Molecular Cloning, Chromosomal Localization, and Expression of Murine Dipeptidyl Peptidase I*

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Dipeptidyl peptidase I (DPPI,1 or cathepsin C) is a lysosomal cysteine protease that belongs to the papain superfamily of proteases (1, 2). DPPI is capable of sequentially removing dipeptides from the amino termini of various peptide substrates (3–7) and, along with other cysteine proteases, is thought to play an important role in intracellular protein degradation and turnover (4, 8, 9). This enzyme has other postulated functions, including involvement in cell growth (10), neunamidase activation (11), and platelet factor XIII activation (12). Several reports have also suggested that DPPI activity, although present in a wide variety of tissues, is significantly higher in cytotoxic lymphocytes and myeloid cells (13–15); in these cells, DPPI localizes to the secretory granule compartment and may be the major enzyme responsible for processing serine proteases from the proenzyme form to the catalytically active form (14, 15). Finally, two different reports have shown that DPPI can directly cleave the activation pro-dipeptides of granzymes A and B (16, 17), serine proteases that are found in the secretory granules of activated cytotoxic lymphocytes (CTL) and natural killer cells (18).

DPPI has been purified from several species (human, rat, bovine, porcine, and goat) (4, 19–23), and the rat and human cDNA sequences have been described (1–2, 24). The amino acid sequences deduced from these cDNAs encode a protein of 462 amino acids (rat) or 463 amino acids (human) with three functional domains: a signal peptide (28 versus 24 residues in rat and human, respectively), an unusually long propeptide (201 versus 206 residues), and the mature region (233 residues in both species). The mature proteins share 87.5% identity with each other, and 28.5–39.5% identity with other members of the papain family (cathepsins H, L, B, O, and K, and papain).

DPPI is initially synthesized as a 55-kDa proenzyme, and is rapidly converted to the two-chain form of the mature enzyme (the enzymes responsible for the proteolytic processing of DPPI are currently unknown). The two chains have molecular masses of 25 kDa (heavy chain) and <10 kDa (light chain) (25). Unlike most other cysteine proteases, which are small monomeric proteins with molecular masses of 20–30 kDa, DPPI exists as an oligomeric enzyme of about 200 kDa, as determined by gel filtration (4, 6, 26, 27). There are differing reports concerning the subunit composition of the enzyme, ranging from dimers to hexamers. Most studies suggest that the monomeric subunit is composed of a heavy chain and light chain (both originating from the mature region of the protein); however, a recent report presented evidence that active human DPPI consists of four identical subunits, each composed of three different polypeptide chains, two of them disulfide-linked (28). Two of the chains showed pronounced similarity to the heavy and light chains of the other papain-like cysteine proteases, whereas the third chain corresponds to the proregion of the enzyme. Thus, it appears that a substantial portion of the proregion remains bound to the mature complex. The regulation of DPPI processing and assembly are unknown.

In this paper, we report the sequence of murine DPPI cDNA, and the genomic organization and chromosomal localization of the gene. We determined that murine DPPI protein is widely distributed, with the highest levels found in the spleen, lung, liver, and small and large intestines. Additionally, our studies...
revealed that DPPI expression and activity are unchanged following in vitro T cell activation.

EXPERIMENTAL PROCEDURES

Molecular Cloning and Sequencing of Murine cDNA—Total RNAs from mouse thymus and spleen were isolated by the guanidinium thiocyanate method as described previously (29). The first strand of cDNA was obtained with reverse transcriptase (Promega) using random hexamer primers. Double-stranded cDNAs were amplified by PCR using Taq polymerase (Perkin-Elmer) using two sets of primers: 1) 5'-AACTGCACTTACCCTGACCTG-3' and 5'-GGCTTCATTGCAGCCACCATA-3' (to amplify the 5' portion of the cDNA); 2) 5'-TGCCCAAGGTTG-3'. Primer set 1 (forward)

Fig. 1, A. The nucleotide and deduced amino acid sequences of murine DPPI. The putative initiation methionine is assigned to the first in-frame ATG codon at position 25. The oligonucleotide primer sets 1 and 2 were used to initially screen thymus and spleen cDNAs. Oligonucleotide primer 3 was used to obtain the 5' untranslated region. This sequence has been submitted to GenBank with accession number U79269. B, comparison of the deduced amino acid sequence of murine DPPI with rat and human sequences. Gaps, indicated by dashes, are introduced to optimize the alignment. Asterisks indicate the conserved cysteine, histidine, and asparagine residues in the active site. Underlined sequences indicate the NH2-terminal sequences of the pro, mature, and light chains obtained by partial protein sequencing of DPPI (isolated from human spleen) (28). Arrowheads indicate the possible amino termini of the propeogen, heavy, and light chains. Bold residues indicate possible glycosylation sites.
B

FIG. 1—continued

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Murine Dipeptidyl Peptidase I

TGATGCGTGA-3' and 5'-CTACAAATTAGGACAGCGATGGATGCG-3' (to amplify the 3' portion of the cDNA). These primers were derived from the rat cDNA sequence. The amplified products were subcloned into the pCR2.0 vector (Invitrogen) and transformed into JM109. Plasmids with appropriate insert lengths were sequenced on an Applied Biosystems model 373 automated sequencer, using vector-based external primers and cDNA-specific primers constructed on the basis of previously obtained sequences.

To obtain the 5'-untranslated region and leader sequence, a mouse thymus cDNA library in λ ZAP was screened using PCR, with primers based on λ ZAP vector sequences and the reverse primer 5'-CGGAGC-GCAATTCTCCCTTGGT-3' (directed against sequences and the reverse primer 5'-CTACAATTTAGGAATCGGTATGGC-3' (to amplify the 3' portion of the cDNA). These primers were derived from the rat cDNA sequence. The amplified products were subcloned into pCR2.0 (Invitrogen) and sequenced.

Molecular Cloning of Murine Genomic DPPI—To obtain the full length genomic DPPI clone, the first 833 bp of the mouse cDNA sequence was used to screen a murine BAC library (Genome Systems, St. Louis, MO). Two clones (BAC 7930 and BAC 7931) hybridized strongly with the cDNA probe. DNA obtained from these clones were digested with EcoRI, and four fragments were subcloned into pUC19 based on the murine cDNA sequence. The amplified products were subcloned into pCR2.0 (Invitrogen) and sequenced.

Chromosomal Localization of DPPI by FISH—Chromosomes obtained from normal mouse spleen cultures were used for fluorescence in situ hybridization (FISH). The DPPI probe (BAC 7930) was labeled with biotin or digoxigenin using the Random-Prime DNA labeling kit (Boehringer Mannheim). The FISH protocol was described in detail elsewhere (31, 32). Slides were pretreated with RNase, denatured in 2 M SSC, 70% (v/v) formamide for 2 min at 70 °C. The DNA probes (200 ng), mouse Cot-1 DNA (Life Technologies, Inc.) in 2 × SSC-50% (v/v) formamide, 10% (w/v) dextran sulfate, 2 × Denhardt's solution, 1% Tween 20 (v/v) were denatured for 5 min at 70 °C, reannealed for 2 h at 37 °C, and hybridized in a humid environment for 18 h at 37 °C. A post-hybridization final wash was performed in 0.1 × SSC at 60 °C. Biotin- and digoxigenin-labeled DNA was detected by fluorescein isothiocyanate-conjugated avidin DCS (Vector Laboratories) and rhodamine-conjugated antidigoxigenin (Boehringer Mannheim), respectively.

Chromosomes were counterstained with propidium iodide or 4,6-diamino-2-phenylindole (DAPI) and examined with a Zeiss Axioshot epifluorescent microscope with a 100-watt mercury lamp. Digital images of selected metaphase spreads were recorded using cooled charge-coupled device camera CH250 (Photometrics, Tucson, AZ) and a filter system consisting of a triple-band-pass beam splitter and emission filters. Excitation of each of fluorochromes was accomplished by single-band-pass excitation filters in a computer-controlled motorized filter wheel. This made it possible to acquire sequential, registration shift-free gray-scale images of two or three fluorochromes (DAPI, fluorescein isothiocyanate, and/or rhodamine). Images were processed and analyzed on an Apple Power Macintosh 8100/100 computer using OnCor's recording and the analytic program Image, as well as NIH's Image and Yale University's Gene Join. To identify individual chromosomes and to assign the location of signal to specific chromosome regions, a method for direct visualization of fluorescent spots on look-up-table (LUT)-inverted digital images of DAPI banded chromosomes was used (33). To confirm the identity of chromosomes, preparations were hybridized (34) with a mouse chromosome 7 painting probe (Cambio, Cambridge, United Kingdom) and previously observed labeled metaphases were rerecorded.

RNA Preparations and S1 Nuclease Protection Assays—RNA was purified from adult 129/SvJ mouse organs or from activated splenocytes using a guanidinium thiocyanate miniprep protocol, as described (29). Since the first exon of DPPI did not contain any unique restriction sites that would permit the construction of a specific S1 probe, we designed a PCR probe spanning the 5'-flanking sequence and the putative first exon, using the primer set 5'-CCCAAAGGCCACTTTCCTTC-3' (forward) and 5'-GAACCTTCTAGGGCCACCCTG-3' (reverse). The 478-bp product was gel-purified and end-labeled using [γ-32P]ATP and polynucleotide kinase as described previously (35). 20 μg of total RNA was hybridized with 100,000 cpm of this labeled probe, and an S1 nuclease protection assay was performed as described previously (35). The specific probes for murine granzyme B and murine β2 microglobulin (β2M) have been described previously (36).

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Primer Extension Analysis—The reverse primer 5'-CAGGAGGTGC-GGAGCGGACGCTGAGCCTCCTCCTG-3' (directed against sequences in exons 1) was end-labeled with [γ-32P]ATP and polynucleotide kinase. 50–100 μg of total cellular RNA was lyophilized with 100,000 cpm of the labeled oligonucleotide, and primer extension was carried out as described previously (37).
Production of Activated Lymphocytes—Splenocytes were either activated with 5 μg/ml ConA (Sigma) supplemented with 50 units/ml recombinant human IL-2 or in a one-way mixed lymphocyte cultures as described previously (38). For the generation of Ad-Lak cells, splenocytes were cultured in the presence of high dose IL-2 (1000 units/ml) for 10 days as described previously (38).

Western Analysis—Organs from 129/SvJ mice and splenocytes harvested from IL-2/ConA activation were suspended in phosphate-buffered saline, and then an equal volume of 2× extraction buffer (2 M NaCl, 50 mM Tris, pH 8.0, 0.2% Triton X-100) was added. The extract was sonicated, and crude cellular debris was removed by centrifugation at 15,000 rpm at 4 °C for 15 min. Thirty μl of this extract was used in each lane of an SDS-PAGE analysis using reducing conditions. Immunoblotting was carried out using a rabbit anti-murine DPPI. Murine anti-DPPI was generated by immunizing rabbits (EL Labs, Soquel, CA) with recombinant DPPI protein (amino acids 230–462) made in Escherichia coli. The IgG fraction of the antiserum was purified and was shown to react specifically with recombinant DPPI protein (data not shown) and tissue extracts. The antibody recognizes the 25-kDa mature form of the DPPI protein, in agreement with a previous report (2). Murine granzyme B antiserum (38) and murine β-actin antiserum (Sigma) were used in control blots. Antibody-protein complexes were detected with a peroxidase-labeled anti-rabbit (DPPI and granzyme B) or anti-mouse (β-actin) antibody, followed by enhanced chemiluminescence (ECL, Amersham).

DPPI Activity Assay—Bovine DPPI, hydroxylamine hydrochloride, Gly-Phe amide, 2-mercaptoethylamine hydrochloride, trichloroacetic acid, and ferric chloride solutions (all from Sigma) were prepared according to manufacturer's directions. Protein concentrations of cell lysates were assayed using the Bio-Rad method (39) and bovine serum albumin as a standard. DPPI activity was assayed in a 0.50-ml reaction mix (pH 6.8) containing 400 mM hydroxylamine, 50 mM Gly-Phe amide, 25 mM 2-mercaptoethylamine, and 100 μl of cell lysate. The reaction was incubated at 37 °C overnight and stopped by the addition of 0.50 ml of 20% trichloroacetic acid and 0.50 ml of 5% ferric chloride solution. The absorbance values at 510 nm for control and test lysates were recorded, and units of DPPI activity/mg of protein were calculated using purified bovine DPPI (Sigma) (assayed simultaneously by the same method over a concentration range of 0.0001–0.1 units) as a standard.

RESULTS

Isolation and Analysis of Murine DPPI cDNA—We used a series of primers based on the rat cDNA sequence and RT-PCR to isolate the cDNA for murine DPPI. A putative TATA box (TATTTTA) and the polyadenylation signal (AATAAA) are underlined. The transcriptional initiation site is denoted as +1. The intron sequences are incomplete and are represented by dotted lines. The sizes of the exons, in base pairs, are indicated at the beginning of each segment. The intron sizes were estimated from PCR reactions. The Cys, His, and Asn residues, which form the active site, are denoted by asterisks. B, schematic representation of murine DPPI gene. Exons are depicted as black boxes. White boxes depict the 5′- and 3′-untranslated regions. The arrow indicates the transcriptional start site. B, BamHI; E, EcoRI; H, HindIII; P, PstI; X, XbaI. Note that this is not a comprehensive restriction map of the gene.

Production of Activated Lymphocytes—Splenocytes were either activated with 5 μg/ml ConA (Sigma) supplemented with 50 units/ml recombinant human IL-2 or in a one-way mixed lymphocyte cultures as described previously (38). For the generation of Ad-Lak cells, splenocytes were cultured in the presence of high dose IL-2 (1000 units/ml) for 10 days as described previously (38).

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RESULTS

Isolation and Analysis of Murine DPPI cDNA—We used a series of primers based on the rat cDNA sequence and RT-PCR to isolate the cDNA for murine DPPI. We were unable to obtain a full-length cDNA using primers that encompassed the entire mature rat coding region. However, using additional primer sets based on the rat sequence, we were able to amplify several 5′ and 3′ fragments from mouse and thymus cDNA, which together encompass most of the mature coding region. PCR fragments from at least three separate reactions for each primer set were subcloned and sequenced to confirm their identity. To obtain the 5′-untranslated region and leader se...
sequence, we used primers based on λ ZAP sequences and primers derived from the mouse sequence to screen a mouse thymus cDNA library. Fig. 1A shows the mouse cDNA and deduced amino acid sequences. The cDNA contains an open reading frame of 1386 nucleotides. The cDNA sequence isolated did not contain a poly(A) tail; however, a putative polyadenylation signal (AAATAA) is found 385 bp from the TAG stop codon in the genomic sequence (Fig. 2A). The mouse sequence in the coding region shares 92.8% and 82.1% identity with the rat and human cDNAs, respectively. The percentage of identity for all three species is highest in the region coding for the mature protein (Table I). The initiation codon was assigned to the first in-frame ATG at position 25; the nucleotide sequence around it (AGCATGG) is nearly a perfect match with the Kozak consensus sequence. The deduced amino acid sequence of the open reading frame encodes 462 amino acids, yielding a calculated molecular mass of 52.41 kDa. Alignment of the deduced amino acid sequence of the mouse DPPI with rat and human DPPI revealed 90.1% and 77.8% sequence identity, respectively. The three amino acid residues that form part of the active site of all papain family members (Cys25, His159, and Asn197, according to the papain numbering system) are conserved in the mouse sequence and across species (Fig. 1B). The mouse sequence in the coding region shares 92.8% and 82.1% identity with the rat and human cDNAs, respectively. The percentage of identity for all three species is highest in the region coding for the mature protein (Table I). The initiation codon was assigned to the first in-frame ATG at position 25; the nucleotide sequence around it (AGCATGG) is nearly a perfect match with the Kozak consensus sequence. The deduced amino acid sequence of the open reading frame encodes 462 amino acids, yielding a calculated molecular mass of 52.41 kDa. Alignment of the deduced amino acid sequence of the mouse DPPI with rat and human DPPI revealed 90.1% and 77.8% sequence identity, respectively. The three amino acid residues that form part of the active site of all papain family members (Cys25, His159, and Asn197, according to the papain numbering system) are conserved in the mouse sequence and across species (Fig. 1B). The mouse sequence in the coding region shares 92.8% and 82.1% identity with the rat and human cDNAs, respectively. The percentage of identity for all three species is highest in the region coding for the mature protein (Table I). The initiation codon was assigned to the first in-frame ATG at position 25; the nucleotide sequence around it (AGCATGG) is nearly a perfect match with the Kozak consensus sequence. The deduced amino acid sequence of the open reading frame encodes 462 amino acids, yielding a calculated molecular mass of 52.41 kDa. Alignment of the deduced amino acid sequence of the mouse DPPI with rat and human DPPI revealed 90.1% and 77.8% sequence identity, respectively. The three amino acid residues that form part of the active site of all papain family members (Cys25, His159, and Asn197, according to the papain numbering system) are conserved in the mouse sequence and across species (Fig. 1B).
tary to mRNA (located in the middle of exon 1) was end-labeled and annealed with 50–100 µg of total cellular RNA derived from mouse spleen or lung tissues. The primer extension product revealed a major band of 150 nucleotides (nt). The strongest band corresponds to the G residue 68 nt upstream from the ATG site, and was designated residue 1 (Fig. 4A). To confirm the location of the 5' end of mRNA, an S1 nuclease protection assay was performed. For a DPPI-specific S1 probe, we generated a double-stranded DNA fragment by PCR using a reverse primer derived from exon 1 and a forward primer derived from the 5'-flanking region. This probe was end-labeled and annealed to 20 µg of total cellular RNA derived from mouse spleen. A probe fragment of 212 nt was protected from S1 digestion by DPPI mRNA. A DNA sequencing ladder, generated using the same exon 1 primer, confirmed that the 5' end of DPPI mRNA is located at this site.

DPPI Is Constitutively Expressed in Many Tissues, and Is Not Up-regulated following Splenocyte Activation—We next examined DPPI protein levels in adult mouse organs using Western blot analysis (Fig. 5) with a polyclonal rabbit anti-DPPI antibody generated against recombinant murine DPPI (see “Experimental Procedures”). The Western blots showed that the 25-kDa mature form of DPPI is detected in almost all tissues, but at varying levels. DPPI is most abundant in lung, liver, spleen, and small and large intestines; the abundance is intermediate in bone marrow, thymus, and stomach, but low in kidney. Heart and brain have very little detectable DPPI protein. S1 nuclease protection assays revealed a similar wide tissue distribution of DPPI mRNA (data not shown).

To determine whether the expression of DPPI is regulated following in vitro lymphocyte activation, we examined DPPI mRNA levels in resting, day 10 Ad-Lak cells (obtained from splenocyte cultures in the presence of high dose IL-2), day 1–5 MLR cultures (Fig. 6), as well as day 2 and day 4 CTL derived from IL-2/ConA-activated splenocytes (Fig. 7A). These RNAs were analyzed using S1 nuclease protection assays with the exon 1 DPPI probe described above, a granzyme B probe (36) (as a marker of lymphocyte activation), and mouse β2 microglobulin (β2M) (as a control for RNA content and quality).
to Western blot analysis. Fig. 7B shows these blots sequentially probed for DPPI, granzyme B, and β2M. The same day 0, 2, and 4 cells were also used in a Western analysis (panel B). The same blot was probed sequentially with polyclonal antisera specific for DPPI, granzyme B, and actin (as a control for protein content). In panel C, these lysates were analyzed for DPPI activity, using purified bovine DPPI as a standard. The day 0 analysis was repeated six times, and the day 2 and 4 analyses were performed using two separate sets of activated lymphocytes. Enzyme activities were normalized for total protein content.

**DISCUSSION**

In this report, we characterized the murine DPPI gene and determined that this gene is highly conserved among species. The murine DPPI gene is localized on chromosome 7 and is a single copy gene. DPPI protein is widely distributed in mouse tissues; its expression is not up-regulated by mitogens or allogeneic stimuli known to activate T lymphocytes.

DPPI exhibits a number of similarities with other lysosomal papain-like cysteine proteases. First, in the mature region of the enzyme, the amino acid sequence of DPPI shares significant homology with the other members of the papain family of cysteine proteases (up to 39% in the case of cathepsin H). It also retains the three amino acid residues that form the active sites of all papain-like proteases. However, unlike other cysteine proteases, DPPI contains a very long proregion, the function of which is still unknown. Although most other cysteine proteases are small monomeric proteins with molecular masses of 20–30 kDa, DPPI exists as an oligomeric enzyme of about 200 kDa. DPPI therefore represents a distinct class of papain-like proteases.
In this report, we have defined the genomic organization of the murine DPPI gene. Southern blot analysis and chromosomal analysis by FISH revealed that DPPI represents a single locus in the mouse genome. The gene was localized by FISH to chromosome 7 at region D3-E1.1. This region has limited homology to human chromosome 15q 11-ter (41–43), but synteny with this region has not yet been established. Several genes have been localized within the same region of chromosome 7. The oncogene product of feline sarcoma virus and the gene for mitochondrial isocitrate dehydrogenase have been localized to the 7 B3-E1 region; their human counterparts are located on chromosome 6p25–24 and 11q21, respectively (41–43). Finally, the mouse hepatic fusion mesoderm development and taupe genes (41–43) are localized within the same region as DPPI.

The murine DPPI gene has seven exons and spans more than 20 kb. Cathepsins B and H both also span more than 20 kb, and comprise 10 and more than 12 exons, respectively (44, 45). In contrast, cathepsin L is a relatively compact gene of 8.5 kb, comprising 8 exons (46). The intron-exon organization of cysteine proteases is only partially conserved (Fig. 8). DPPI, and cathepsins H and L share the common intron insertion position that isolates the exon containing the active site cysteine residue (47). This highly conserved domain is split by an intron in cathepsin B (44). DPPI also shares one common intron insertion site with cathepsin B (intron 6-exon 7 for DPPI and intron 5-exon 6 for cathepsin B). In all four genes, the active site histidine and asparagine residues are found within one exon, although the exon on which they reside, and the positions of the intron-exon insertion sites, differ from gene to gene (44, 47). These similarities suggest that these cysteine proteases arose from a common ancestral gene, but that their structures have evolved to include intron losses and insertions.

Several studies have suggested that DPPI activity is enriched in 

in vitro and 

in vivo activated cytotoxic lymphocytes (14). In addition, DPPI activity was found to colocalize with serine protease activity (such as granzyme A tryptase activity) in the specialized granule fraction of CTL (14). Several studies have also demonstrated the ability of DPPI to directly cleave the prodipeptide of various serine proteases, thereby activating them (16, 17). To examine whether the expression of DPPI is regulated during 

in vitro lymphocyte activation, we examined DPPI mRNA and protein levels, and DPPI activity (using a defined synthetic substrate) in resting splenocytes, or splenocytes activated with various protocols (Figs. 6 and 7). To our surprise, we found that DPPI mRNA were not up-regulated in Ad-Lk cells, MLR-derived cells, or IL-2/ConA-activated cells. Moreover, the protein levels and enzymatic activities seem to correlate with mRNA expression.

The discrepancy between these findings and previously published data (13–15) (measuring DPPI enzymatic activity only) may be due to several factors. In our study, we used unfractionated splenocytes; activated CD8+ T cells could potentially contain higher levels of DPPI activity than resting splenocytes or activated CD4+ T cells and B lymphocytes. However, we were also unable to find a significant increase in DPPI levels in either MLR or Ad-Lk cells, both of which contain relatively pure populations of activated cells. Also, in one study, the elevated levels of DPPI activity were not detected in all activated populations of CD8+ T cells (14). In fact, the level of DPPI activity in 

in vitro activated CD8+ T cells was unchanged compared with resting CD8+ T cells, which agrees with our data. Furthermore, despite the wide variations in levels of DPPI activity in various populations of 

in vitro and 

in vivo activated CTLs, these T cells all exhibited similar levels of cytolytic activity (14). These results suggest that DPPI is not up-regulated during the activation of T cells, but that its expression is constitutive.

These data do not alter the hypothesis that DPPI plays an important role in the posttranslational processing of serine proteases. Lytic granules (and the serine proteases contained within them) are not found in resting T cells, but are synthesized after T cell activation. The serine proteases undergo proteolytic processing during their intracellular transport and are packaged into secretory granules (with the prodipeptides presumably already cleaved). DPPI therefore may interact with the serine proteases shortly after they leave the Golgi apparatus, although the exact subcellular compartment where this processing takes place is not yet established.

The availability of DPPI probes and antisera will enable us to further study the functions of DPPI in normal and pathological conditions. Expression of purified rDPPI protein will also help to establish the role of this protease in granzyme processing. Finally, the creation of a loss of function mutation in mice, using homologous recombination techniques, should clarify the importance of this enzyme in vivo.

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