Tryptase 4, a New Member of the Chromosome 17 Family of Mouse Serine Proteases*

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Genomic blot analysis raised the possibility that uncharacterized tryptase genes reside on chromosome 17 at the complex containing the three genes that encode mouse mast cell protease (mMCP) 6, mMCP-7, and transmembrane tryptase (mTMT). Probing of GenBank’s expressed sequence tag data base with these three tryptase cDNAs resulted in the identification of an expressed sequence tag that encodes a portion of a novel mouse serine protease (now designated mouse tryptase 4 (mT4) because it is the fourth member of this family). 5′- and 3′-rapid amplification of cDNA ends approaches were carried out to deduce the nucleotide sequence of the full-length mT4 transcript. This information was then used to clone its ~5.0-kilobase pair gene. Chromosome mapping analysis of its gene, sequence analysis of its transcript, and comparative protein structure modeling of its translated product revealed that mT4 is a new member of the chromosome 17 family of mouse tryptases. mT4 is 40–44% identical to mMCP-6, mMCP-7, and mTMT, and this new serine protease has all of the structural features of a functional tryptase. Moreover, mT4 is enzymatically active when expressed in insect cells. Due to its 17-mer hydrophobic domain at its C terminus, mT4 is a membrane-anchored tryptase more analogous to mTMT than the other members of its family. As assessed by RNA blot, reverse transcriptase-polymerase chain reaction, and/or in situ hybridization analysis, mT4 is expressed in interleukin-5-dependent mouse eosinophils, as well as in ovaries and testes. The observation that recombinant mT4 is preferentially retained in the endoplasmic reticulum of transiently transfected COS-7 cells suggests a convertase-like role for this integral membrane serine protease.

A complex of genes resides on human chromosome 16p13.3 that encodes the homologous tryptases a1, a2, b1, bII, bIII, transmembrane tryptase (TMT)/tryptase γ, and eosinophil serine protease-1 (Esp-1)§ (1–7). The corresponding complex of genes resides on the syntenic region of mouse chromosome 17 at the interface between bands 17A3.3 and 17B1 (7–11). Although our previous genomic blot analysis suggested the presence of additional mouse tryptase genes in the family (7), the only genes and/or transcripts cloned so far at the mouse complex are those that encode mouse mast cell protease (mMCP) 6, mMCP-7, and mouse TMT (mTMT).

The amino acid sequences of mMCP-6 and mMCP-7 are ~75% identical, and the mast cells (MCs) in the skin and skeletal muscle of BALB/c mice express both tryptases (12, 13). Nevertheless, in vivo and in vitro studies have established that these two homologous serine proteases are metabolized quite differently during allergic reactions (12). Moreover, they are functionally distinct. Because fibrinogen is a physiologic substrate of mMCP-7 (14), this tryptase appears to help prevent the deposition of fibrin/platelet clots in inflammatory sites so that the various granulocytes and lymphocytes in the blood are not physically prevented from entering an inflamed mouse tissue site. Recent data have suggested that mTMT-7 also regulates eosinophil extravasation in tissues.§ MCs are essential for combating bacterial infections in the peritoneal cavity (15–17). Because peritoneal MCs express mMCP-6 (18), because the complement factors C3a and C5a induce peritoneal MCs to degranulate (19), and because recombinant mMCP-6 is a potent and selective inducer of neutrophil extravasation into the peritoneal cavity (20), mMCP-6 appears to play an important and protective role in bacterial infections. The function of mTMT is not yet known, but its C-terminal hydrophobic domain (7) probably causes its prolonged retention at the plasma membrane when mTMT+ MCs are immunologically activated.

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1 The abbreviations used are: TMT, transmembrane tryptase; ER, endoplasmic reticulum; hEsp-1, human eosinophil serine protease-1; FISH, fluorescent in situ hybridization; MC, mast cell; mMCP, mouse MC protease; mT4, mouse tryptase 4; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; mTesp, mouse testicular serine protease; RT-PCR, reverse transcriptase-PCR; UTR, untranslated region; EST, expressed sequence tag; kb, kilobase pairs; bp, base pairs; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; IL, interleukin; PAGE, polyacrylamide gel electrophoresis; PNGase F, peptide N-glycosidase F.

2 Genomic sequencing of the 35.7-kb cosmid clone 352F10 by D. O. Ricke and co-workers (GenBank™ direct submission, accession number AC005361) resulted in the initial identification and chromosomal localization of the 4.6-kb human gene in 1998 that others (4, 5, 44) designated as the hEsp-1/testisin gene in 1999.

3 R. L. Stevens, unpublished observations.
Because our previous genomic blot analyses suggested the presence of undiscovered tryptase-like genes in the mouse genome and because mouse chromosome 17A3.3 to 17B1 has not been sequenced in its entirety, we used a gene-hunt approach in the present study to identify a new member of this important family of serine proteases. We now describe the isolation, characterization, and expression of the fourth member of the mouse tryptase family of serine proteases.

**EXPERIMENTAL PROCEDURES**

**Cloning of the mT4 cDNA and Gene**—The nucleotide sequences of the mTMT, mMCP-6, and mMCP-7 transcripts were used as templates to search for novel, but related, mouse tryptase-like ESTs in the GenBankTM data base. Sequence analysis of the mouse testis-derived clone AI236140, obtained from the “Integrated Molecular Analysis of Gene Expression” consortium, revealed that its insert corresponded to a portion of what appeared to be a novel serine protease. Based on the deduced nucleotide sequence of this clone, 5′- and 3′-rapid amplification of cDNA ends (RACE) approaches were carried out on a pool of testis cDNAs (CLONTECH) to deduce the nucleotide and amino acid sequences of the full-length transcript. The 5′-RACE reaction was carried out with 5′-GACCTCAATACCTTCCACG-3′ and the anchor oligonucleotide 5′-CCATCTAATAGCGTCTTGGGCCG-3′. The resulting DNA products were purified on a 1% agarose gel, subcloned into pCR2.1 (Invitrogen, Carlsbad, CA), and the inserts in two of the arbitrarily selected clones were sequenced. The 5′-RACE was carried out with the RLM-RACE kit (Ambion, Austin, TX). The mT4-specific primer 5′-GACCTGAGTCCGATGTAACCATATG-3′ and the outer primer 5′-GCTGAGGCTGAGATGACACCTG-3′ were used in the first PCR. One microliter of the generated product was then used as a template in the second PCR step with the mT4-specific primer 5′-CCACGAGCTGCTGTTGCTTC-3′ and the inner primer 5′-CCGCGATCCCGAAGCTTGGGCTGAGTTGAAATC-3′. The resulting products were purified and subcloned, and the inserts in six of the arbitrarily selected clones were sequenced.

Because human tryptase genes so far cloned in this family are >6 kb in size, a long range nested PCR approach was used to isolate and characterize the mT4 gene from BALB/c mice. The oligonucleotides 5′-GCCAACCTGGGCTGACACGCTG-3′ and 5′-GTCAGTGAC- TACAGTGTTGGGCCTAGCCG-3′ were used in the initial 30-cycle PCR, whereas the oligonucleotides 5′-ATGGCGCTATTAAATCACTCTTAT- GGAC-3′ and 5′-GCCTGAGCACCCCACTTGCGCATC-3′ were used in the subsequent 50-cycle reaction. The PCR products (25 μl each) were cloned into the pCR2.1 vector (Invitrogen) and the inserts in six of the resulting plasmids were purified on 1% agarose gels and subcloned into pBluescript II KS (Stratagene, La Jolla, CA) containing a 3′-P-labeled 536-bp probe corresponding to the 5′ end of the mT4 cDNA. The clone was cloned into a phagemid vector. The resulting two plasmid DNA samples were linearized with EcoRI and XhoI and subcloned into the vector pBluescript II KS. The inserts in the resulting plasmid DNA samples were sequenced.

**Gene—** For the nonradioactive in situ hybridization approach, the mT4-specific oligonucleotide 5′-CTATTTGTGAGCTGTTGGGAATGCGTGTTGAGTTTCCAG-3′ was labeled with digoxigenin-alkaline phosphatase with a commercially available 3′ end labeling kit (Roche Molecular Biochemicals). BALB/c mouse testis was fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at 4°C. The preparation was washed twice with PBS containing 2% dimethyl sulfoxide and then dehydrated and embedded in JB4 glycolmethacrylate according to the manufacturer’s instructions (Polysciences Inc., Warrington, PA). Sections were cut on a Reichert-Jung Supraicut microtome (Leica, Deerfield, IL) at 5-μm thickness and picked up on glass slides. The slides were incubated sequentially for 15 min at 37°C in 0.025% trypsin containing 2 mM calcium chloride and then for 30 min in Target Retrieval solution (Dako, Carpinteria, CA) at 95°C. After the slides were washed in 2×SSC at each temperature, they were incubated in prehybridization solution containing 50% formamide, 4× SSC, 1% Denhardt’s solution, sonicated salmon sperm DNA, and 10% dextran sulfate at 42°C for 30 min. The prehybridization solution was removed, 50 μl of hybridization buffer containing the digoxigenin-labeled oligonucleotide was added, and each specimen was incubated overnight at 42°C. The next morning, each slide was washed twice with 2× SSC, once with 1× SSC, and once with 0.5× SSC, each for 20 min at room temperature and then incubated in streptavidin-biotin complex for 30 min at room temperature. The slides were washed twice with Tris-buffered saline, incubated with streptavidin complex conjugated to horseradish peroxidase, and washed twice again with Tris-buffered saline. Color development was performed according to the manufacturer’s instructions (Dako). In this analysis, cells that contained abundant levels of mT4 mRNA stain brown.

A radioactive in situ hybridization approach was used to confirm the obtained data with the above digoxigenin-alkaline phosphatase-labeled probe. For this second type of in situ hybridization analysis, nucleotides 1–536 of the mT4 cDNA (Fig. 1) were subcloned into pCR2.1 in both the sense and antisense directions. The resulting two plasmid DNA samples were linearized with SpeI (New England Biolabs) and transcribed with T7 RNA polymerase (Promega, Madison, WI) in the presence of [α-32P]UTP (PerkinElmer Life Sciences) to generate antisense and sense radiolabeled RNA probes. Tissue sections were placed on slides, deparaffinized, fixed in 4% paraformaldehyde in PBS, and treated with proteinase K. After washing in 0.5× SSC, the sections were covered with 50 μl of hybridization solution (50% deionized formamide, 0.3 M NaCl, 0.3× SSC, 5 mM EDTA, 1× Denhardt’s solution, 0.5 mg/ml tRNA, 20 μg/ml dithiothreitol, and 20 mM Tris-HCl, pH 8.0) and incubated for 2 h at 55°C. [32P]-Labeled antisense or sense RNA probes (3 million cpm/ml) were added to the hybridization solution, and the sections were incubated for an additional 12–18 h at 55°C. The resulting sections were washed for 20 min in 2× SSC, 10 μM β-mercaptoethanol, and 1 mM EDTA and then were exposed to RNase A (10 μg/ml) for 30 min at room temperature. To minimize the possibility of nonspecific binding of the
radiolabeled probe, each section was incubated for an additional 2 h at 60 °C in 0.1× SSC, β-mercaptoethanol, and 1 mM EDTA. The resulting sections were dehydrated, dipped in photographic emulsion NTB2 (Kodak), and stored at 4 °C. After 7 days of exposure, the radiolabeled sections were developed, and in these sections were counterstained with hematoxylin and eosin. In this way, moderate black silver granules appear over those cells that contain abundant levels of mT4 mRNA.

**Expression of mT4 Protein in COS-7 Cells and Insect Cells and Protein Modeling of Its Translated Mature Product**—To address whether or not the mT4 transcript encodes an enzymatically active, membrane-anchored protease, the entire coding region of mT4 was cloned in the expression vector pcDNA3.1/V5-His-TOPO (Invitrogen). This expression vector was chosen because the resulting product will contain the 14-mer V5 peptide (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Val) and its C terminus, thereby allowing its detection with anti-V5 antibody. Vector lacking an insert was used as a negative control in the transfection experiments. African green monkey, SV40-transformed kidney COS-7 cells (line CRL-1615, 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 × 10⁵ cells/well in 6-well plates 24 h prior to transfection. After the cells were transfected, they were trypsinized, and portions of the resulting cells were placed in 24-well plates containing 11-mm coverslips for immunofluorescence microscopy. The remainder of the cells were placed in 12-well plates for SDS-PAGE/immunoblot analysis. Conditioned media and cells were collected 24 and 48 h post-transfection.

For immunofluorescence microscopy, transfected COS-7 cells grown on coverslips were washed once with PBS, fixed in 4% paraformaldehyde for 10 min, and permeabilized in methanol for 10 min. The treated cells were washed three times with PBS. They were then exposed to 10% donkey serum in PBS for 1 h at room temperature to prevent nonspecific binding of the relevant mouse and rabbit antibodies. After this step, the cells were stained with mouse anti-V5 antibody (Invitrogen) in the absence or presence of a mixture of rabbit anti-calnexin and anti-calreticulin antibodies (StressGen, Victoria, British Columbia, Canada). Calnexin and calreticulin reside in the endoplasmic reticulum (ER). Thus, antibodies directed against these two proteins were used to confirm the ER location of recombinant mT4 protein in the transfectants. Cy3-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) was used to detect the mT4-V5 fusion protein, whereas Cy5-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch) was used to detect calnexin and calreticulin. Stained cells were viewed with a Nikon Eclipse 800 microscope. Images were digitally captured using a CCD-SPOT RT camera and compiled using Adobe Photoshop software.

For SDS-PAGE/immunoblot analysis, samples of the transfected COS-7 cells and their conditioned media were boiled in SDS sample buffer containing β-mercaptoethanol. After electrophoresis, the resolved proteins were blotted onto polyvinylidene difluoride membranes (Bio-Rad). Each protein blot was exposed to Tris-buffered saline (15 ml) containing 5% non-fat milk, 0.1% Tween 20, 0.5% goat serum, and 3 μg of mouse anti-V5 antibody (Invitrogen) for 2 h at room temperature. After each blot was washed 3 times with Tris-buffered saline containing 10% donkey serum in PBS for 1 h at room temperature to prevent nonspecific binding of the relevant mouse and rabbit antibodies. After this step, the blots were incubated with a 1:1000 dilution of a stock solution of horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) for 1 h at room temperature. The immunoreactive proteins were detected by chemiluminescence (5% non-fat milk, 0.1% Tween 20, 0.5% goat serum, and 3 μg of mouse anti-V5 antibody (Invitrogen)) for 2 h at room temperature. The resulting digest was then subjected to SDS-PAGE/immunoblot analysis.

As noted under "Results," mT4 can be generated in COS-7 cells using the above expression strategy. Unfortunately, because mT4 remains tightly associated with the ER membrane of the cell, contaminating ER membranes cannot be removed and isolated. In this way, it will not be possible to activate convertively the generated recombinant protein. We previously generated pseudomyxom forms of mMCP-6 and mMCP-7 in High Five insect cells that in each instance contain an enteropeptidase-susceptible sequence between the natural propeptide and catalytic domain (14, 20). Because each pseudomyxom also contains the FLAG peptide Asp-Tyr-Lys-Asp-Asp-Val-Glu-Glu-Glu-Lys (Roche Molecular Biochemicals) was used to determine whether or not insect cell-derived mT4 is enzymatically active. In this assay, a 200-μl sample of the eluate from the above immunoaffinity column was added to 50 μl of 5 mM calcium chloride and 10 mM Tris- HCl, pH 5.0, containing or lacking 0.4 unit of enterokinase (New England Biolabs); the sample was then incubated at 37 °C for 2 h. To evaluate the enzymatic activity of insect material, 10 μl of 0.1% Triton X-100, pH 7.8, containing 10 mM calcium chloride and 200 μg of resorufin-labeled casein was used. After an 18-h incubation at 37 °C, each reaction was stopped by the addition of 480 μl of 5% trichloroacetic acid, followed by a 10-min centrifugation at >8,000 × g. A 400-μl sample of the resulting supernatant was mixed with 600 μl of 0.5 M Tris-HCl, pH 8.8, and the absorbance was measured at 574 nm. Because low molecular weight resorufin-labeled peptides released from its substrate are not precipitated by the trichloroacetic acid step, the concentration of resorufin-labeled peptides in the supernatant is directly proportional to the general proteolytic activity present in the sample. Trypsin (Sigma) was used as a positive control in this enzymatic assay.

A three-dimensional model of the extracellular domain of mature mT4 (i.e., residues 1–256) was built by MODELLER (27, 28) as described for other MC proteases (7, 12, 29, 30). The primary template for comparative protein structure modeling was the crystal structure of human trypsin βII (31). Within this region, the amino acid sequences of the two tryptases are 39% identical.

**RESULTS**

**Cloning and Sequence Analysis of mT4 cDNA**—When the mTMT, mMCP-6, and mMCP-7 cDNAs were used as templates to screen GenBank’s EST data base, an EST (accession number AI326140) that was somewhat homologous to all three target tryptases. The relevant EST was obtained from the Integrated Molecular Analysis of Gene Expression consortium, and nucleotide sequence analyses of the cDNA clones in the clone revealed that it encoded a portion of a serine protease (designated mT4 because it is the fourth member of this family of serine proteases) not previously described. 5′- and 3′-RACE approaches (Fig. 1A) were therefore carried out to deduce the nucleotide sequence of the missing portion of the transcript.
The deduced amino acid sequence of its cDNA suggested that mT4 could be initially translated as an ~36-kDazymogen consisting of 324 amino acids (Fig. 1B). Hydrophathy plot analysis (Fig. 1C) disclosed that mT4 possesses an unusual hydrophobic domain in its C terminus at residues 251–267. Phylogenetic analysis (Fig. 2A) of all known mouse proteins revealed that mT4 is most closely related to mTMT. However, the degree of identity is only 44%. Every tryptase in this family has an N-terminal sequence of Ile-Val-Gly-Gly when its propeptide is removed (Fig. 2B). Because the zymogen form of mT4 also possesses this sequence near its initially translated N terminus, it is predicted that the 54-residue propeptide is cleaved at the Arg-Ile site indicated in Fig. 1B. In its mature form, the protein portion of the catalytic domain of mT4 is predicted to have a molecular mass of ~30 kDa. However, because the mature protein contains four potential N-linked glycosylation sites at Asn116, Asn123, Asn156, and Asn229, post-translationally modified mT4 is expected to be somewhat larger in size in vivo.

When transfected into COS-7 cells, the level of immunoreactive mT4 in the conditioned media of the transfectants was below detection by SDS-PAGE/immunoblot analysis (Fig. 3A). Thus, very little, if any, recombinant mT4 was constitutively released from the transfectants. The fact that mT4 was preferentially recovered in the microsomal fraction of the cell lysates (Fig. 3B) confirmed that mT4 is a membrane-anchored protein. Immunohistochemical analysis of the transfectants revealed that mT4 was preferentially retained in the calexin/calreticulum-enriched ER (Fig. 3C). The fact that immunoreactive mT4 was not released after trypsin treatment (data not shown) confirmed that very little, if any, mT4 targets to the plasma membrane in transfected COS-7 cells. As assessed by SDS-PAGE analysis, an immunoreactive protein of ~40 kDa was identified in COS-7 cell transfectants that shifted to ~35 kDa after PNGase F treatment (Fig. 3C). Based on the magnitude of this change in its molecular weight, mT4 contains more than one N-linked glycan.

By using resorufin-labeled casein as a substrate, the level of proteolytic activity in the lysates of the mT4-expressing COS-7 cells was significantly greater than that in the lysates of control non-transfectants (data not shown). However, the amounts of active enzyme were low, and recombinant mT4 remained tightly associated with the membrane of the ER (Fig. 3). Thus, a bioengineered form of mT4 possessing the FLAG peptide at its C terminus was expressed in High Five insect cells (Fig. 4). In contrast to what occurs when similar FLAG derivatives of mMCP-6 (20) and mMCP-7 (14) are expressed in insect cells, the FLAG derivative of mT4 was not constitutively secreted from the insect cells. Nevertheless, the recombinant protein could be purified from the lysates of its expressing cells using the immunoadfinity column (Fig. 4A). Even though the amount of protein in fractions 5 and 6 of the eluate of the column was below detection by Coomassie Blue staining of a duplicate gel, the amount of protein in fraction 4 was identified in COS-7 cell transfectants that shifted to ~35 kDa after PNGase F treatment (Fig. 4C). Based on the magnitude of this change in its molecular weight, mT4 contains more than one N-linked glycan.
The overall fold of mature mT4 is predicted to be similar to that of most serine proteases (Fig. 5A). For example, like all other functional serine proteases, mT4 possesses the conserved triad (i.e. His41, Asp93, and Ser194) in its putative catalytic site. Like mTMT, mT4 lacks a number of the Pro and Tyr residues (31) that are needed for human tryptase βII, mMCP-6, and mMCP-7 to form tetramers. However, mT4 possesses the functionally important surface Trp-rich domain found in all other tryptases in this family. The presence of Asp^{188}, Gly^{215}, and Gly^{225} in mT4 also strongly suggests that it is a tryptase.

Nevertheless, the seven loops that form its substrate-binding cleft are unique (Figs. 2B and 5B). For example, residue substitutions in loops 3 and A in mT4 are predicted to result in shape differences relative to human tryptase βII (Fig. 5B).

Although mature mT4 has an overall net −5 charge at neutral pH, it is predicted to have two positively charged surface regions at diametrically opposite ends of the folded protein (Fig. 5A).

Arg^{33}, Arg^{34}, and Arg^{243} reside in one region, whereas...
ated product is isolated and nonglycosylated forms of mT4, respectively. The deglycosylated product is 35 kDa because it contains the additional V5 and His tags at its C terminus. A, mT4-expressing COS-7 cells were stained with a mouse monoclonal antibody directed against the V5 peptide (D), or antibodies directed against all three epitopes (P). Yellow color in F indicates co-localization of mT4 with calnexin and calreticulin. Based on this double staining approach, a substantial portion of the expressed mT4 is anchored in the ER membrane of the cell.

His<sup>162</sup>, Lys<sup>165</sup>, Lys<sup>166</sup>, Arg<sup>170</sup>, and Arg<sup>223</sup> reside in the other region.

**Nucleotide Sequence and Chromosomal Location of the mT4 Gene**—Although two DNA fragments were detected when a blot containing AvrII-, HindIII, or BglII-digested mouse genomic DNA was probed with the mT4 cDNA (Fig. 6A), subsequent nucleotide sequence analysis revealed that these findings were due to the presence of internal enzyme restriction sites within the gene. Thus, a single gene encodes mT4. The fact that no additional DNA fragment was observed when the genomic blot was probed at moderate stringency (data not shown) suggests that no closely related gene is present in the mouse genome.

The initial FISH analysis revealed that the mT4 gene resides on the proximal region of a small-sized chromosome believed to be mouse chromosome 17. Based on that data, a second experiment was conducted in which a probe specific for the telomeric region of chromosome 17 was co-hybridized with the mT4-containing genomic clone. This experiment resulted in the specific labeling of the telomere and the proximal portion of mouse chromosome 17. Measurements of 10 specifically labeled chromosomes 17 demonstrated that the mT4 gene is located at a position that is 23% of the distance from the heterochromatic-euchromatic boundary to the telomere of the chromosome. This area corresponds to the interface between bands 17A3 and 17 B1 (Fig. 6B) where the mMCP-6, mMCP-7, and mTMT genes reside. When 50 metaphase cells were analyzed, 77 exhibited specific labeling.

With a long range PCR approach, the entire mT4 gene was isolated and sequenced. The mT4 gene is ~5.0 kb in size and contains 6 exons (Fig. 7A). All exon/intron splice sites conformed to the GT/AG rule for other eukaryotic genes (33). Exons 1–6 consist of 126, 27, 166, 284, 155, and 237 bp, respectively, whereas introns 1–5 consist of 101, 291, 599, 2717, and 269 bp, respectively. The exon/intron organization of the mT4 gene differs somewhat from that of the other three mouse genes in this family (Fig. 7B). For example, although the mT4 transcript is similar in size to that of the other three mouse MC tryptase transcripts, its gene is larger due primarily to intron 4. The mT4 gene also has six exons, whereas the mTMT and mMCP-7 genes have five exons.

**Expression of mT4 in Immune and Non-immune Cells**—As assessed by RNA blot analysis, the level of mT4 mRNA was below detection in normal mouse bone marrow (Fig. 8A). Transgenic mice that have been induced to express abnormally high levels of IL-5 exhibit a constitutive eosinophilia (21). Although barely detectable amounts of mT4 mRNA were found in the bone marrow of IL-5 transgenic mice, larger amounts of this transcript were present in the IL-5-dependent eosinophils purified from the transgenic animals (Fig. 8A). In confirmation of these data, mT4 mRNA also could be detected in the jejunum of a T. spiralis-infected BALB/c mouse (Fig. 8B) precisely when the number of eosinophils are maximal in this tissue (34). mT4 mRNA could not be detected by RNA blot analysis in normal heart, brain, spleen, lung, liver, skeletal muscle, and kidney (Fig. 8C), as well as ear, tongue, stomach, and intestine (data not shown). Because these tissues contain substantial numbers of MCs, mT4 is the only member of its family that is not preferentially expressed in MCs. The level of mT4 mRNA also was below detection in day-7–17 mouse embryos (Fig. 8C). However, using an RT-PCR approach, mT4 mRNA was de-

![Image](74x561 to 272x729)

**FIG. 3. SDS-PAGE/immunoblot and immunohistochemical analysis of mT4-expressing 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 mT4 possessing the immunogenic V5 peptide at its C terminus (right lanes). Forty eight hours later, samples of the resulting conditioned media supernatants (S) and lysates of the cell pellets (P) were analyzed for the presence of recombinant protein with anti-V5 antibody. B, lysates of mT4-expressing COS-7 cells were fractionated to determine whether or not mT4 is a membrane-anchored protein. Shown is the immunoblot analysis of the membrane- (MF) and cytosol (CP)-enriched fractions. C, a sample of the cell lysates of mT4-expressing COS-7 cells was incubated 1 h in the absence (−) or presence (+) of PNGase F prior to SDS-PAGE/immunoblot analysis to determine whether or not mT4 contains N-linked glycans. Molecular mass markers are shown on the left. The arrow and the open arrowhead on the right point to glycosylated and nonglycosylated forms of mT4, respectively. The deglycosylated product is ~35 kDa because it contains the additional V5 and His6 peptides at its C terminus. D–F, mT4-expressing cells were stained with a mouse monoclonal antibody directed against the V5 peptide (D), or a mixture of rabbit antibodies directed against calnexin and calreticulin (E), or antibodies directed against all three epitopes (F). Yellow color in F indicates co-localization of mT4 with calnexin and calreticulin. Based on this double staining approach, a substantial portion of the expressed mT4 is anchored in the ER membrane of the cell.

![Image](354x495 to 508x730)

**FIG. 4. Evaluation of the enzymatic activity of insect cell-derived recombinant mT4.** A, recombinant mT4-FLAG was purified from the lysates of High Five insect cells using an immunoaffinity chromatography approach. The soluble proteins in lysates of mT4-FLAG-expressing cells were applied to an anti-FLAG antibody column. After the column was washed extensively, the pH of the buffer was changed to elute the bound recombinant protein. B, fractions 5 and 6 of the immunaffinity column were pooled and evaluated for their enzymatic activity before (−) and after (+) enterokinase (EK) treatment. Trypsin and activating buffer containing enterokinase alone were used as positive and negative controls, respectively, in this casein-susceptibility assay. As assessed by Coomassie Blue staining of a duplicate gel, the amount of protein in fractions 5 and 6 was below detection. Thus, the amount of trypsin used in the depicted experiment exceeds that of recombinant mT4.
detected in the testes and ovaries of adult mice (Fig. 8D). As assessed by two different in situ hybridization methods, mT4 is transiently expressed relatively late in spermatogenesis during the cap phase of acrosome formation (Fig. 9).

**DISCUSSION**

While at least six distinct tryptase genes reside at a complex on human chromosome 16 (1–3, 6, 7), only three corresponding genes have been identified so far on the syntenic region of mouse chromosome 17 (7–11). We now describe a new mouse gene in this family that is related to the genes that encode mTMT, mMCP-6, and mMCP-7.

The mT4 cDNA (Fig. 1) and gene (Fig. 7) encode a 324-residue polypeptide having a 54-residue prepropeptide and a 17-residue, C-terminal hydrophobic domain. When transiently expressed in COS-7 cells, mT4 remains cell-associated (Fig. 3). Thus, as predicted based on analysis of its cDNA and gene, translated mT4 is a membrane-anchored serine protease. Like the transmembrane protease angiotensin-converting enzyme (35), the C-terminal hydrophobic domain of mT4 is flanked by Asp and Arg residues. Because angiotensin-converting enzyme (35), prostasin (36), and acrosin (37) can be released from cells.

**Fig. 5.** Three-dimensional model of the catalytic portion of mature mT4 based on the crystal structure of human tryptase βII. A, a three-dimensional model of the catalytic domain of mature mT4 was created. Shown is the overall structure of residues 1–256 of mT4. Because human tryptase βII lacks a C-terminal hydrophobic domain, the short membrane-spanning domain in mT4 was not modeled. The active site residues (His41, Asp93, and Ser194) are represented as green sticks. The free Cys residue 113 is shown in orange. The side chains of the residues (His162, Lys165, Lys166, Arg170, Arg220, Arg223, Arg23, Arg34, and Arg33) that consist of the two positively charged surface regions are shown as blue sticks. The C-α atoms of the conserved residues (Trp12, Trp14, Trp206, Trp214, and Trp236) that form the hydrophobic domain opposite the substrate-binding cleft are shown as red spheres. Two of the conserved residues (Trp206 and Trp174) in the domain are hidden in this view. The figure was created with the programs Molscript (50) and Raster3D (51). The general orientation of the mT4 model is similar to that of the mMCP-6 (12), mMCP-7 (30), and mTMT (7) models in our previous publications. B, the putative substrate-binding cleft of mT4 was analyzed at a higher resolution using the modeling approach. The 7 loops that form the substrate-binding cleft of mT4 are marked A–D and 1–3 and are superimposed on the corresponding loops of human tryptase βII. The loops in mT4 and human tryptase βII are shown in red and blue, respectively; the active site residues are shown as green sticks.

**Fig. 6.** Genomic blot analysis and chromosomal location of the mT4 gene. A, a blot containing mouse genomic DNA digested with EcoRI, DraI, BamHI, AvaII, EcoRV, BglII, or HindIII was probed under conditions of high stringency with a radiolabeled 536-bp fragment derived from the 5’ end of the mT4 cDNA. DNA fragments of known molecular weight (HindIII-digested lambda DNA) are indicated on the left of the blot. As noted in Fig. 7A, the mT4 gene contains internal sites that are susceptible to AvaII, HindIII, and BglII. B, the chromosome location of the mT4 gene was determined by FISH analysis. The fluorescent-labeled, mT4-containing BAC clone hybridized specifically to a small-sized chromosome (arrow, left panel) that was subsequently shown to be chromosome 17. The location (arrow) of the mT4 gene on this chromosome is more clearly indicated in the right panel.
by a proteolytic processing mechanism, the possibility has not been ruled out that under certain situations mT4 undergoes a similar post-translational processing event in vivo to cause its release from cells.

The zymogen form of mT4 contains 10 Cys residues. Based on the crystal structure of human tryptase βII (31), Cys 26, Cys 42, Cys 127, Cys 160, Cys 179, Cys 190, Cys 200, and Cys 218 are predicted to form 4 disulfide bonds in the catalytic portion of the mature, properly folded protease (Fig. 5A). One of the additional Cys resides at residue 299 in the propeptide; the other resides at residue 113. Although Cys113 is not present in mMCP-6 or mMCP-7 (Fig. 2B), a corresponding Cys is present in the two-chained proteases factor XI (38), plasma kallikrein (39), and acrosin (40). Because this Cys forms a disulfide bond with a Cys residue in the propeptide of each zymogen, mature mT4 could be a two-chain serine protease consisting of a 33-residue, non-catalytic N-terminal chain covalently linked to the larger sized catalytic C-terminal chain.

Although its physiologic substrate(s) was not deduced in this initial study, mT4 is enzymatically active when expressed in FIG. 7. Structure of the mT4 gene. A, the nucleotides that consist of the six exons and five introns of the mT4 gene were deduced and are shown in upper and lowercase letters, respectively. The exons are boxed, and the deduced amino acid sequence of the translated product is indicated, as well as the components of the catalytic triad (●). The putative polyadenylation signal site in exon 6 is underlined. The BglII and AvaII restriction sites in introns 3 are italicized. B, the exon/intron organization of the mT4 gene was compared with that of the mTMT, mMCP-6, and mMCP-7 genes. Boxes (●) indicate exons. The size of each gene is indicated on the right. H, D, and S refer to the catalytic triad amino acids in each tryptase.

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insect cells (Fig. 4). mT4 has several features that indicate that it probably exhibits trypsin-like activity in vivo. For example, all trypsinic serine proteases possess an Asp six residues N-terminal of the catalytic Ser residue. This Asp is needed for interaction of the serine protease with the P1 Lys or Arg residue in the susceptible substrate (41). Not only does mT4 have the conserved Asp at residue 188 (Figs. 1, 2, 5, and 6), but also this residue is predicted to reside at the base of the substrate-binding cleft. As in other trypsinic proteases, Gly215 and Gly225 are present in mT4. Based on the crystal structures of rat trypsin (42) and human tryptase \( \beta \) II (31), Gly 225 is conserved because it contacts Asp 188 at the base of the substrate-binding cleft. Gly 215 resides in loop 2 (Fig. 5), and this surface loop helps define the substrate specificity of the serine protease (42). Although human tryptase \( \alpha \) I is an exception (25), trypsinic enzymes have a Gly at the corresponding site presumably because this small-sized amino acid residue facilitates entry of the bulky P1 residue of the substrate into the pocket of the enzyme.

When properly folded, a hydrophobic domain consisting of eight Trp residues forms on the surface of every MC tryptase opposite that of the substrate-binding cleft (31, 43). Expression/site-directed mutagenesis studies revealed that this domain is of functional importance in the maturation of mMCP-7 (26). The observation that mT4 contains these conserved residues (Trp12, Trp14, Trp15, Trp128, Trp132, Trp206, Trp214, and Trp236) (Fig. 2B) and that they reside on the appropriate surface region (Fig. 5A) is further evidence that mT4 is a functional tryptase in vivo.
Genomic blot analysis (Fig. 6A) revealed that there is only one mT4-like gene in the mouse genome, and FISH analysis (Fig. 6B) revealed that this new gene resides on mouse chromosome 17 quite close to the mMCP-6, mMCP-7, and mTMT genes. Two transcripts that differ slightly in their size were seen in the testis (Fig. 8C). Although the possibility has not been ruled out that the mT4 transcript can undergo alternative splicing in the testis, this seems unlikely because analysis of its gene (Fig. 7A) predicts that a functional enzyme would not be generated if any one of the 6 exons is deleted. Because sequence analysis of the eight RACE products failed to reveal a differentially spliced transcript, a more likely explanation for the RNA blot data is that different transcription-initiation sites are utilized regions.

The deduced amino acid sequence of mT4 is <45% identical to that of mMCP-6, mMCP-7, and mTMT (Fig. 2). Although mT4 is most homologous to human Esp-1 (hEsp-1), the sequence identity is still only 68%, and mT4 and hEsp-1 differ in a number of ways. At the protein level, hEsp-1 lacks the 3-residue cytoplasmic tail found in mT4 (Fig. 1B). Cytoplasmic tails often regulate intracellular routing of membrane proteins. hEsp-1 is a plasma membrane-anchored protease (4, 44). The fact that recombinant mT4 is unable to reach the plasma membrane in transfected COS-7 cells implies a regulatory role for the three cytoplasmic residues in the ER retention of mT4. The membrane-spanning domains of mT4 and hEsp-1 also differ substantially in their length and primary amino acid sequences, as do their prepropeptides. More important, the amino acid sequences that consist of 6 of the 7 loops that form the substrate-binding clefts of mT4 and hEsp-1 are very different (Figs. 2B and 5B). Thus, the preferred substrate specificities of these two tryptases are most certainly distinct in vivo. Although the mT4 (Figs. 8C and 9) and hEsp-1 (44) transcripts are present in abundance in the testis, their precise location in this tissue also differs. hEsp-1 has been reported to be expressed exclusively by primary spermatocytes before their first meiotic division (44). In contrast, as assessed by two different in situ hybridization methods, mT4 is transiently expressed in secondary spermatocytes (Fig. 9). Although RNA blot (Fig. 8A) and RT-PCR (Fig. 8B) data indicate that IL-5-dependent mouse eosinophils express mT4, the level of the mT4 transcript in vivo must play a more critical role in this biologic process. Although mouse testicular serine protease (mTesp)-1 (47), mMCP-2 (47), and mTesp-4 (48) were recently cloned from mouse testis and found to reside in the allosomal compartment of sperm, nothing is known about their in vivo functions and substrate specificities. The observation that each of these proteases possess an Arg residue in its propeptide adjacent to the N-terminal Ile in the mature enzyme suggests that an undefined tryptic-like enzyme is required for the proteolytic processing of their propeptides. Membrane-anchored, tryptic-like convertases such as human furin/PACE and yeast Kex2 play important roles in the post-translational processing of a diverse array of biologically active proteins (49). Because mT4 is a membrane-anchored serine protease that resides in the ER of transfected COS-7 cells and because this serine protease is predicted to possess tryptic-like enzymatic activity in vivo, this protease probably plays an important convertase-like role in the maturation of certain families of proteins.

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