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Human Tryptase ϵ (PRSS22), a New Member of the Chromosome 16p13.3 Family of Human Serine Proteases Expressed in Airway Epithelial Cells*

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Probing of the GenBank™ expressed sequence tag (EST) data base with varied human tryptase cDNAs identified two truncated ESTs that subsequently were found to encode overlapping portions of a novel human serine protease (designated tryptase ϵ or protease, serine S1 family member 22 (PRSS22)). The tryptase ϵ gene resides on chromosome 16p13.3 within a 2.5-Mb complex of serine protease genes. Although at least 7 of the 14 genes in this complex encode enzymatically active proteases, only one tryptase ϵ-like gene was identified. The trachea and esophagus were found to contain the highest steady-state levels of the tryptase ϵ transcript in adult humans. Although the tryptase ϵ transcript was scarce in adult human lung, it was present in abundance in fetal lung. Thus, the tryptase ϵ gene is expressed in the airways in a developmentally regulated manner that is different from that of other human tryptase genes. At the cellular level, tryptase ϵ is a major product of normal pulmonary epithelial cells, as well as varied transformed epithelial cell lines. Enzymatically active tryptase ϵ is also constitutively secreted from these cells. The amino acid sequence of human tryptase ϵ is 38–44% identical to those of human tryptase α, tryptase βI, tryptase βII, tryptase βIII, transmembrane tryptase/tryptase γ, marapsin, and Esp-1/testisin. Nevertheless, comparative protein structure modeling and functional studies using recombinant material revealed that tryptase ϵ has a substrate preference distinct from that of its other family members. These data indicate that the products of the chromosome 16p13.3 complex of tryptase genes evolved to carry out varied functions in humans.

Serine proteases play important roles in embryonic development, immunity, blood coagulation, fertilization, fibrinolysis, hormone activation, food digestion, and other biological processes. Six gene clusters have been identified in the human genome that contain four or more serine protease genes at chromosomes 6q26–27 (1), 7q35 (2), 14q11.2 (3, 4), 16p13.3 (5–7), 19p13.3 (8, 9), and 19q13.4 (10). The protease gene cluster at the chromosome 16p13.3 locus contains the genes that encode human tryptase α, tryptase βI, tryptase βII, tryptase βIII, transmembrane tryptase (TMT)[1]/tryptase γ, marapsin,[2] and eosinophil serine protease-1 (Esp-1)/testisin/PRSS21 (5–7, 11–16). Caughey and co-workers (5) concluded that human tryptase βII and βIII probably are allelic variants of the same gene. Thus, the human chromosome 16p13.3 locus contains at least six distinct genes that encode enzymatically active serine proteases. The corresponding mouse tryptase locus resides at chromosome 17A3.3-B1 and contains the genes that encode mMCP-6, mMCP-7, mTMT, and tryptase 4 (mT4)/Prss21 (6, 17–21).

hEsp-1 is expressed predominantly by premeiotic germ cells in the testis, but its expression at the mRNA level is down-regulated in testicular tumors (16). Eosinophils also contain low levels of this tryptic-like transcript (14). The mast cells (MCs) that reside in varied human connective tissues express varied combinations of tryptases α, βI, βII, βIII, and TMT (6, 11–13, 22). hTMT is also expressed in varied tumor cell lines and in the intestine (6). Human tryptases α, βI, βII, and βIII, and mouse tryptases mMCP-6 and mMCP-7 are stored in the MC secretory granules as tetramers ionically bound to serglycin proteoglycans (23–27). It has been concluded that the tetramer unit evolved to physically restrict the substrate specificities of these six serine proteases, to prevent their rapid entrapment and inactivation by α macroglobulins, and/or to vary the extent a protein is degraded by the different combination of tryptase monomers.

Using comparative protein structure modeling based on the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AAG35070 and AF321182.

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[1] The abbreviations used are: TMT, transmembrane tryptase; hTMT, human TMT; EK, enterokinase; Esp-1, eosinophil serine protease-1; EST, expressed sequence tag; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MC, mast cell; MCP, mast cell protease; mMCP, mouse MCP; *p*NA, *p*-nitroanilide; SPL, serine protease-like; TBS, Tris-buffered saline; and UTR, untranslated region.

[2] Although the human serine protease designated as marapsin had not been described in any scientific publication at the time of submission of this tryptase ϵ manuscript, the nucleotide sequence of its cDNA was released at GenBank™ by Fortunato, Dando, Rawlings, and Barrett on March 31, 2001.

crystallographic structure of pancreatic trypsin, Johnson and Barton (28) first noted that tetramer-forming tryptases have conserved Pro-, Tyr-, and Trp-rich domains on their surfaces. Analysis of its crystallographic structure revealed that the four monomers of a homotypic complex of human tryptase βII physically interact with one another via their Pro- and Tyr-rich domains (29). hTMT and hEsp-1 lack the conserved Pro- and Tyr-rich domains. Instead, they contain membrane-spanning domains at their C termini. Thus, these two members of the chromosome 16p13.3 family of serine proteases are unable to form the tetramer unit. Despite the lack of conservation of the Pro, Tyr, and membrane-spanning domains, all proteases encoded by the genes residing at chromosome 16p13.3 contain the Trp-rich domain. Expression/site-directed mutagenesis studies carried out on recombinant mMCP-7 revealed that the Trp domain is required for the conversion of the inactive zymogen into an enzymatically active tryptase (27). The presence of the Trp-rich domain in all mouse and human tryptases indicates the development >100 million years ago of a mechanism that is still used today in mammals to activate the varied members of the tryptase superfamily.

Tryptase-specific cDNAs and/or antibodies have been invaluable reagents for identifying MCs in tissues, for understanding MC development and fate, and for evaluating the consequences of MC activation in normal and diseased states (22, 26, 30–39). Nevertheless, because enzymatically active recombinant tryptases have not been generated until recently (27, 40–43), the physiologic and pathologic roles of these serine proteases are just beginning to be elucidated. We generated recombinant human tryptases α, βI, and βII, and recombinant mouse tryptases mMCP-6, mMCP-7, and mT4, to address the substrate specificity and function of these homologous serine proteases (21, 40–43). Even though the primary amino acid sequences of tryptases α and βII are 93% identical, it is now apparent that these two human MC proteases are functionally distinct due, in part, to a single amino acid difference in one of the seven loops that form their substrate-binding clefts (42). Administration of recombinant human tryptase βI or mMCP-6, but not human tryptase α, confers protective immunity to MC-deficient W/W^v mice during a *Klebsiella pneumoniae* infection of the lung (43). This effect is mediated, in part, by the ability of human tryptase βI and mMCP-6 to induce the selective extravasation of neutrophils into the bacteria-infected lungs. mMCP-7 regulates the extravasation of eosinophils (43). Because the α chain of fibrinogen is a physiologic substrate of mMCP-7 (40), this tryptase also appears to play a role in the prevention of fibrin/platelet clots in inflammatory sites.

Because of the potential roles of the MC proteases in asthma and other inflammatory diseases, low molecular weight inhibitors have been generated by pharmaceutical industries in an attempt to inactivate what was previously thought to be only one functional tryptase in the lungs of asthma patients. The first generation of tryptase inhibitors turned out to be nonspecific. For example, the most studied low molecular weight tryptase inhibitor, APC-366, actually inhibits pancreatic trypsin better than a human lung MC tryptase (44). The ability to create a second generation of more specific tryptase inhibitors is highly dependent on our knowledge of how many tryptase-like genes exist in humans and where and when these genes are expressed. This knowledge becomes attainable, in part, with the generation of the draft sequence of the human genome. We now describe the isolation, characterization, expression, and substrate specificity of the seventh functional member of the human chromosome 16p13.3 family of serine proteases. Our finding that human tryptase ε/PRSS22[3] is abundantly expressed in epithelial cells, in the adult trachea and esophagus, and in fetal lung suggests that this new human tryptase plays an important role in the airways.

EXPERIMENTAL PROCEDURES

Cloning of the Human Tryptase ε cDNA—The nucleotide sequences of the human tryptase βI and TMT transcripts were used as templates to search for novel but related human tryptase-like ESTs in the GenBank™ data base. Sequence analyses of EST clones BE907706 and AI249364 (derived from the human pancreas) revealed that their inserts correspond to overlapping portions of a new serine protease cDNA. Based on the deduced nucleotide sequences of the two truncated clones, a PCR approach was carried out on a pool of adult pancreas cDNAs and a pool of fetal kidney cDNAs (CLONTECH, Palo Alto, CA) to isolate full-length human tryptase ε cDNAs. The oligonucleotides used in these PCRs were 5′-CCAGCCATGGTGGTTTCTGGAGCG-3′ and 5′-CTGGCCGCCTTTCAGATCCGAGC-3′. The resulting products were purified on 1% agarose gels and subcloned into pCR2.1 (Invitrogen, Carlsbad, CA), and the inserts in four arbitrarily selected clones were sequenced.

Expression of Human Tryptase ε at the mRNA Level—To identify which human tissues contain the highest levels of tryptase ε mRNA, multi-tissue blots (CLONTECH) containing ~2 μg of poly(A)$^+$ RNA from varied tissues were probed under conditions of high stringency with a radiolabeled 653-bp probe corresponding to nucleotides 431–1083 in the human tryptase ε cDNA. After the probe was random-primed with [α-^{32}P]dCTP using the *Redi*prime kit (Amersham Biosciences, Inc.), it was hybridized to each RNA blot at 65 °C for 2 h in QuickHyb solution. Each blot was washed twice at room temperature for 20 min in 2× SSC containing 0.1% SDS and then twice at 65 °C for 20 min in 0.2× SSC containing 0.1% SDS before being exposed to BIOMAX film for 5–7 days. After the bound probe was removed, the blot was reprobed with a β-actin cDNA (CLONTECH) to evaluate the amount of RNA loaded in each lane.

A PCR approach also was used to screen human transformed cell lines, tumor xenografts (CLONTECH), primary human airway epithelial cells (Clonetics, San Diego, CA), and primary microvascular endothelial cells (Clonetics) for the presence of human tryptase ε mRNA. The oligonucleotides 5′-CCAGCCATGGTGGTTTCTGGAGCG-3′ and 5′-GAGCCCTGGCTCGGTGCCCTGAG-3′ were used to generate the relevant 938-bp fragment. The obtained products were analyzed by gel electrophoresis, gel-purified, and sequenced to confirm the identity of the PCR products. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH)-derived oligonucleotides (CLONTECH) were used as a positive control in these transcript studies.

Analysis of the Serine Protease Gene Cluster on Human Chromosome 16p13.3—The nucleotide sequences of the cDNAs that encode tryptase βI, βII, and α, TMT/tryptase γ, Esp-1/testisin, marapsin, and tryptase ε were aligned against the Human Genome Project's sequence of chromosome 16p13.3 using the "blastn" algorithm available at the National Center for Biotechnology Information Web site. Because human tryptases βI and βII are 98% identical (12, 13) and because Ricke and coworkers were unable to identify the first two exons of the human tryptase ε gene in their analysis of the chromosome 16-derived cosmid clone 325D7 (GenBank™ accession no. AC003965), multiple tryptase ε-like genes could reside at chromosome 16p13.3. Thus, the draft sequence of the entire human chromosome 16 was searched using the translated "tblastn" algorithm for additional serine protease-like genes that encode peptide sequences flanking the catalytic triad His, Asp, and Ser residues in all functional serine proteases (*i.e.* WVLTAAHC, DIAL, or GDSGGPL; the residues forming the catalytic triad are underlined).

Protein Structure Modeling of Human Tryptase ε—A three-dimensional model of residues −10 to +243 of human tryptase ε was built by MODELLER (45–47) as described for other mouse and human tryptases (6, 21, 25–27). The templates used in the comparative modeling were the crystallographic structures of human tryptase βII (Protein Data Bank code 1A0L) (29) and the proenzyme domain of human plasminogen (Protein Data Bank code 1QRZ) (48). Within the modeled regions, the amino acid sequence identities are 37 and 38%, respectively.

[3] The nucleotide and amino acid sequences of our human tryptase ε/PRSS22 cDNA (GenBank™ accession no. AF321182) were released to the public on November 28, 2000. Although the gene is known as *PRSS22* in the current draft of the human genome, the HUGO Gene Nomenclature Committee has indicated that it might be redesignated as *PRSS26*.

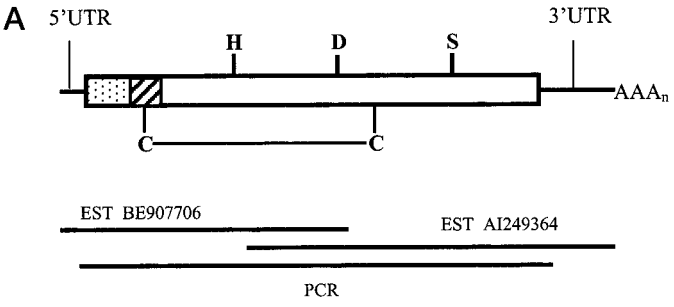

FIG. 1. **Cloning of human tryptase ϵ cDNAs.** *A,* a computer search of the GenBank™ EST data base resulted in the identification of two truncated ESTs that encode the indicated portions of the human tryptase ϵ transcript. Using this information, a PCR approach was carried out on adult pancreas and fetal kidney cDNAs to isolate multiple cDNAs that correspond to the entire coding region of human tryptase ϵ. The diagram highlights the putative 5′-UTR, hydrophobic leader/signal peptide (*stippled area*), propeptide (*cross-hatched area*), the mature catalytic domain with its active-site triad amino acids His, Asp, and Ser (denoted as *H, D*, and *S*), 3′-UTR, poly(A) tail, and the Cys^{-9}–Cys^{112} disulfide bond that links the propeptide and catalytic domain. *B,* the nucleotide and amino acid sequences of the human tryptase ϵ transcript were deduced. The *N*-linked glycosylation site (Asn^{21}, *circled*), components of the catalytic triad (*solid boxes*), translation-initiation site (*), translation-termination site (−), polyadenylation regulatory sequence (*underlined*), hydrophobic signal peptide (*single bracket*), and propeptide (*double bracket*) are indicated. Nucleotide numbering begins at the 5′-UTR of the isolated cDNA; amino acid *numbering* (*bracketed* at *left*) begins with the mature protein.

B

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   1                    *                cgcatcgccctgggtctctcgagcctgctgcctgct
[-49]                     M  V  V  S  G  A  P  P  A  L  G  G  G  C  L  G  T  F
  37  cccccgccccaccagccatggtggtttctggagcgcccccagccctgggtgggggctgtctcggcaccttca

[-31]  T  S  L  L  L  L  A  S  T  A  I  L  N  A  A  R  I  P  V  P  P  A  C  G
 109  cctccctgctgctgctggcgtcgacagccatcctcaatgcggccaggatacctgttcccccagcctgtggga

[ -7]  K  P  Q  Q  L  N  R  V  V  G  G  E  D  S  T  D  S  E  W  P  W  I  V  S
 181  agccccagcagctgaaccgggttgtgggcggcgaggacagcactgacagcgagtggccctggatcgtgagca

[ 18]  I  Q  K  (N) G  T  H  H  C  A  G  S  L  L  T  S  R  W  V  I  T  A  A  H
 253  tccagaagaatgggacccaccactgcgcaggttctctgctcaccagccgctgggtgatcactgctgcccact

[ 42]  C  F  K  D  N  L  N  K  P  Y  L  F  S  V  L  L  G  A  W  Q  L  G  N  P
 325  gtttcaaggacaacctgaacaaaccatacctgttctctgtgctgctgggggcctggcagctggggaaccctg

[ 66]  G  S  R  S  Q  K  V  G  V  A  W  V  E  P  H  P  V  Y  S  W  K  E  G  A
 397  gctctcggtcccagaaggtgggtgttgcctgggtggagccccaccctgtgttatcctggaaggaaggtgcct

[ 90]  C  A  D  I  A  L  V  R  L  E  R  S  I  Q  F  S  E  R  V  L  P  I  C  L
 469  gtgcagacattgccctggtgcgtctcgagcgctccatacagttctcagagcgggtcctgcccatctgcctac

[114]  P  D  A  S  I  H  L  P  P  N  T  H  C  W  I  S  G  W  G  S  I  Q  D  G
 541  ctgatgcctctatccacctccctccaaacacccactgctggatctcaggctgggggagcatccaagatggag

[138]  V  P  L  P  H  P  Q  T  L  Q  K  L  K  V  P  I  I  D  S  E  V  C  S  H
 613  ttcccttgccccaccctcagaccctgcagaagctgaaggttcctatcatcgactcggaagtctgcagccatc

[162]  L  Y  W  R  G  A  G  Q  G  P  I  T  E  D  M  L  C  A  G  Y  L  E  G  E
 685  tgtactggcggggagcaggacagggacccatcactgaggacatgctgtgtgccggctacttggaggggagc

[186]  R  D  A  C  L  G  D  S  G  G  P  L  M  C  Q  V  D  G  A  W  L  L  A  G
 757  gggatgcttgtctgggcgactccgggggccccctcatgtgccaggtggacggcgcctggctgctggccggca

[210]  I  I  S  W  G  E  G  C  A  E  R  N  R  P  G  V  Y  I  S  L  S  A  H  R
 829  tcatcagctggggcgagggctgtgccgagcgcaacaggcccggggtctacatcagcctctctgcgcaccgct

[234]  S  W  V  E  K  I  V  Q  G  V  Q  L  R  G  R  A  Q  G  G  G  A  L  R  A
 901  cctgggtggagaagatcgtgcaaggggtgcagctccgcgggcgcgctcaggggggtggggccctcagggcac

[258]  P  S  Q  G  S  G  A  A  A  R  S  -
 973  cgagccagggctctggggccgccgcgctcctagggcgcagcgggacgcggggctcggatctgaaaggcgg
1045  ccagatccacatctggatctggatctgcggcggcctcgggcggtttccccgccgtaaataggctcatctac
1117  ctctacctctgggggcccggacggctgctgcggaaaggaaaccccctccccgacccgcccgacggcctcagg
1189  ccccgcctccaaggcatcaggccccgcccaacggcctcatgtccccgcccccacgacttccggccccgcccc
1261  gggccccagcgcttttgtgtatataaatgttaatgatttttataggtatttgtaaccctgcccacatatctt
1333  atttattcctccaatttcaataaattatttattctcaaaaaaaaaaaaaa
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Generation of a Tryptase ϵ-specific Antibody and Use of the Antibody to Evaluate the Expression of Human Tryptase ϵ at the Protein Level—The anti-peptide approach we used previously to obtain mMCP-6 (26)-, mMCP-7 (35)-, and hTMT (6)-specific antibodies in rabbits was used to generate antibodies that recognize human tryptase ϵ in SDS-PAGE/immunoblot studies. The 17-mer peptide Glu-Asp-Ser-Thr-Asp-Ser-Glu-Trp-Pro-Trp-Ile-Val-Ser-Ile-Gln-Lys-Asn corresponding to residues 5–21 in mature tryptase ϵ is not present in any protein in the GenBank™ protein data base. The model of the three-dimensional structure of tryptase ϵ predicts that residues 5–21 protrude from the surface of the folded tryptase. Thus, antibodies were raised in rabbits against the synthetic peptide by Genemed Synthesis (San Francisco); the resulting antibodies were purified using a peptide affinity column.

Recombinant human tryptases ϵ, α, βI, and TMT were used in preliminary control studies to evaluate the specificity of the anti-tryptase ϵ antibodies. H358 cells contain tryptase ϵ mRNA but not SW579 cells. Thus, to evaluate whether tryptase ϵ is translated and constitutively exocytosed, the two epithelial cell lines were cultured in 10 ml of serum-free Opti-MEM I culture medium (Life Technologies, Inc.) for 24–48 h. The resulting conditioned medium was placed in 7-kDa cut-off Slide-A-Lyzer dialysis cassettes (Pierce). After an overnight dialysis against 0.1 M ammonium bicarbonate, each dialysate was freeze-dried and reconstituted in 0.5 ml of phosphate-buffered saline. An aliquot of the reconstituted sample was boiled in SDS sample buffer containing or lacking β-mercaptoethanol. After electrophoresis, the resolved proteins were blotted onto polyvinylidene difluoride membranes (Bio-Rad). Each protein blot was exposed to Tris-buffered saline (TBS; 15 ml) containing 5% nonfat milk, 0.1% Tween 20, 0.5% goat serum, and 1 μg/ml of rabbit anti-tryptase ϵ antibody for ~17 h at room temperature. After the blot was washed three times with TBS containing 0.1% Tween 20, it was exposed to TBS (15 ml) containing 5% nonfat milk, 0.1% Tween 20, 0.5% goat serum, and a 1000-fold dilution of a stock solution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) for 1 h at room temperature. The immunoreactive proteins were then visualized using a chemiluminescence kit (Geno Technologies, St. Louis, MO) and BioMax MR film (Eastman Kodak).

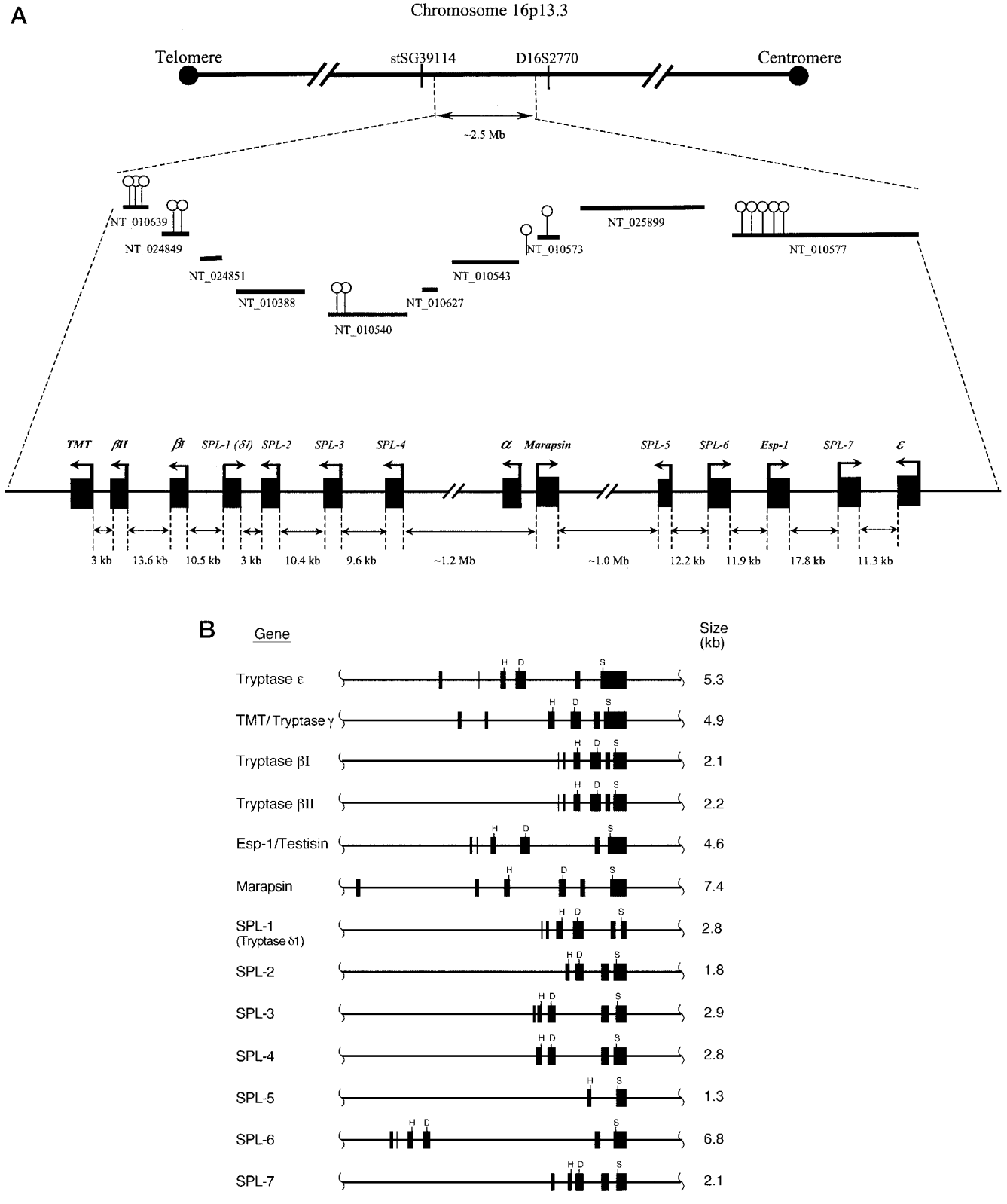


FIG. 2. **Location of the tryptase ϵ gene on human chromosome 16p13.3.** *A,* diagram of human chromosome 16p13.3 spanning the sequence tag sites stSG39114 and D16S2770. All nonoverlapping contigs that span the ~2.5-Mb serine protease cluster are shown. A *circle-with-a-stick* denotes the presence of a serine protease/serine protease-like gene on the contig. Shown are the relative positions and spacing of the seven genes (*bold italics*) that encode the enzymatically active serine proteases within the cluster as well as the seven additional SPL genes (*italics*) that are likely to encode enzymatically inactive proteins. The *SPL-1* gene was initially designated by Pallaoro and co-workers (5) as the "mMCP-7-like human" gene; it was then designated as the tryptase δ1 gene. The tryptase ϵ gene resides on NT 010577. The exact position of the tryptase α gene remains to be determined but it probably resides between the *SPL-4* and marapsin genes. In each instance, the *single arrow above* the gene denotes its orientation in the chromosome. Only the serine protease genes and their related family members are shown in this region of chromosome 16p13.3. *B,* comparison of the exon/intron organization of the human *tryptase ϵ*, *tryptase βI*, *tryptase βII*, *Esp-1*, *marapsin*, and seven *SPL* genes. *Boxes* indicate exons. The size of each gene is indicated on the *right*. *H*, *D*, and *S* refer to positions of the codons that encode the catalytic triad amino acids in each serine protease gene.

TABLE I
Evaluation of serine protease-like (SPL) genes 1–7 at human chromosome 16p13.3

Seven genes are present at chromosome 16p13.3 that encode enzymatically active serine proteases. While seven additional genes are present at this locus, SPL-1 to SPL-7 are predicted to encode serine protease-like proteins that are enzymatically inactive. Noted are some of the properties of the latter genes and their products if these genes are transcribed and their transcripts are translated. RACE, rapid amplification of cDNA ends.

Serine protease-like gene	Contig	EST	Tissue source	Note
SPL-1 (tryptase δ1)	NT 024849	None	None	Premature stop codon in exon 5, resulting in the deletion of loop 2 that forms a portion of the substrate binding cleft
SPL-2	NT 024849	None	None	Stop codon in exon 3 shortly after the catalytic Asp residue
SPL-3	NT 010540	None	None	Stop codon in exon 4 shortly after the catalytic Asp residue
SPL-4	NT 010540	None	None	Stop codon in exon 2 shortly after the catalytic His residue
SPL-5	NT 010577	BG762809	Cutaneous melanoma	Contains only two exons; one of the missing exons encodes the catalytic Asp residue
SPL-6	NT 010577	AW243584	Pancreas Adenocarcinoma	No "IVGG" in exon 3, the indicated EST also contains intron 4
SPL-7	NT 010577	AA400057	Testis	Defective splicing between exon 3 and exon 4 results in the shift of reading frame in all ESTs, which in turn results in a premature stop codon; prematured stop codons were also found in 5′ RACE products.
		AA400181	Testis	
		AA400107	Testis	
		AA625677	Testis	
		AA904710	Mixed (lung, testis, B-cell)	
		AA400289	Testis	
		AA757002	Testis	
		AA400704	Testis	
		AA400045	Testis	
		AA401517	Testis	
		AA401525	Testis	

As noted below, bioengineered forms of recombinant human tryptase ϵ also were generated that contained either the V5 peptide (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr) or the FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) at their C termini. Goat anti-mouse immunoglobulin G (Bio-Rad) was used as the secondary antibody when SDS-PAGE/immunoblots containing these recombinant proteins were probed with mouse anti-V5 (Invitrogen) or anti-FLAG (Sigma) antibody.

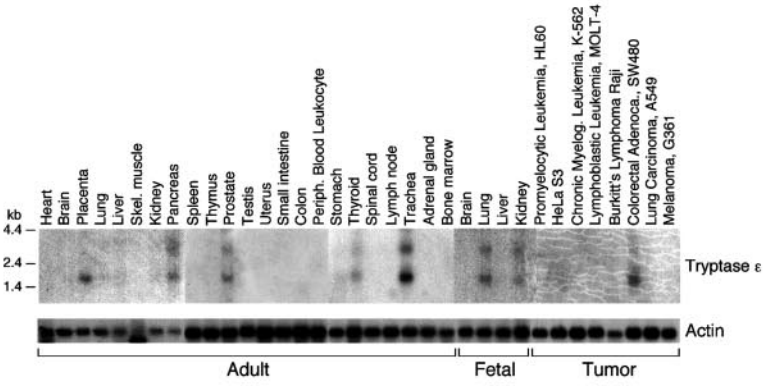
Evaluation of the Substrate Specificities of Naturally Occurring and Recombinant Human Tryptase ϵ—Aliquots of the conditioned media of H358 and SW579 epithelial cells were evaluated for the presence of tryptase-like activity using the chromogenic substrate H-D-Leu-Thr-Arg-*p*NA. More definitive proof that human tryptase ϵ is a functional protease was obtained using recombinant material generated in COS-7 cells and High Five insect cells. For COS-7 cell expression, the entire coding region of the human tryptase ϵ cDNA was placed in the expression vector pcDNA3.1/V5-His-TOPO (Invitrogen). This expression vector was chosen because the resulting product contains the 14-mer V5 peptide at its C terminus, thereby allowing the detection of the recombinant product with anti-V5 antibody. Vector lacking an insert served as a negative control in these transfection experiments. African green monkey, SV40-transformed kidney COS-7 cells (line CRL-1651; American Type Culture Collection (ATCC) Manassas, VA) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Transient transfections were performed with SuperFect (Qiagen, Valencia, CA), according to the manufacturer's instructions. Cells were plated at a density of 2×10^5 cells/well in 6-well plates 24 h prior to transfection. Twenty-four h after transfection, the cells were washed and then cultured in serum-free, Opti-MEM I medium for another 48 h before the conditioned medium and cell pellets were collected. This was done in an attempt to minimize the inactivation of any secreted mature tryptase ϵ by an endogenous protease inhibitor present in the fetal calf serum.

Recombinant tryptase ϵ also was generated in High Five insect cells using the expression system we had developed previously to generate recombinant mouse tryptases mMCP-6, mMCP-7, and mT4 and recombinant human tryptases α, βI, and βII (21, 40–43). Insect cells lack the post-translational processing mechanism needed to remove the propeptide of a tryptase zymogen (25). Thus, the five-residue peptide Asp-Asp-Asp-Asp-Lys was placed in between the natural propeptide of human tryptase ϵ and the first Val residue in its catalytic domain to allow activation of the recombinant protein by enterokinase (EK). The 8-mer FLAG peptide also was placed at the C terminus of tryptase ϵ so that the resulting pseudozymogen could be purified easily from the insect cell-conditioned medium using an anti-FLAG antibody immunoaffinity column. Tryptase ϵ^+ insect cells were cultured at room temperature in serum-free Xpress medium for 6–7 days. The resulting conditioned media were collected and generally ~750 ml were loaded at 4 °C onto a 1-ml column containing anti-FLAG M2 antibody (Sigma). After the immunoaffinity column was washed with 250 ml of TBS (pH. 7.0), 0.1 M glycine-HCl (pH 3.5) buffer was added to elute-bound pro-tryptase ϵ. Ten 1-ml fractions were collected into tubes containing 20 μl of 1 M Tris-HCl, pH 8.0. Samples of the resulting fractions were then analyzed by SDS-PAGE for the presence of Coomassie Blue$^+$ proteins and immunoreactive tryptase ϵ using anti-FLAG antibody (Invitrogen). Pro-tryptase ϵ-enriched fractions were pooled and the protein content estimated using the micro-BCA protein assay reagent kit (Pierce).

Many mouse and human tryptases contain *N*-linked glycans (35, 49, 50), and expression/site directed mutagenesis studies carried out with mMCP-7 have revealed that these glycans are important in the thermal stability of this protease (27). Analysis of the predicted primary amino acid sequence of human tryptase ϵ revealed one potential *N*-linked glycosylation site at Asn^{21}. Thus, to determine whether tryptase ϵ contains an *N*-linked glycan, a sample of the conditioned medium from the transfected COS-7 cells was incubated with PNGase F (New England Biolabs) according to the manufacturer's suggested conditions. The resulting digest was then subjected to SDS-PAGE/immunoblot analysis.

The substrate specificities of naturally occurring human tryptase ϵ, recombinant human tryptase ϵ, and recombinant human tryptase βI were compared using the chromogenic substrates H-D-Leu-Thr-Arg-*p*NA, Boc-Leu-Gly-Arg-*p*NA, Bz-Phe-Val-Arg-*p*NA, H-Gly-Arg-*p*NA, Z-Arg-Arg-*p*NA (Bachem, King of Prussia, PA), and tosyl-Gly-Pro-Arg-*p*NA (Sigma). Recombinant tryptase βI was obtained as described previously (43). Purified insect cell-derived pro-tryptase ϵ was activated as described previously for other recombinant tryptase pseudozymogens by incubating ~30 μg of the purified recombinant protein for 2 h at 37 °C in 10 mM Tris-HCl buffer (pH 5.5) supplemented with 5 mM calcium chloride and ~0.4 units of EK (New England Biolabs). One μl of each chromogenic substrate (50 μg) was then added to 150 μl of 10 mM Tris-HCl buffer (pH 8.0) containing ~6 μg of activated insect cell-derived human tryptase ϵ or human tryptase βI. After a 1-h incubation at 37 °C, the proteolytic activity of the sample was measured as a change in optical density at 405 nm using an enzyme-linked immunosorbent assay plate reader. Buffer containing EK alone or pro-tryptase ϵ alone were used as negative controls. Each enzymatic assay was done in duplicate. In separate dose and kinetic experiments, 1 μl of H-D-Leu-Thr-Arg-*p*NA was added to reaction tubes containing 150 μl of buffer and either 3, 6, or 9 μg of activated tryptase ϵ or 1 μg of pancreatic trypsin (Sigma). Each enzymatic assay was done in triplicate. Conditioned media from H358 and SW579 epithelial cells as well as conditioned media from the COS-7 cell transfectants were analyzed

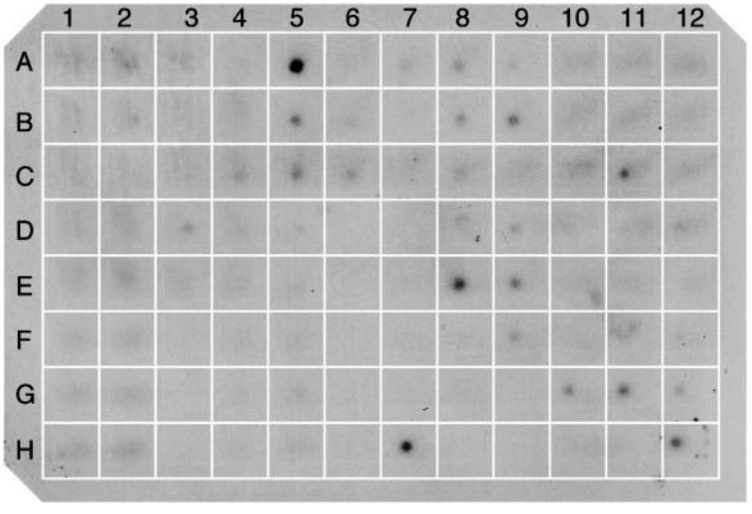
A



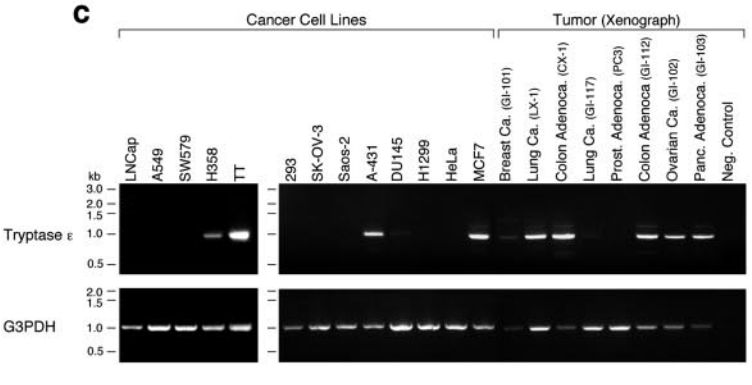
B

	1	2	3	4	5	6	7	8	9	10	11	12
A	whole brain	cerebellum, left	substantia nigra	heart	esophagus	colon, transverse	kidney	lung	liver	leukemia, HL-60	fetal brain	yeast total RNA
B	cerebral cortex	cerebellum, right	accumbens nucleus	aorta	stomach	colon, desending	skeletal muscle	placenta	pancreas	HeLa S3	fetal heart	yeast tRNA
C	frontal lobe	corpus callosum	thalamus	atrium, left	duodenum	rectum	spleen	bladder	adrenal gland	leukemia, K-562	fetal kidney	*E. coli* rRNA
D	parietal lobe	amygdala	pituitary gland	atrium, right	jejunum		thymus	uterus	thyroid gland	leukemia, MOLT-4	fetal liver	*E. coli* DNA
E	occipital lobe	caudate nucleus	spinal cord	ventricle, left	ileum		peripheral blood leukocyte	prostate	salivary gland	Burkitt's lymphoma, Raji	fetal spleen	Poly r(A)
F	temporal lobe	hippo-campus		ventricle, right	ilocecum		lymph node	testis	mammary gland	Burkitt's lymphoma, Daudi	fetal thymus	human C_0t-1 DNA
G	p. g.* of cerebral cortex	medulla oblongata		inter-ventricular septum	appendix		bone morrow	ovary		colorectal adeno-carcinoma, SW480	fetal lung	human DNA 100 ng
H	pons	putamen		apex of the heart	colon, ascending		trachea			lung carcinoma, A549		human DNA 500 ng

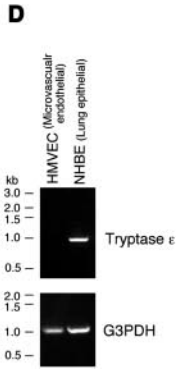
* paracentral gyrus



C



D



in a similar manner. The epithelial and COS-7 cell transfectants were cultured for 24–48 h in serum-free conditioned media. Fifty-μl samples of the resulting conditioned media (COS-7 cells) or concentrated dialysates (epithelial cells) were added to separate reaction wells containing H-D-Leu-Thr-Arg-*p*NA in 50 μl of 10 mM Tris-HCl buffer, and the samples were then incubated for 6–16 h at 37 °C.

RESULTS

Cloning and Sequence Analysis of Human Tryptase ϵ cDNAs and Analysis of the Serine Protease Gene Cluster on Human Chromosome 16p13.3—When the human tryptase βI and TMT cDNAs were used as templates to screen the GenBank™ EST data base, two truncated ESTs were identified that were somewhat homologous with the target cDNAs. The relevant ESTs were obtained from the IMAGE (Integrated Molecular Analysis of Genomes and their Expression) consortium. Further nucleotide sequence analysis of their entire inserts revealed that they encoded different portions of a novel human serine protease (now designated as human tryptase ϵ or PRSS22) (Fig. 1*A*). Based on the sequences of these truncated ESTs, PCR approaches were carried out to isolate the entire coding region of the primary tryptase ϵ transcript expressed in two different tissues (Fig. 1*B*). The full-length tryptase ϵ cDNA consists of ~1400 nucleotides, and its 5′- and 3′-untranslated regions (UTRs) consist of at least 53 and 380 nucleotides, respectively. Analysis of the tryptase ϵ gene (data not shown) revealed a TATA box ~30 bp upstream of the nucleotide sequence noted in Fig. 1*B*. Thus, the 5′-UTR of the tryptase ϵ transcript in some cells could be 30 bp larger. The putative translation-initiation codon conforms to that of most eukaryotic transcripts.

An analysis of all nucleotide sequences deposited in GenBank™ revealed that the human tryptase ϵ gene resides on the chromosome 16p13.3-derived cosmid clone 325D7 (GenBank™ accession no. AC003965). Although Ricke and co-workers correctly predicted that a serine protease-like gene resided on their cosmid clone, these investigators were unable to identify the first two exons of the gene that encode the N-terminal 47 amino acids of the translated product. It was therefore incorrectly concluded that the serine protease-like gene on cosmid clone 325D7 was not transcribed. Alternatively, if transcribed, the gene did not encode an enzymatically active protease.

The failure of the Human Genome Project to identify the first two exons of the tryptase ϵ gene raised the possibility that multiple undiscovered tryptase ϵ-like genes reside on human chromosome 16p13.3. The human genes that encode tryptases α, βI, βII, TMT, marapsin, Esp-1, and tryptase ϵ reside within a 2.5-Mb sequence that is covered by 10 nonoverlapping contigs (Fig. 2*A*). When this region of the human genome was screened for nucleotide sequences that encode highly conserved amino acid sequences flanking the catalytic triad of known serine proteases, seven additional genes containing the target features were identified (Fig. 2*A*). These serine protease-like (SPL) genes (designated *SPL-1–SPL-7*) contain premature stop codons, lack exons, or encode proteins that lack critical residues (Table I). If expressed, all seven SPL genes should encode catalytically inactive proteases. Thus, no additional human tryptase ϵ-like gene appears to be present at the examined locus. The exon/intron organizations and the sizes of the chromosome 16p13.3 family of serine protease and SPL genes are depicted in Fig. 2*B*. The tryptase ϵ gene is the second largest gene in the complex.

Expression of Tryptase ϵ at the mRNA Level in Human Tissues, Primary Cells, and Transformed Cell Lines—As assessed by RNA blot analysis, tryptase ϵ mRNA is present in abundance in the esophagus and trachea of adult humans (Fig. 3, *A* and *B*). The placenta, pancreas, prostate, and thyroid contain lower levels of the tryptase ϵ transcript, but the level of tryptase ϵ mRNA is below detection in most tissues by blot analysis. These results were confirmed by PCR analysis of CLONTECH tissue cDNA panels (data not shown). As assessed by RNA blot (Fig. 3*A*) and PCR (data not shown) analysis, the levels of the tryptase ϵ transcript are below detection in normal brain, heart, spleen, liver, skeletal muscle, kidney, stomach, and intestine. Most of these tissues contain substantial numbers of MCs. Because the steady-state levels of the tryptase ϵ transcript are extremely low in these tissues, as well as in the HMC-1 MC line (data not shown), tryptase ϵ does not appear to be expressed in MCs. The finding that the steady-state level of the tryptase ϵ transcript is much higher in fetal lung than in

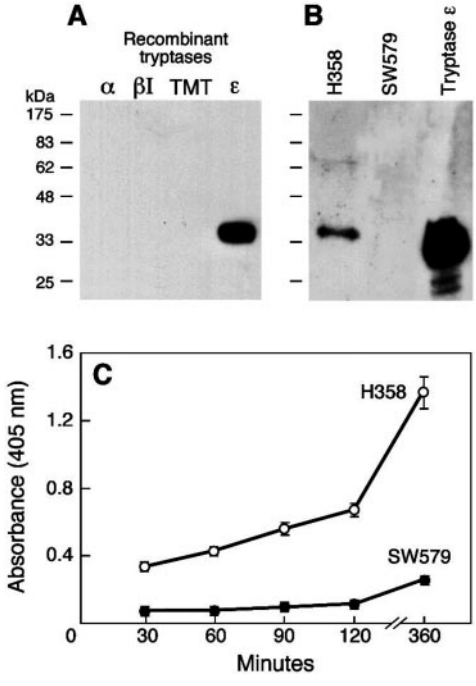


FIG. 4. **Generation of tryptase ϵ-specific antibodies and demonstration that the tryptase ϵ transcript is translated and the resulting product is constitutively exocytosed from epithelial cells.** *A*, SDS-PAGE/immunoblot containing similar amounts of the recombinant human tryptases α, βI, and TMT were probed with anti-tryptase ϵ antibodies. Only recombinant tryptase ϵ was recognized by the anti-peptide antibodies. *B*, H358 cells contain tryptase ϵ mRNA but not SW579 cells. Thus, dialyzed and concentrated 24-h serum-free conditioned media derived from these two epithelial cell lines were subjected to SDS-PAGE/immunoblot analysis with anti-tryptase ϵ antibodies. Recombinant tryptase ϵ (*right lane*) was used as a positive control. *C*, conditioned media derived from human cell lines H358 (○) and SW579 (■) were assayed for their relative tryptase activity using the chromogenic substrate H-D-Leu-Thr-Arg-*p*NA.

Fig. 3. **Expression of the tryptase ϵ transcript in human tissues and cell lines.** *A,* membranes containing poly(A)$^+$ RNA isolated from varied human tissues and cell lines were probed under conditions of high stringency with a ^{32}P-labeled tryptase ϵ probe (*upper panel*) and then with a ^{32}P-labeled β-actin probe (*lower panel*). RNA size markers are indicated on the *left*. *B,* a second multi-tissue blot containing additional human tissues mRNA was probed under conditions of high stringency with a ^{32}P-labeled tryptase ϵ probe (*lower panel*). The *upper panel* shows the tissue origin of each sample evaluated in the *lower panel*. *C*, a cDNA panel derived from the indicated transformed cell lines and tumor xenografts was used to evaluate the expression of the tryptase ϵ and G3PDH transcripts. *D*, primary human bronchial epithelial (*NHBE*) and human microvascular endothelial cells (*HMVEC*) were evaluated for tryptase ϵ and G3PDH mRNA using a reverse transcriptase-PCR approach.

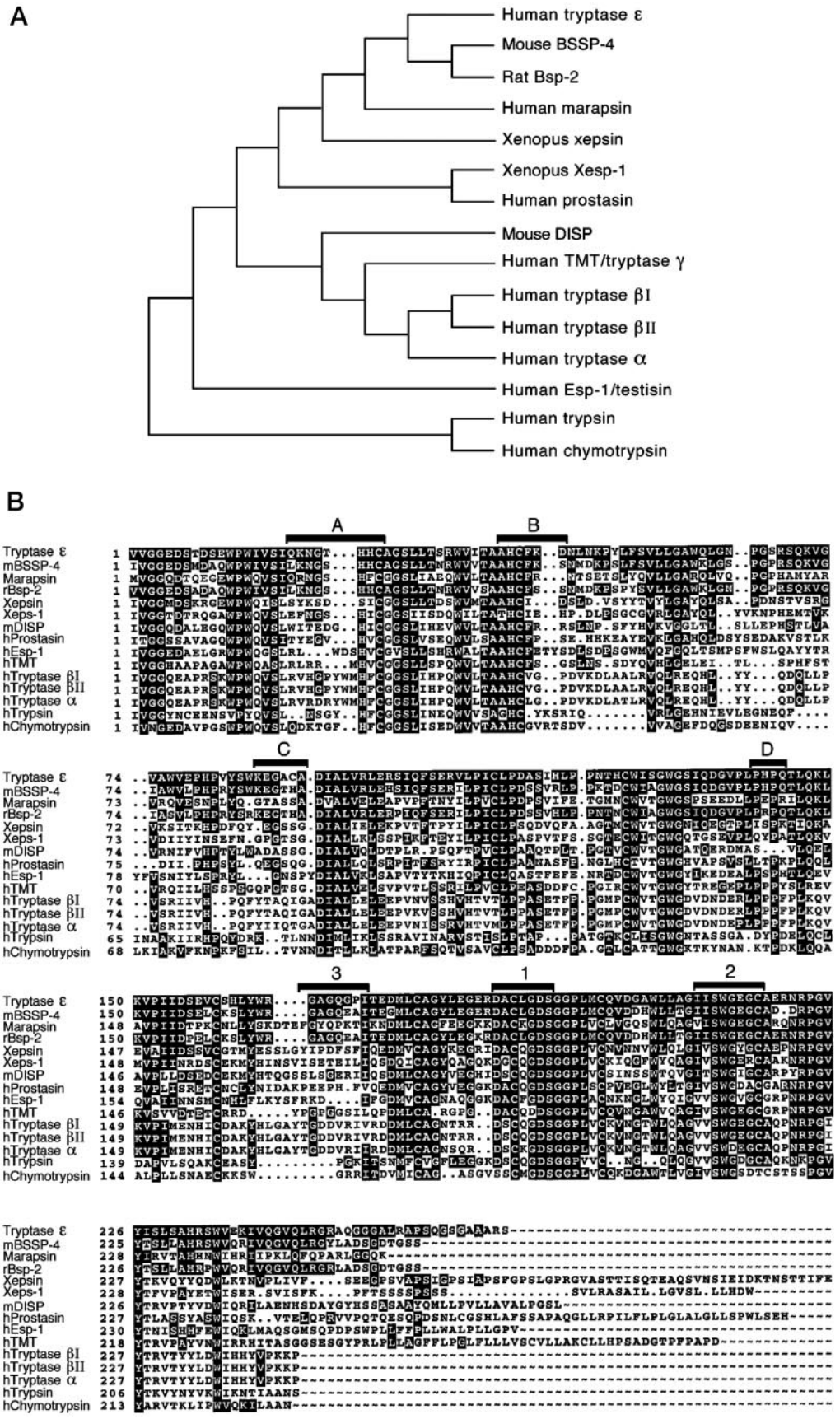


FIG. 5. **Comparison of the amino acid sequences of human tryptase ϵ and related human, *Xenopus*, rat, and mouse serine proteases.** *A*, the amino acid sequences (GenBank™ accession numbers are in parentheses) of human TMT (AF175522), Esp-1 (AB031329), marapsin (AJ306593), prostasin/PRSS8 (L41351), pancreatic trypsin 1/PRSS1 (M22612), chymotrypsin (M24400), tryptase βI (NM003294), tryptase βII (NM024164), and tryptase α (NM003293); *Xenopus* embryonic serine protease 1 (Xesp-1; AB038496) and epidermis specific serine protease (Xepsin; 018694); rat Bsp-2 (RRA5642); and mouse Distal intestinal serine protease (mDISP; AJ243866) and Bssp-4 (AB010778) were extracted from the GenBank™ data base. The C-terminal 62 residues of Xepsin are not shown in the alignment. A dendrogram was generated by

TABLE II
Comparison of the chromosome 16p13.3 human serine proteases

Indicated are the percent identities at the protein level between the seven functional members of the human chromosome 16p13.3 family of serine proteases.

	Tryptase ϵ	Tryptase α	Tryptase βI	Tryptase βII	TMT	Marapsin	Esp-1
Tryptase ϵ	100	40	40	40	43	50	38
Tryptase α		100	93	92	49	44	40
Tryptase βI			100	99	48	43	40
Tryptase βII				100	48	43	40
TMT					100	48	44
Marapsin						100	43
Esp-1							100

adult lung (Fig. 3, *A* and *B*) also indicates that tryptase ϵ is expressed in the airways in a developmentally regulated manner. Using a reverse transcriptase-PCR approach, the tryptase ϵ transcript was detected in the transformed epithelial cell lines MCF7 (ATCC line HTB-22), A-431 (ATCC line CRL-1555), H358 (ATCC line CRL-5807), and TT (ATCC line CRL-1803) but not in the SW579 epithelial cell line (Fig. 3*C*). The transcript also was detected in varied human tumor xenografts of epithelial origin maintained in nude mice. Because the accumulated data raised the possibility that tryptase ϵ is a product of certain normal epithelial cells, primary lung epithelial cells were examined for the presence of tryptase ϵ mRNA. As noted in Fig. 3*D*, the tryptase ϵ transcript was detected in pulmonary epithelial cells but not in microvascular endothelial cells.

Analysis of Human Tryptase ϵ at the Protein Level—Because tryptase ϵ is present in H358 cells but not SW579 cells, H358 cells were used to determine whether the tryptase ϵ transcript is ever translated in epithelial cells. Using a newly developed tryptase ϵ-specific antibody (Fig. 4*A*), immunoreactive tryptase ϵ was found in the conditioned medium of the H358 cell line but not the SW579 cell line (Fig. 4*B*). The additional finding that the conditioned medium of the H358 epithelial cell line contains an activity that can cleave the chromogenic substrate H-D-Leu-Thr-Arg-*p*NA (Fig. 4*C*) indicates that some populations of human epithelial cells constitutively secrete enzymatically active tryptase ϵ.

Human tryptase ϵ is initially translated as an ~34-kDa zymogen (Fig. 4*B*). Phylogenetic analysis (Fig. 5*A*) of all known proteins in the GenBank™ data base revealed that human tryptase ϵ is 81, 81, 50, 49, and 42% identical to mouse brain-specific serine protease 4 (mBssp-4), rat brain serine protease 2 (rBsp-2), human marapsin (Table II), *Xenopus* Xepsin, and *Xenopus* Xesp-1, respectively. All mature tryptases have an N-terminal sequence of Ile/Val-Val-Gly-Gly (Fig. 5*B*). Because the zymogen form of human tryptase ϵ also possesses this sequence, its prepropeptide consists of 49 amino acids (Fig. 1*B*). In its mature form, the catalytic domain of unmodified tryptase ϵ monomer has a molecular mass of ~30 kDa.

The overall three-dimensional structure of mature tryptase ϵ is predicted to be similar to other serine proteases (Fig. 6*A*). For example, like all other functional serine proteases, tryptase ϵ possesses the conserved triad amino acids (*i.e.* His^{41}, Asp^{93}, Ser^{193}) in its putative catalytic site. Tryptase ϵ lacks a number of the Pro and Tyr residues needed for human tryptase βII, mMCP-6, and mMCP-7 to form tetramers. For example, the Pro-Pro-Phe-Pro sequence that is essential for the interaction of two human tryptase βII monomers corresponds to His-Pro-Gln-Thr in human tryptase ϵ at residues 142–145. Although these data indicate that tryptase ϵ cannot form the tetramer unit, this new human serine protease possesses the seven conserved Trp residues at positions 12, 14, 127, 131, 205, 213, and 235. Like human tryptase βII, Trp^{12}, Trp^{14}, Trp^{127}, and Trp^{205} are predicted to be spatially close on the surface of folded tryptase ϵ (Fig. 6*A*). The prepropeptide of tryptase ϵ does not resemble that of its other family members. Nevertheless, the presence of the critical Trp residues in its catalytic domain suggests that the activation of the tryptase ϵ zymogen also requires the conserved Trp-rich domain.

The presence of Asp^{187}, Gly^{214}, and Gly^{224} in tryptase ϵ initially suggested that this serine protease possesses tryptic-like activity. However, further analysis of its substrate-binding cleft also suggested that tryptase ϵ has a unique substrate preference. For example, residue substitutions in loops A, C, and 3 in tryptase ϵ are predicted to result in shape changes relative to that of human tryptase βII (Fig. 6*B*). Mature tryptase ϵ has a net −2 charge at neutral pH, and this serine protease does not appear to have a heparin-binding domain. In addition, its overall electrostatic potential (Fig. 6*C*) does not resemble that of tryptase βII (Fig. 6*D*).

When transfected into COS-7 cells, immunoreactive tryptase ϵ was recovered in the conditioned medium of the transfectants. As assessed by SDS-PAGE analysis, an immunoreactive protein of ~37 kDa was identified in the transfectants that shifted to ~33 kDa after PNGase F treatment (Fig. 7*A*). Thus, the one potential *N*-linked glycosylation site at Asn^{21} is utilized. When SDS-PAGE analysis of the COS-7 cell conditioned medium was carried out under nonreducing conditions, two immunoreactive proteins of ~33 and ~66 kDa often were observed (Fig. 7*B*). The latter finding suggests that tryptase ϵ sometimes can exist as a Cys–Cys-linked homotypic dimer, at least when artificially expressed in COS-7 cells.

Evaluation of the Enzymatic Activity of Natural and Recombinant Human Tryptase ϵ—Because the H358 epithelial cell line probably produces multiple proteases, the preliminary finding that its conditioned medium contained an activity that could cleave H-D-Leu-Thr-Arg-*p*NA (Fig. 4) did not conclusively prove that the relevant protease was tryptase ϵ. Thus, two different transfection approaches were used to address this issue definitively. As noted above, tryptase ϵ was readily expressed in COS-7 cells (Fig. 7*A*). Using the H-D-Leu-Thr-Arg-*p*NA substrate, the level of proteolytic activity in the serum-free conditioned medium obtained from the tryptase ϵ-transfectants was low but was significantly greater than in the conditioned medium of the control transfectants (Fig. 7*C*). Because this chromogenic substrate is relatively specific for tryptic-like proteases, the novel cDNA described in this study does indeed encode a functional tryptase. Moreover, the enzymatic activity initially identified in the conditioned medium of

the Genetics Computer Group (GCG) program "Distances" using the unweighted pair group with arithmetic mean algorithm. *B,* the amino acid sequences of the serine proteases were aligned using the PILEUP program of the GCG software package. Identical amino acids are *shaded*. *Numbering* (*left*) begins at the first residue in the mature portion of each protease. The seven loops (designated *A, B, C, D, 1, 2,* and *3*) predicted to form the substrate-binding cleft of each serine protease are indicated (*bars*).

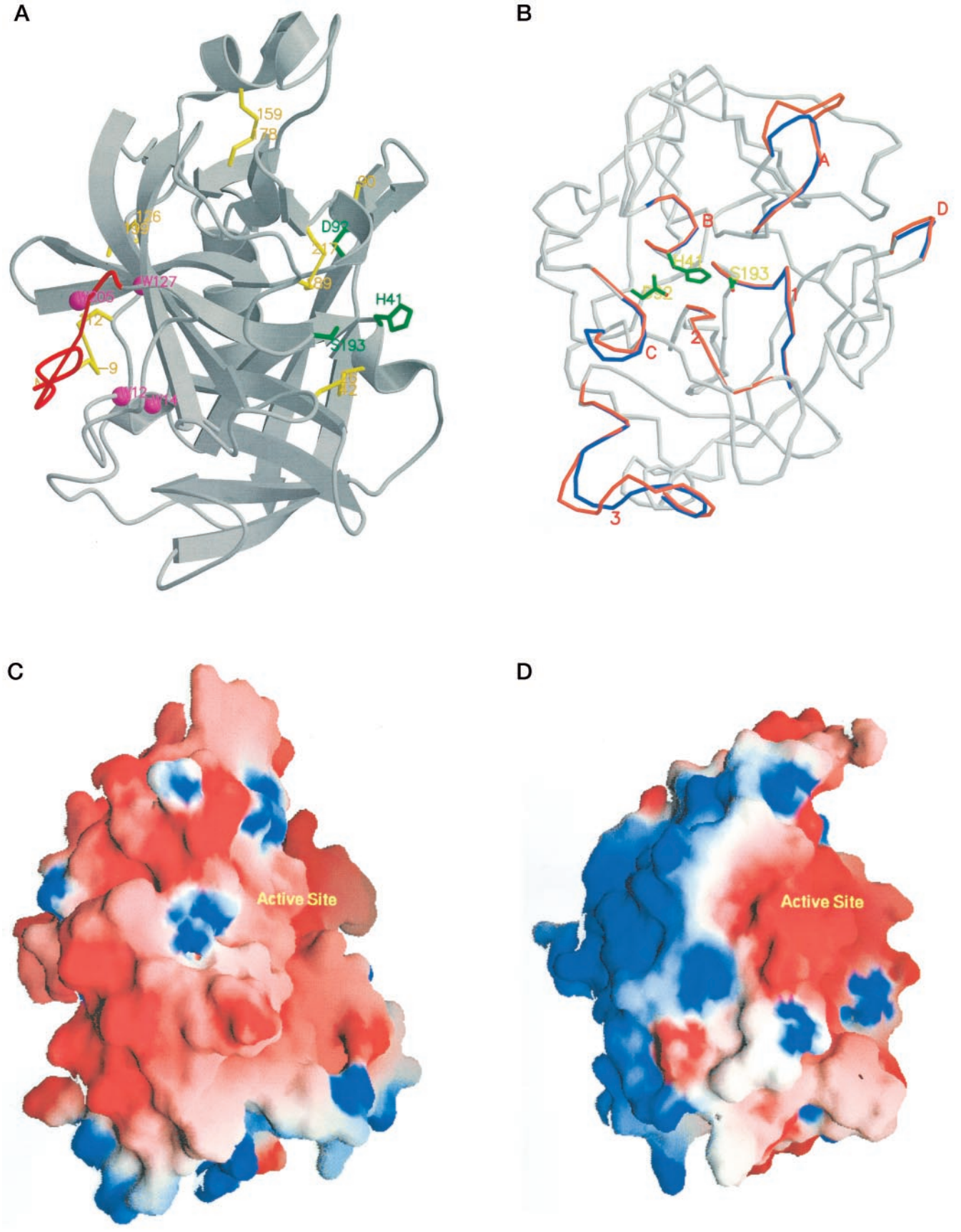


FIG. 6. **Three-dimensional models of human tryptase ϵ.** *A,* a three-dimensional model of residues −10 to +243 of human tryptase ϵ was calculated based on the crystallographic structures of mature human tryptase βII and the proenzyme domain of human plasminogen. The active-site residues (His^{41}, Asp^{93}, and Ser^{193}) are represented as *green sticks*. Cys residues and their disulfide bonds are shown in *yellow*. The covalently attached propeptide is shown in *red*. The C-α atoms of the conserved Trp residues (*i.e.* Trp^{12}, Trp^{14}, Trp^{127}, Trp^{205}) that are spatially close to each other, on the surface away from the active site, are shown as *pink spheres*. The plot was created with the programs Molscript (58) and Raster3D (59). The orientation of the tryptase ϵ model is similar to that of the mouse and human tryptase models reported in our previous

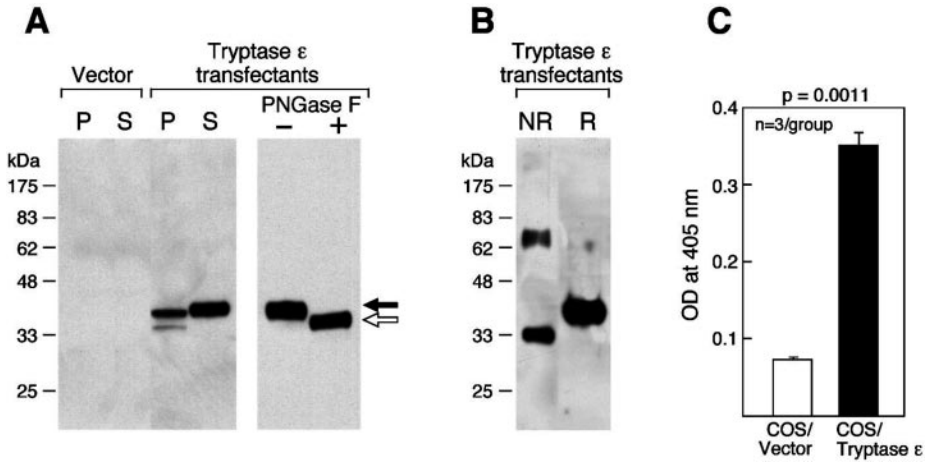


FIG. 7. **Evaluation of the enzymatic activity and carbohydrate status of recombinant human tryptase ϵ generated in COS-7 cells.** *A,* COS-7 cells were transfected with expression vector alone (*left lanes*) or expression vector containing an insert that encodes a bioengineered form of tryptase ϵ possessing the immunogenic V5 peptide at its C terminus (*right lanes*). 48 h later, samples of the resulting serum-free conditioned media/supernatants (*S*) and lysates of the cell pellets (*P*) were evaluated for the presence of recombinant tryptase ϵ using anti-V5 antibody. A sample of the conditioned media derived from tryptase ϵ-expressing COS-7 cells was incubated for 1 h in the absence (−) or presence (+) of PNGase F prior to SDS-PAGE/immunoblot analysis to determine whether recombinant tryptase ϵ contains an *N*-linked glycan when expressed in COS-7 cells. The *closed* and *open arrow* on the *right* point to glycosylated and deglycosylated forms of tryptase ϵ, respectively. The deglycosylated product is ~34 rather than 30 kDa because the recombinant protease contains the additional V5 and 6xHis peptides at its C terminus. *B*, serum-free conditioned medium containing tryptase ϵ was subjected to SDS-PAGE under nonreducing (*NR*) or reducing (*R*) conditions; the resulting protein blot was probed with anti-V5 antibody. Molecular size markers are shown on the *left*. *C*, the ability of comparable amounts of the serum-free conditioned media derived from vector- and tryptase ϵ-transfected COS-7 cells to cleave the substrate H-D-Leu-Thr-Arg-*p*NA was evaluated. Three independent experiments were performed. In each experiment, the enzymatic assay was performed in triplicate. The *p* value is indicated for these data.

H358 cells appears to be due to tryptase ϵ rather than a contaminating protease.

To better define the substrate preference of tryptase ϵ, a bioengineered form of the human protein possessing the FLAG peptide at its C terminus and an EK susceptible site in between the putative native propeptide and mature catalytic domain of human tryptase ϵ was expressed in High Five insect cells. Insect cells constitutively secreted human pro-tryptase ϵ and the recombinant protein could be quickly purified from the serum-free conditioned medium using the immunoaffinity column (Fig. 8*A*). The resulting protein also could be activated by EK without inducing rapid autolysis of the resulting protein (Fig. 8*B*). Using various chromogenic substrates, the substrate specificities of recombinant human tryptase ϵ and tryptase βI were found to be quite different. Whereas recombinant tryptase βI preferred tosyl-Gly-Pro-Arg-*p*NA over H-D-Leu-Thr-Arg-*p*NA (Fig. 8*D*), recombinant tryptase ϵ preferred H-D-Leu-Thr-Arg-*p*NA over tosyl-Gly-Pro-Arg-*p*NA (Fig. 8, *C* and *D*).

DISCUSSION

Tryptase ϵ (Fig. 1) represents a new member of the human chromosome 16p13.3 family of serine proteases. Analysis of a 2.5-Mb region of human chromosome 16p13.3 revealed the presence of seven genes that encode the enzymatically active serine proteases marapsin, Esp-1/testisin/PRSS21, and tryptases α, βI, βII, ϵ, and TMT/γ (Fig. 2). The tryptase ϵ gene apparently was missed by the automated gene assignment of the Human Genome Project, presumably because the first two exons of the tryptase ϵ gene do not closely resemble those in other serine protease genes. Although no additional tryptase ϵ-like gene was identified, seven additional serine protease-like genes (designated *SPL-1–SPL-7*) reside at chromosome 16p13.3 (Fig. 2). Thus, the serine protease gene cluster at chromosome 16p13.3 represents the second largest cluster of protease genes in the human genome. *SPL-1–SPL-7* appear to encode enzymatically inactive serine proteases (Table I). Azurocidin (51) is a well described neutrophil protein that exhibits potent antibiotic activity against Gram-negative bacteria; it also regulates monocyte/macrophage chemotaxis, survival, and differentiation. Although azurocidin is 44% identical to human neutrophil elastase, it is not enzymatically active because of mutations in two of the three residues that should comprise its "charge relay system" (52). Based on the azurocidin example, it presently cannot be concluded that the SPL genes encode nonfunctional proteins. Nevertheless, the functional tryptase ϵ gene resides downstream of the *SPL-7* gene. Two *Xenopus* proteases are more similar to human tryptase ϵ than the other members of its family (Fig. 5*A*). These findings suggest that the tryptase ϵ gene was the first to develop at the locus on human chromosome 16p13.3.

The expression of tryptase ϵ is much more restricted than that of its other family members. This finding is another explanation of why no full-length tryptase ϵ cDNA had been described previously. The esophagus and trachea of adult humans contain the highest steady-state levels of tryptase ϵ mRNA (Fig. 3, *A* and *B*). Although low levels of tryptase ϵ were detected in some adult human lung preparations (Fig. 3*B*), higher levels of this protease transcript were found in fetal lung. Tryptase ϵ therefore appears to be expressed in the human lung in a developmentally regulated manner. Human MCs express tryptases α, βI, βII, βIII, and TMT/tryptase γ (6, 7,

publications. *B*, the putative substrate-binding cleft of tryptase ϵ was analyzed at a higher resolution. The seven loops that form the substrate-binding cleft of tryptase ϵ are marked *A–D* and *1–3* (*blue*) and are superimposed on the corresponding loops of mature human tryptase βII (*red*). The active-site residues are shown as *green sticks. C* and *D,* comparison of the electrostatic potentials on the surfaces of human tryptase ϵ (*C*) and human tryptase βII (*D*) at pH 7.0. The two human tryptases are orientated similarly to show the substantial difference in their electrostatic potentials. The *blue-* and *red-colored* patches on the surface of each tryptase correspond to the regions with a net concentration of positive (Arg and Lys) and negative (Asp and Glu) charges, respectively. The program GRASP (60) was used to prepare *panels C* and *D*. The active sites in the human tryptase ϵ and tryptase βII monomers are also indicated (*orange*).

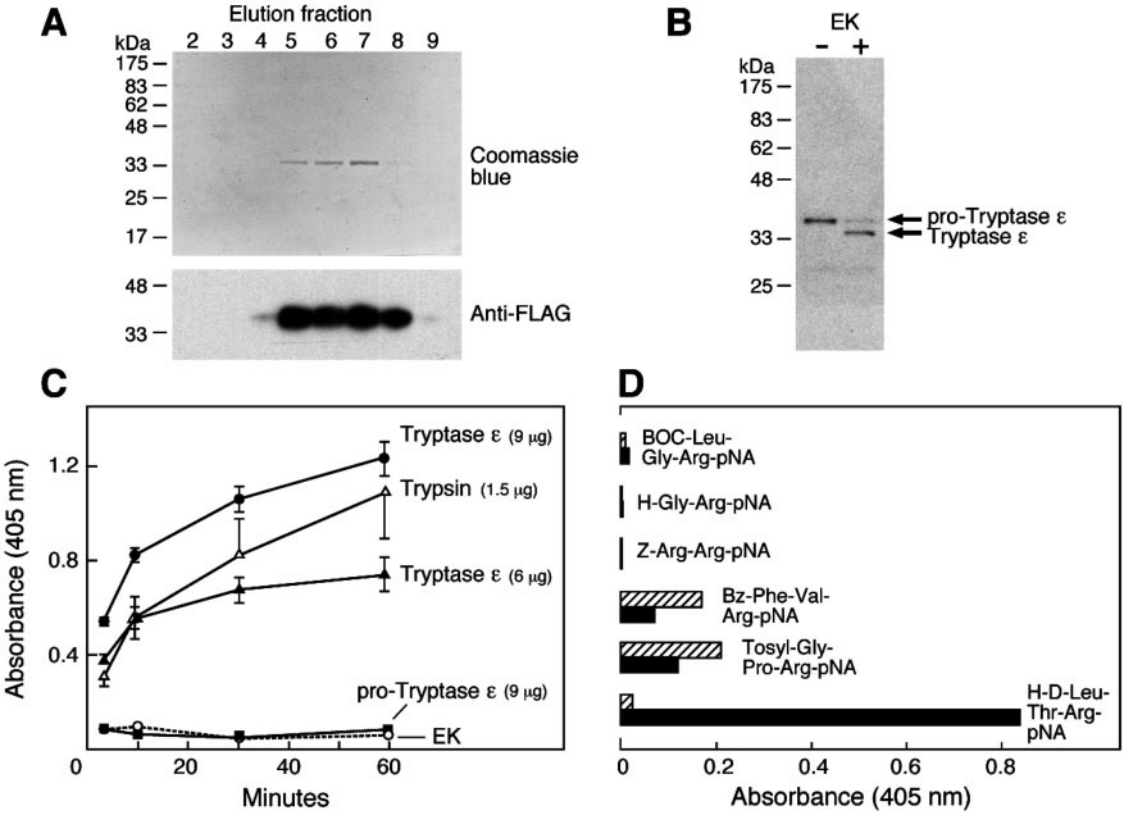


FIG. 8. **Expression, purification, and enzymatic activity of insect cell-derived recombinant human tryptase ϵ.** *A,* a pseudozymogen form of human tryptase ϵ was expressed in insect cells that contained the FLAG peptide at its C terminus and an EK-susceptible site in between the natural propeptide and catalytic portion of the mature tryptase. The protein was purified from the conditioned media using an immunoaffinity column. Shown in the *upper portion* of *A* are the Coomassie Blue-stained proteins in the eluate of the column. Anti-FLAG antibody was used in the *lower portion* of *A* to confirm that the major ~35-kDa protein in the eluate fractions is recombinant pro-tryptase ϵ. *B,* pro-tryptase ϵ was incubated in the absence (−) or presence (+) of EK, and the resulting digests were subjected to SDS-PAGE/immunoblot analysis. EK is able to remove the bioengineered propeptide in such a way that it does not cause rapid autolysis of the recombinant tryptase. *C*, pro-tryptase ϵ (9 μg (■)) and EK-containing activation buffer lacking (○) or containing tryptase ϵ (6 (▲) or 9 μg (●)) were evaluated for their ability to cleave H-D-Leu-Thr-Arg-*p*NA in a kinetic manner. Pancreatic trypsin (1.5 μg (Δ)) was used for comparison. The depicted data are the mean ± S.D. of an experiment carried out in triplicate on the same day. *D,* comparable amounts of activated recombinant human tryptase ϵ (*solid bars*) and human tryptase βI (*hatched bars*) were evaluated in terms of their ability to cleave the indicated six substrates.

11–13). The level of tryptase ϵ was below the detection threshold in cultured HMC-1 mast cells. In addition, the tryptase ϵ transcript was not detected in a number of MC-enriched tissues such as the heart (Fig. 3*B*). hTMT mRNA is abundant in the jejunum (6), whereas hEsp-1 mRNA is abundant in the testis (16). As noted in Fig. 3*B*, tryptase ϵ mRNA was below detection in both tissues. The accumulated data indicate that human tryptase ϵ is not coordinately expressed with any member of its family.

The tryptase ϵ transcript was detected in a number of transformed human epithelial cell lines (Fig. 3*C*). Chromosome instability is a common occurrence in cancerous cells, and the hTMT gene is transcribed in numerous transformed cell lines (6). Although the instability of chromosome 16p13.3 could contribute to the high levels of tryptase ϵ mRNA in certain epithelial cell lines (Fig. 3*C*), nontransformed bronchial epithelial cells express this new human serine protease (Fig. 3*D*). Thus, human tryptase ϵ is a normal product of nontransformed airway epithelial cells. Yamaoka and co-workers (53) cloned a human type II membrane protein (designated HAT) that possesses tryptic-like activity from the serous glands of bronchi and trachea. Because tryptase ϵ is not HAT, MCs and airway epithelial cells are not the only cell types in the human lung that produce tryptic-like proteases. APC-366 and other low molecular weight protease inhibitors have been generated in an attempt to regulate the tryptases that are stored in the secretory granules of human lung MCs (44). Because one cannot easily interpret pulmonary data with an inhibitor that inactivates multiple lung tryptases, the discovery of tryptase ϵ and its expression in the airway epithelium greatly impacts future clinical trials with protease inhibitors carried out on patients with asthma and other lung disorders.

At the mRNA and protein levels, human tryptase ϵ is more similar to the mouse and rat brain-specific serine proteases mBssp-4 and rBsp-2 (54) than to any tryptase so far cloned from mouse, rat, or human MCs. The steady-state levels of human tryptase ϵ were below detection in varied brain samples (Fig. 3, *A* and *B*). Thus, tryptase ϵ is not the human orthologue of mBssp-4 or rBsp-2. Alternately, substantial divergence in its expression pattern occurred during the last 40–100 million years of evolution.

Human tryptase ϵ is initially translated as an ~34-kDa zymogen (Fig. 4) that consists of 317 amino acids (Fig. l). The initial 32 residues likely represent the signal peptide of the translated protein. Thus, removal of these hydrophobic residues in the endoplasmic reticulum results in a zymogen that possesses a 17-mer propeptide and a 268-mer catalytic domain. The overall amino acid sequence of the proenzyme domain of tryptase ϵ does not resemble that of the other members of its family. Surprisingly, it resembles that of plasminogen. For example, the Cys-Gly-Lys-Pro-Gln sequence at residues −9 to −5 in pro-tryptase ϵ is also present in the corresponding domain of plasminogen as is the Arg-Val-Val-Gly-Gly cleavage site at residues −1 to +4.

Although the overall three-dimensional structure of the catalytic domain of tryptase ε is predicted to be similar to that of other serine proteases (Fig. 6*A*), tryptase ε lacks the Tyr- and Pro-rich domains present in tryptases α, βI, βII, and βIII (28, 29). Tryptase ε probably is unable to form the tetramer unit observed by Pereira and co-workers (29) in their crystallographic structure of tryptase βII. Tryptase ε also lacks the C-terminal hydrophobic domain found in hEsp-1 (14) and hTMT (6). Thus, it is highly unlikely that tryptase ε is a membrane-associated protease *in vivo*. In support of this conclusion, H358 cells (Fig. 4) and transfected COS-7 cells (Fig. 7) and insect cells (Fig. 8) constitutively secrete tryptase ε into the conditioned media. The mature domain of human tryptase βII contains 8 Cys residues that form four intramolecular disulfide bonds (29). Because the corresponding Cys residues are present in the mature domain of human tryptase ε (Figs. 1 and 6*B*), the same four intramolecular disulfide bonds (*i.e.* Cys^{26}–Cys^{42}, Cys^{126}–Cys^{199}, Cys^{159}–Cys^{178}, and Cys^{189}–Cys^{217}) are presumed to be present in tryptase ε (Fig. 6*A*). However, tryptase ε differs from tryptase βII in that it has two additional Cys residues at positions 90 and 112. There is also a Cys residue at position −9 in the propeptide. The disulfide partner of this propeptide-localized Cys can be predicted based on the crystallographic structure of plasminogen. Plasminogen, the zymogen precursor of plasmin, is a single chain protein consisting of 791 amino acids. It contains five kringle domains followed by the proenzyme domain. The crystallographic structure of the proenzyme domain of plasminogen (48) revealed that Cys^{548}, residing 14 amino acids upstream of the N-terminal Val in the enzymatically active portion of the protein, forms a disulfide bond with Cys^{666}. Because of this additional disulfide bond, the five kringle domains are retained once plasminogen is converted to plasmin by cleavage of the Arg^{561}–Val^{562} bond in the precursor protein. The amino acid sequences at residues −9 to −13 and residues +112 to +114 in human tryptase ε are identical to the corresponding residues in plasminogen. Because it is likely that Cys^{-9} forms a disulfide bond with Cys^{112}, mature tryptase ε appears to be a two-chain protease analogous to plasmin. Although the Cys^{-9}–Cys^{112} disulfide bond causes the 17-residue propeptide to remain bound to the activated tryptase, it remains to be determined whether or not the propeptide influences the substrate specificity of tryptase ε.

When expressed in COS-7 cells (Fig. 7*A*) but not insect cells (data not shown), some tryptase ε is secreted as a homotypic dimer. If Cys^{112} forms an intramolecular disulfide bond with Cys^{-9}, the intermolecular disulfide bond linking two monomers must involve Cys^{90} on each monomer. Human cytomegalovirus protease is a serine protease that dimerizes (55), as does the tryptase ε homologue, dog proteinase 3 (56). However, the dimerization of dog proteinase 3 does not involve a Cys residue equivalent to Cys^{90} in tryptase ε. In addition, Cys^{90} is only two positions upstream of the Asp in the catalytic triad of the protease. The presence of the intermolecular disulfide bond involving two Cys^{90} residues probably hinders access of large proteins or peptides to the active site of the dimer. We therefore suspect that tryptase ε homotypic dimers are probably rare *in vivo*.

The substrate specificities of recombinant human tryptases α and βII are very different despite their overall 93% amino acid sequence identity (42). In contrast, the substrate specificities of recombinant human tryptases βI and βII are very similar if not identical (57). The presence of Asp^{187}, Gly^{214}, and Gly^{224} in tryptase ε initially suggested that this human serine protease possesses tryptic-like activity. Thus, the substrate specificities of recombinant tryptases α, βI, and ε were compared. Naturally occurring (Fig. 4) and recombinant (Figs. 7 and 8) tryptase ε cleaved H-D-Leu-Thr-Arg-*p*NA much more effectively than tosyl-Gly-Pro-Arg-*p*NA. In contrast, recombinant tryptase βI cleaved tosyl-Gly-Pro-Arg-*p*NA much more effectively than H-D-Leu-Thr-Arg-*p*NA (Fig. 8). We have previously shown that recombinant tryptase α cannot cleave either D-Leu-Thr-Arg-*p*NA or tosyl-Gly-Pro-Arg-*p*NA effectively (42). Thus, the substrate specificities of tryptase ε, βI, and α are quite different despite the fact that these human tryptases are 40–93% identical (Table II). Amino acid sequence alignment (Fig. 5*B*) and comparative protein structure modeling (Fig. 6*B*) predict that the different enzymatic activities of tryptase ε and βI are caused by substantial residue changes in loops A, C, and 3 that comprise the substrate-binding cleft of each protease. The physiologic substrates of human tryptase ε remain to be determined. Nevertheless, it is now apparent that a primordial serine protease gene at chromosome 16p13.3 duplicated repeatedly during evolution to give rise to multiple tryptic-like serine proteases in humans, which possess distinct tissue distributions and substrate specificities.

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