of PCR-generated amplimers from HMC-1 cells reveals the same sequence predicted from amplimers of human lung cDNA. These sequences, which cover 70% of the protein sequence predicted from BAC genes, is 99.1–97.8% identical with that of \( \gamma \)- and \( \gamma \)-tryptases, respectively (see Fig. 1) in 224 residues of overlap. Blotting of electrophoresed human mRNA from a number of tissues does not yield strong hybridization signals (not shown) with \( \gamma \)-tryptase probes, suggesting that overall levels of \( \gamma \)-tryptase transcript are low (although detectable by PCR), perhaps because \( \gamma \)-tryptase is expressed in a small subpopulation of cells in the tissues surveyed. However, \( \gamma \)-tryptase mRNA is detected by standard blotting of mRNA from the 5C6 subclone of HMC-1 cells.
Localized a transcript of I-tryptase via PCR from BACs (not shown). The identification of γ-tryptase mRNA in a mast cell line suggests that mast cells may be among the subset of cells expressing γ-tryptases.

Localization of γ-tryptase genes

Previously, this laboratory identified two BAC clones containing clustered α/β-tryptase genes localizing to chromosome 16p13.3 (23). Overlapping portions of these BACs are homologous but not identical. Hypothesizing that additional tryptase-like genes lie in these two BACs (324 and 48), we examined the sequence of these regions. Amplifiers obtained from UV, ovary, prostate, small intestine, colon, and spleen but none from PBL or thymus. Amplimers obtained from γ-tryptase genes via PCR using EST- and cDNA-based primers. Each BAC yields a strong band of amplimers, one of which was subcloned and sequenced, confirming the presence of genomic sequence containing the exon sequence predicted by γ-tryptase ESTs. To localize γ-tryptase genes within each BAC, radiolabeled probes based on cloned fragments of γ-tryptase were hybridized with BAC DNA digested singly and in combination with HindIII, NotI, and EcoRI. In each BAC clone, the results localized a γ-tryptase gene to a particular HindIII fragment bracketing a NotI site splitting the gene, as shown in Fig. 4. The NotI site in the two γ-tryptase genes was localized by sequencing genomic fragments generated by PCR. The transcriptional orientation of each γ-tryptase gene was determined by DNA blotting using radiolabeled probes derived from the 5′ side of the established NotI site. The γ-tryptase gene on BAC 324 lies in a region covered by a subcloned HindIII fragment (λ324D) found previously to contain the βI gene (23). On BAC 48, the nearest tryptase neighbor is the βII gene, which resides on an adjacent HindIII fragment (λ48 M) rather than on the same HindIII fragment due to the presence of a HindIII site not present in the otherwise homologous region of BAC 324. As shown in Fig. 5, DNA blots of HindIII-digested BAC 324, BAC 48, and full genomic DNA reveal that the HindIII isoforms predicted by the two BACs both are present in some but not all individuals, suggesting that γI and γII are alleles of each other. By inference, based on the homologies between BAC 324 and 48 depicted in Fig. 4, other allelic pairs of tryptases are βII/βIII, αII/βI, and mMCP-7-like III/II, respectively.

Based on examination of γ-tryptase ESTs and sequenced portions of the BAC-derived genes, the nearest neighboring gene on the 3′ flank is an α1H T-type Ca2+ channel gene identical or closely related to CACNAIH. In the case of the BAC 324 γI-tryptase gene, the 3′ untranslated region possibly overlaps with that of CACNAIH, which is in the opposite transcriptional orientation, as depicted in Fig. 4. CACNAIH, which has been localized to chromosome 16p13.3 by fluorescent in situ hybridization (42). A mouse homologue (Cacnaih) has been mapped to chromosome 17 in a region that is homologous with a portion of human chromosome 16p13.3 (42). This syntenic region also harbors two mouse tryptase genes, Mcpt6 and Mcpt7 (43). At least two genomic fragments encoding parts of CACNAIH were identified in database searches. One of these (GenBank AL031703.9) aligns with one end of BAC 324 based on shared restriction patterns and a segment of near-identity with BAC 324’s sequenced T7 end, as shown in Fig. 4, allowing extension of the γI-tryptase locus beyond the BAC terminus. A second GenBank sequence (fragment 10 of unfinished chromosome 16-derived contig AL031715.1) contains additional CACNAIH–related exons and aligns elsewhere on BAC 324, as shown in Fig. 4. The 5′ end of CACNAIH predicted by cDNA cloning was not found in the AL031703.9 sequence; therefore, the extended BAC 324 sequence in Fig. 4 does not include the entire gene. However, AL031703.9 contains the well-known minisatellite MS205 (also known as DS165309), which is highly polymorphic and localizes to 16p13.3, ~1.2 Mb from 16pter (44). MS205, which has been used to explore the origin of human populations (45), is ~30 kb from the γI-tryptase gene.

BAC 48, which contains the γII-tryptase gene, also contains an α1H T-type Ca2+ channel gene, based on sequencing of the γII gene itself (see below) and of the BAC insert’s SP6 end (see Fig. 4), which contains part of a late CACNAIH-like exon in the sequenced portion. This finding strengthens the finding of homology between the two BACs based on restriction mapping and alignment of tryptase genes. BAC 48’s T7 end extends beyond the area of homology with BAC 324. The sequence of the T7 end is almost identical with that of fragment 5 of a partially sequenced contig (GenBank AL031711.16), which encodes three tryptase-like genes, MP-2, MP-7, and mastin, as shown in Fig. 4. All three genes have multiple flaws, e.g., premature stop codons, faulty intron-exon junctions (not shown). None have closely related ESTs. Thus, they are likely to be pseudogenes. The mastin gene, whose exons are most closely related to the tryptase-like gene mastin characterized in dogs, is discussed in more detail below.

γ-tryptase gene sequence and organization

The BAC 324 and 48 γ-tryptase genes, termed γI- and γII-tryptase, respectively, were fully sequenced to reveal their relationship to the cDNAs in EST databases and in DNA amplified by PCR. The GenBank accession numbers of the γI and γII genes are AF191031 and AF195508, respectively. In these genes, intron-exon splice junctions were identified using open reading frames and cDNA alignments by application of the “GT . . . AG” rule for initiating and ending introns and by referring to patterns of intron phase and placement in known tryptases and in other serine protease genes, as described (23). As revealed in Fig. 5, we find that fragment 12 of the unfinished chromosome 16-derived contig AL031715.1 deposited in the HTGS database contains the partial sequence.
The gene sequence predicted from portions of the genome sequence deposited in GenBank by other investigators. These findings indicate that the gene is flanked by a b-tryptase gene and from blotting of full genomic DNA is that there is one genomic site with multiple alleles. As shown in Fig. 6 and discussed in the accompanying legend, the organization of yII introns and exons more closely resembles that of the human prostasin gene (46) than of a/b-tryptases, which are more compact genes featuring only five protein-coding exons, with exon 1 consisting entirely of the 5’ untranslated region. Prostasin and the yI-tryptase genes each distribute DNA encoding the prepropeptide and yI-T-type Ca2+ channel gene. These loci appear to have arisen from duplications of portions of chromosome 16.

FIGURE 4. Map and alignment of tryptase loci on chromosome 16p13.3. y-Tryptase genes I and II localize to homologous 16p13.3 loci. These loci are aligned here based on similarities of restriction maps generated using HindIII (H), EcoRI (E), and NolI (N), as well as on similarities in sequenced portions of each locus. The map of each locus is primarily based on characterization of BAC 324 and 48, as shown. Portions of each BAC were subcloned as HindIII restriction fragments, as indicated by the lambda clones below each BAC. The sequenced ends of each BAC were used to generate probes, which reinforced the alignment. Each locus was extended by overlap with portions of chromosome 16 sequenced by other groups. The GenBank accession and version numbers of these sequences (e.g., AL031703.9) are given. Where aligned sequence corresponds to fragments of unfinished contigs, the fragment number is indicated in parentheses. Bold lines indicate portions of the BAC that were sequenced. Arrows indicate the predicted direction of gene transcription and the approximate extent of the gene. The thin lines on the sides of the aII and bII genes indicate the extent of sequenced flanking regions. Dotted lines indicate the gene sequence predicted from portions of the genome sequence deposited in GenBank by other investigators. These findings indicate that the yI-tryptase gene is flanked by a bI-tryptase gene and an aI-II T-type Ca2+ channel gene and is within 30 kb of the highly polymorphic minisatellite, MS205. The yII gene is flanked by a bIII-tryptase gene and a aI-II T-type Ca2+ channel gene. These loci appear to have arisen from duplications of portions of chromosome 16.

FIGURE 5. Detection of y-tryptase genes by genomic DNA blotting. BAC 48, BAC 324, and full human genomic DNA were digested with HindIII, electrophoresed in agarose, blotted, then hybridized with a radiolabeled, 250-bp probe corresponding to a portion of exon 6 on the 3’ side of the NolI site (see Fig. 4). As shown by the autoradiogram on the left side of the figure, BACs 48 and 324 yield bands of ~9 kb and 18 kb, respectively, as predicted by the restriction maps in Fig. 4. The right side of the figure shows autoradiograms of similarly blotted and probed genomic DNA from pooled human kidney (Clontech) and peripheral blood leukocytes of three unrelated individuals in lanes 1–4, respectively. DNA from pooled kidney and from one individual yields two hybridizing bands (~9 and 18 kb). DNA from two of the individuals yields only the 18-kb band. These data suggest that full genomic DNA contains genes corresponding to both yI- and yII-tryptase in some, but not all, individuals and supports the hypothesis that yI and yII are allelic variants of the same gene.
Human mastin gene organization, localization, and amino acid sequence

The sequence of a putative human homologue (not shown) of dog mastin was predicted from the GenBank-derived genomic sequence. Intron splice junctions were predicted by reference to dog mastin cDNA (27) and patterns of intron phase and placement in known tryptase genes (22, 23) by examination of open reading frames and by application of “GT...AG” rules for intron ends, as noted above in connection with γ-tryptase genes. As shown in Fig. 4, genomic sequences more homologous to dog mastin than to any other known cDNA were identified in fragment 5 of partially sequenced contig AL031711.16, which overlaps extensively with BAC 48’s T7 end. However, no closely related human ESTs were found. The gene itself is flawed by faulty splice junctions, by mutation of Ser to Asn (which would lead to catalytically inactive protein if the gene were expressed), and intronic insertions of Alu repetitive sequences. Thus, this mastin-homologous gene is a pseudogene, which may explain the absence of human ESTs and the failure of polyclonal antisera raised against dog mastin (28) to identify homologues in human tissues and cell lines (Ref. 29 and our unpublished results). Two additional apparent tryptase pseudogenes (MP-2 and MP-7; see Fig. 4) lie in the same fragment of AL031711.16. BAC 48’s T7 end terminates at a HindIII site in a minisatellite in the middle of MP-2 pseudogene intronic sequence. Thus, both BACs and their flanks are rich in tryptase-like genes and pseudogenes. Based on the sequence acquired so far, these regions of chromosome 16 are also richly endowed with various repeats, at least one of which, MS205, is highly polymorphic. DNA instability in the neighborhood of these repeats may contribute to general instability in the region, facilitating duplications, gene conversion events (23), and proliferation of pseudogenes.

γ-Tryptase protein expression

Polyclonal Abs raised against a portion of the γ-tryptase catalytic domain sequence recognize a protein of ~31 kDa in extracts of HMC-1 5C6 cells, as shown in Fig. 7. This band is distinct from those of β-tryptases based on its smaller apparent size, narrower banding pattern, and lack of reactivity with antisera raised against purified human lung mast cell tryptase (not shown). These results suggest that one or more γ-tryptases are expressed in 5C6 cells and predict that mast cells are a source of this enzyme. The detection of γ-tryptase mRNA in a variety of tissues by PCR, but not by conventional mRNA blotting, is consistent with the expression of γ-tryptase in a subpopulation of cells found in many tissues, such as mast cells. No difference in migration of the immunoreactive band was found in the presence or absence of a reducing agent, suggesting the absence of disulfide-linked oligomerization characteristic of some tryptase-like enzymes, such as mastin (29). The lack of a reduction in size in the presence of reducing agents suggests that the predicted disulfide-linked propeptide remnant is absent in the mature enzyme or that it is too small to produce detectable differences in electrophoretic migration between reduced and unreduced samples.

FIGURE 6. Comparison of gene structures. A, A comparison of structures of γ-tryptase, prostatin, and α/β-tryptase genes. B, A reduced-scale comparison of A showing the relationship between BAC 324 βIII and γII genes, which have the same transcriptional orientation and are ~2.5 kb apart. Exons of γ-tryptase, prostatin, and α/β-tryptase genes are numbered and indicated by boxes (untranslated; prepro; catalytic domain; transmembrane tail). Lines represent introns and flanking regions. The location of codons encoding the His (H), Asp (D), and Ser (S) catalytic triad residues common to serine proteases are indicated. Each intron is labeled with its phase (0, I, or II). Precise lengths of γ-tryptase gene untranslated regions are not yet determined. Note the similarity in phase and placement of introns in γ-tryptase and prostatin genes, both of which contain a prepro sequence divided among three exons. The γ-tryptase and prostatin genes also both contain an extended 3′ open reading frame, which encodes a putative transmembrane segment and small cytoplasmic tail. α/β-Tryptase genes (e.g., β, βIII, αII) differ in that the 5′ untranslated region is separated from the first protein-coding exon by an intron, the prepro sequence is divided among just two exons, and there is no C-terminal transmembrane tail. These findings suggest a close evolutionary relationship between the γ-tryptase and prostatin genes.
As shown in Fig. 8, our polyclonal anti-γ-tryptase Abs recognize scattered, granulated mononuclear cells in human airway submucosa. The distribution and appearance of these cells is typical of tissue mast cells, which is consistent with the identification of immunoreactive γ-tryptase in the 5C6 subclone of the HMC-1 line of mast cells. The granular pattern of staining suggests the possibility that immunoreactive γ-tryptase resides in secretory granules.

γ-Tryptase homology model

Models of the γ-tryptase catalytic domain based on the established structure of its closest crystallized relative, βII-tryptase, are depicted in Fig. 9. These models reveal that the segment Cys\(^{192}\), Pro\(^{206}\), which is among the most distinctive compared with otherwise similar relatives (see Fig. 1), forms a predicted surface loop that is far enough removed from the classic serine protease substrate binding site that it is unlikely to interact directly with polypeptide targets in the vicinity of the scissile bond. Because this is the peptide segment against which antisera were raised, it is unlikely that our anti-γ-tryptase Ig will block access to potential substrate substrates. The models also show that the sole predicted N-glycosylation site is closer to the catalytic active site. However, this glycosylation site, which is not conserved in α/β-tryptases, prostatasin, or testisin, lies outside of the classic serine protease-extended peptide binding site. Carbohydrate attached at this site could interfere with bulky protein substrates but probably not with smaller peptide substrates. The models also predict that the two cysteines (145 and 154) that we are unable to pair with other cysteines based on the βII-tryptase crystal structure are not close enough to form a disulfide linkage with each other. Both lie on predicted surface loops and could potentially form intersubunit bonds with other catalytic subunits. However, there is no evidence of this in HMC-1 immunoblots. As noted above, we predict that Cys\(^{145}\) (Cys\(^{122}\) in chymotrypsinogen) may remain linked to cleaved remnants of propeptide by analogy to chymotrypsin. The model also predicts that the putative membrane-anchored C-terminal hydrophobic segment attaches to the catalytic domain’s back side, where it is less likely to interfere with substrate access to the active site.

Relation of γ-tryptases to transmembrane tryptase

While this manuscript was under review, Stevens and colleagues published a report of a similar gene, termed transmembrane tryptase (47). Their gene contains one fewer exon and is ~0.8 kb shorter than our γ genes. However, the deduced amino acid sequence of transmembrane and γ-tryptase catalytic domains is 98–99% identical, suggesting that the enzymes may be allelic variants of each other. Partial characterization by Stevens and colleagues of a gene locus containing a mouse homologue closely related to that predicted from our murine ESTs suggests that the mouse gene has a relationship to the neighboring mouse tryptase mMCP-6 gene that is similar to the relationship between the human γII and βIII tryptase genes we describe here. Interestingly, Abs raised against transmembrane tryptase recognize mast cells in human skin and intestine, thereby supporting our hypothesis that γ-tryptase is expressed in mast cells based on our identification of immunoreactive protein in human airway cells and in a mast cell line. If, like other human tryptases, γ-tryptases are expressed in most mast cells, then γ-tryptase expression in tissues will be widespread. The immunological significance of γ-tryptase expression by mast cells is not yet clear and awaits further characterization of this novel gene product’s biogenesis and physical and enzymological properties. If γ-tryptases, like mast cell β-tryptases, are activated intracellularly, stored in secretory granules, and secreted in response to Ag-bound IgE, they may aggravate the local pathology of allergic inflammation by hydrolyzing proteins in the vicinity of degranulated mast cells. In this regard, if γ-tryptases remain membrane-anchored after activation and release, they may remain attached to the cell surface, thus ensuring that proteolytic effects
FIGURE 9. Molecular models of \( \gamma \)-tryptase. A homology model of human \( \gamma \) catalytic domain was constructed using Swiss-Model. Coordinates of human \( \beta \)III tryptase (1AOL) (34) served as template for the model. Images were generated using RasMac (version 2.6-ucb.1, available at http://mc2.chem.berkeley.edu/rasmol/v2/2.6/). The top pair of ribbon models begin with Ile \( ^{38} \) and end in Ser \( ^{272} \) at the C-terminus. The N and C termini are colored orange. The active site faces front in the left panel views, with the catalytic serine colored red in space-filling mode. The right panels show the back of the protease rotated 180° on a vertical axis compared with the front views. For emphasis, side chains are shown for Leu \( ^{173} \)-Tyr \( ^{177} \) (involved in oligomerization in \( \alpha \)/\( \beta \)-tryptases; colored green), Cys \( ^{192} \)-Pro \( ^{106} \) (the peptide against which Abs were raised; colored blue), Asn \( ^{85} \) (a consensus N-glycosylation site; cyan), and cysteines 145 and 154 (neither of which is found in \( \alpha \)/\( \beta \)-tryptases; yellow). The same color scheme applies to the space-filling models in the lower half of the figure. The predicted \( \gamma \)-tryptase C-terminal hydrophobic tail is not present in \( \beta \)-tryptase and therefore is not modeled here. Note that it is expected to extend from the back of the protease, allowing the active site to face away from the membrane. In the front view, the putative substrate binding site extends roughly vertically above and below the active site Ser \( ^{222} \).


