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Cloning and expression of the recombinant mouse natural killer cell granzyme *Met-ase-1*

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Abstract *Met-ase-1* is a 30 000 M_r serine protease (granzyme) that was first isolated in the cytolytic granules of rat CD3$^-$ large granular lymphocytes. We screened a mouse genomic library with rat *Met-ase-1* cDNA, and obtained bacteriophage clones that contained the mouse *Met-ase-1* gene. The mouse *Met-ase-1* gene comprises five exons spanning approximately 5.2 kilobases (kb) and exhibits a similar structural organization to its rat homologue and a family of neutrophil elastase-like serine proteases. Mouse *Met-ase-1* mRNA was only detected in total cellular and poly A mRNA of mouse CD3$^-$ GM1$^+$ large granular lymphocytes derived from splenocytes stimulated with IL-2 and the mouse NK1.1$^+$ cell line 4–16. Spleen T-cell populations generated by Concanavalin A stimulation and a number of mouse pre-NK and T cell lines did not express mouse *Met-ase-1* mRNA. The 5′ flanking region of the mouse *Met-ase-1* gene also shares considerable regions of identity with the 5′ flanking region of the rat *Met-ase-1* gene. A 3.3 kb segment of 5′ sequence flanking the mouse *Met-ase-1* gene was inserted upstream of the chloramphenicol acetyltransferase reporter gene and this construct transiently transfected into a variety of mouse and rat large granular lymphocyte leukemia and T-cell lines. The transcriptional activity of the mouse *Met-ase-1* 5′ flanking region was significant in the RNK-16 large granular lymphocyte leukemia, strongest in the 4–16 mouse NK1.1$^+$ cell line, and weak in several mouse pre-NK cell lines. Reverse transcriptase polymerase chain reaction of mouse large granular lymphocyte mRNA was used to derive the full-length coding sequence for mouse *Met-ase-1*. The predicted hexapropeptide of mouse *Met-ase-1* (Asn^{-6} to Gln^{-1}), was deleted by polymerase chain reaction mutagenesis to enable expression of active mouse *Met-ase-1* in mammalian COS-7 cells. Northern blot analysis and protease assays of transfected COS cell lysates against a panel of thiobenzyl ester substrates formally demonstrated that the mouse *Met-ase-1* gene encodes a serine proteinase that hydrolyzes substrates containing a long narrow hydrophobic amino acids like methionine, norleucine, and leucine in the P_1.

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession number L76741

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Introduction

Until recently, granzyme genes were known to encode just three enzyme activities (tryptase (trypsin-like), Asp-ase (after Asp residues) and chymase (chymotrypsin-like) specificities; Jenne and Tschopp 1988 a). With the development of model synthetic peptide substrates, we purified a novel serine protease, that cleaves after methionine (designated *Met-ase-1* (*Met-1*), from the cytotoxic granules of the rat large granular lymphocyte (LGL) cell leukemia, RNK-16. Isolation and cloning of the cDNA encoding rat *Met-1* revealed rat *Met-1* (*RMet-1*) to be a unique granzyme with a predicted amino acid sequence less than 45% identical to any other member of the serine protease family (Smyth et al. 1992). A cDNA clone encoding a human LGL-specific *Met-1* (*HMet-1*) was obtained using the *RMet-1* cDNA clone (Smyth et al. 1993). Unlike other members of the granzyme family which are highly expressed in activated peripheral T cells, *RMet-1* and *HMet-1* have a restricted expression in cells of CD3$^-$ LGL phenotype. Thus far, *Met-1* gene transcripts have not been detected in other resting or activated primary cell populations or cell lines including thymocytes, CD4$^+$ and CD8$^+$ T cells, B cells, myelomonocytic cells, granulocytes, or in a variety of non-lymphoid tissues (Smyth et al. 1992, 1993, 1995 a; Smyth and coworkers, unpublished data).

The evolution of a subfamily of *Met-1* granzymes distinct from those previously described (Jenne and Tschopp 1988 a) has been suggested by chromosomal

gene mapping studies. The *HMet-1* gene (*GZMM*) is located on chromosome 19p13 (Baker et al. 1994; Pilat et al. 1994) and the equivalent mouse *LMet1* gene is located on a syntenic region of chromosome 10 (Pilat et al. 1994; Thia et al. 1995). Interestingly, both genes are closely linked to a cluster of neutrophil elastase-like serine proteases (Zimmer et al. 1992). In this study we cloned and characterized the mouse *Met-1* gene (*MMet-1*) in order to determine whether: 1) *MMet-1* has a unique gene organization compared with other granzyme family members; 2) *MMet-1* and *RMet-1* share similar 5′ flanking sequences; and 3) *MMet-1*, like *HMet-1* and *RMet-1*, can cleave substrates C-terminal to Met, Nle, and Leu amino acids.

Materials and methods

Isolation of genomic mouse Met-1 clones

A full-length cDNA transcript encoding the RMet-1 protein (Smyth et al. 1992), was radiolabeled by random priming (Sambrook et al. 1989) and used to screen a mouse genomic library constructed in λ fix II (Stratagene, La Jolla, CA) using standard protocols (Sambrook et al. 1989). A single reactive phage clone was plaque purified, the phage DNA was isolated, and a number of overlapping mouse genomic DNA fragments, including 0.65 kilobase (kb) *Pst* I, 3.0 kb *Bam* HI, and 5.5 kb *Eco* RI, were subcloned into the pBluescript KS$^+$ (KS$^+$) plasmid vector (Stratagene). To elucidate the 5′ sequence of *MMet-1*, we assumed a high level of identity between *RMet-1* and *MMet-1* sequences and *RMet-1* primers:
5′-TGGCTGTAGTTGCTGCCC-3′ (sense, –192, Smyth et al. 1995b)
5′-TGCCCACAGTGTTTTCAG-3′ (antisense, +54)
were used to generate a polymerase chain reaction (PCR) product derived from the *MMet-1* genomic clone, MGM#4, that encoded 192 base pair (bp) 5′ untranslated (UT) and the entire sequence of exon I. This PCR product (P-1) was cloned into the pCR3 vector (Invitrogen, San Diego, CA) and sequenced. A second PCR product (P-2) was derived from MGM#4 using *RMet-1* and *MMet-1* primers:
5′-CTAAGCTTGCGGCCGCACACCCTCACTGTCTGAC-3′ (sense, –3246, Smyth et al. 1995b)
5′-TGTCTAGACCTGGCACAGGATCAGTG-3′ (antisense)
This product included a further 3.4 kb of 5′ flanking region and was also cloned into pCR3 and pCAT basic (see below).

Isolation of mouse Met-1 cDNA

MMet-1 cDNA was isolated by reverse transcriptase (RT)-polymerase chain reaction (PCR) from total cellular RNA derived from interleukin-2 (IL-2)-activated BALB/c splenocytes. The sequence of the *MMet-1*-specific primers used to generate the *MMet-1* cDNA were determined as follows. The 3′ sequence of the *MMet-1* cDNA was determined from the *MMet-1* genomic *Eco* RI DNA fragment (also including 3′ UT cDNA). An 18-mer 3′ oligonucleotide was prepared that included the TGA termination codon and a *Not* I restriction site;
5′-CTCTGCGGCCGCTCAGACCAAAGATTGGGG-3′.
A 27-mer 5′ oligonucleotide was synthesized that comprised the first 18 nucleotides of sequence encoding translated *MMet-1* and an *Xho* I restriction site;
5′-CTCTCTCGAGATGGAGGTCTGCTGGTC-3′.
Total cellular RNA was prepared from 10^7 cells in RNAzol (Cinna-Biotecx Labs, Houston, TX; Chomczynski and Sacchi 1987). Single-stranded cDNA synthesis was performed on 2 μg total RNA with random hexamers (0.1 μg) and 200 units M-MLV RT (Gibco BRL, Life Technologies, Melbourne, Australia). PCR amplification was carried out in 100 μl using 30% of the cDNA reaction with 20-pmole of primers, 0.2 mM dNTP, 1.25 mM $MgCl_2$, and 1.5 units *Taq* polymerase (Gibco). Amplification involved 30 cycles of 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min, followed by a single cycle of 95 °C for 5 min, 52 °C for 1 min, and 72 °C for 7 min using a DNA Thermal Cycler (Perkin Elmer-Cetus, Norwalk, CT). The PCR product was cloned into the CDM8 plasmid vector using the *Xho* I/*Not* I sites of the polylinker region. Sequencing of the gene was performed in both orientations using synthetic oligonucleotides, the dideoxy method (Sanger et al. 1977), and single-stranded and double-stranded templates (Chen and Seeburg 1985).

Nucleotide sequencing

The entire final sequence was determined from both strands using the following primers (depicted in Figure 1A). The approximate size of introns 1 and 2 were estimated by restriction enzyme mapping and PCR using primers from 5′ exon 1 and intron 2 or exon 3, respectively. DNA sequence alignments were performed following comparison of the *MMet-1* nucleotide sequence against GenBank, EMBL, and accessed individual granzyme sequences. An analysis of potential transcriptional regulatory elements in the *MMet-1* 5′ flanking region was performed by using SIGNAL SCAN (Prestridge 1991), and lineups with *RMet-1* and *HMet-1* 5′ flanking regions were generated using BESTFIT (GCG Sequence Analysis Software Package, Genetics Computer Group, Madison, Wisconsin). Numbering of nucleotides refers to the translational start codon ATG = +1.

Genomic Southern analysis

High M_r genomic DNA was extracted using guanidinium isothiocyanate, as reported previously (Smyth et al. 1995b). Twenty micrograms of DNA was digested with 50 units of the designated restriction enzyme (New England BioLabs, Beverly, MA) overnight at 37 °C in the manufacturer's restriction enzyme buffer. DNA was then subjected to electrophoresis in a 1.0% agarose gel, transferred to Nytran membrane (Schleicher and Schuell, Keene, NH), and hybridized to the *MMet-1* cDNA in hybridization buffer at 42 °C. Southern blots were washed (final, 0.2× standard sodium citrate, 0.1% sodium dodecyl sulfate and exposed to film as previously described (Smyth et al. 1995b).

Spleen cell culture

Resting spleen cells were those immediately taken from BALB/c mice prior to culture. Spleen cells from BALB/c mice (1 to 5×10^5/ml) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco) and either 100 units/ml IL-2 (a generous gift from Chiron Corp.) or Concanavalin A (ConA; 1 μg/ml; Sigma Chemical, St. Louis, MO). After 7 days, the cells were harvested for Northern analysis. IL-2 stimulated cultures were predominantly CD3$^-$ GM1$^+$ while ConA-treated cultures were CD3$^+$ GM1$^{+/-}$ (data not shown).

Northern analysis

Total cellular RNA was obtained from the following cell lines and tissues. The mouse/rat cell lines: RNK-16, a rat LGL leukemia; YAC-1, a mouse T cell lymphoma; and EL-4, a mouse T-cell lymphoma were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco). The mouse pre-NK cell lines SC-1 and f8 (a generous gift from L. Ratner, Washington University, St. Louis, MO; Grossman et al. 1995) and the mouse myeloma cell line, NS-1, were maintained in DME with the same additives. The mouse cytotoxic T cell line, CTLLR8 (Palladino et al. 1983), and mouse pre-NK cell line, f197 (L. Ratner), were maintained in DME, additives and 100–250 units/ml IL-2. The mouse NK1.1$^+$ NK cell line, 4–16, was generously provided by T. Ley (Washington University, St. Louis, MO) and was maintained in RPMI, additives, 15 mM Hepes, and 250 units/

ml IL-2. Mouse $CD3^-$ $GM1^+$ LGL were generated from mouse BALB/c splenocytes by IL-2 stimulation for 7 days, $CD3^+$ T cells were generated by culture in ConA for 7 days. Total cytoplasmic RNA was purified from all cells (Chomczynski and Sacchi 1987). Poly A^+ mRNA was isolated from 100 μg of total cellular RNA using a PolyAttract mRNA isolation system (Promega, Madison, WI). The entire mRNA was used for Northern analysis or 20 μg of total cellular RNA was subjected to electrophoresis on a 1.0% agarose formaldehyde gel, then transferred to Nytran. Blots were hybridized to ^{32}P-labeled *MMet-1* cDNA, mouse granzyme B cDNA (CCP1; Lobe et al. 1986a), mouse 18S rRNA or human γ-actin (Gunning et al. 1983) cDNA as described previously (Thomas 1983). The blots were then exposed to Kodak X-OMAT AT film for 1 to 5 days at –70 °C.

CAT vectors

An *MMet-1* gene promoter fragment was derived by PCR and cloned upstream of a promoterless and enhancerless chloramphenicol acetyl transferase (CAT) gene in *Hin* dIII and *Xba* I restriction sites (pCAT-basic; Promega). Oligonucleotide primers used for PCR were:

5′-CTAAGCTTGCGGCCGCACACCCTCACTGTCTGAC-3′
(5′ *Hin* dIII – sense)

5′-TGTCTAGACCTGGCACAGGATCAGTG-3′
(3′ *Xba* I – antisense)

The annealing temperature for the PCR was 2–4 °C below the predicted melting temperature. PCR products were gel purified, digested with *Hin* dIII and *Xba* I, and ligated into *Hin* dIII-/*Xba* I-digested pCATbasic. Sequencing of clones was performed as above. The RSVCAT plasmid was obtained from H. Young (NCI-Frederick Cancer Research and Development Center, Frederick, MD). The pCAT basic plasmid was used as a promoterless and enhancerless control construct. All plasmid DNA for electroporation was purified by two rounds of banding on CsCl gradients.

Transient transfection

Exponentially growing cells were washed and resuspended at 5×10^7 cells/ml in Dulbecco's modified Eagle medium (DMEM) with 10–20 μg plasmid DNA. Cells (250 μl suspension) were electroporated at 960 μF and 260 V (RNK-16, 4–16, f8, f197, SC-1) or 240 V (CTLLR8, YAC-1, NS-1, EL-4; Gene-pulser, Bio-Rad Laboratories, Richmond, CA). After incubation for 10 min on ice, the cells were diluted with warm medium and cultured in a humidified CO_2 incubator. CAT enzyme produced by transfected cells was assayed quantitatively using a sandwich enzyme linked immunoadsorbent assay (ELISA) technique (CAT-ELISA kit, Boehringer Mannheim, Sydney, Australia) as previously described (Smyth et al. 1995b). COS-7, an African Green Monkey kidney cell line, was maintained in DMEM (Gibco) supplemented with 10% fetal calf serum, 5×10^{-5} M β-mercaptoethanol, 2 mM glutamine, 0.1 mg/ml streptomycin, 100 units/ml penicillin. COS-7 cells were transfected in 10 cm plastic dishes with 20 μg of plasmid DNA, using the DEAE-dextran method, as described (Aruffo and Seed 1987). Cells were harvested after 72 h, washed, lysed and the lysate analyzed for proteolytic activity and by northern blotting.

Expression vector construction and mutagenesis of propeptide

The cDNA encoding full-length *MMet-1* was subcloned in the sense orientation into the mammalian expression vector CDM8 (Invitrogen, San Diego, CA). Deletion of the eighteen nucleotides (encoding residues Asn^{-6} to Gln^{-1}) of the *MMet-1* cDNA was performed by PCR splice overlap extension (SOE). For PCR-SOE, internal *MMet-1* oligonucleotide primers were as follows:

[–6 to –1 mutant (hd)]
5′-GCAGCAGGCATCATTGGGGGTCGAGA-3′ (sense)
5′-CCCAATGATGGCTGCTGCCCACAGTGTT-3′ (antisense)

External oligonucleotide primers and for *MMet-1* PCR-SOE were derived from flanking CDM8 sequences bordering the unique *Xho* I and *Not* I sites

5′-GTCTCCACCCCATTGACG-3′ (5′ *Xho* I from CDM8)
5′-CTCTGTAGGTAGTTTGTCC-3′ (3′ *Not* I from CDM8)

The annealing temperature for the PCR-SOE was 2–4 °C below the predicted melting temperature of the overlapping DNA fragments. SOE products were gel purified, digested with *Xho* I and *Not* I, and ligated into *Xho* I-/*Not* I-digested CDM8. Sequencing of PCR-SOE CDM8 clones was performed using synthetic oligonucleotide primers, and the entire sequence of each PCR-generated clone was verified from both strands.

Assay of protease activities

α-N-benzyloxycarbonyl-L-lysine-thiobenzylester (BLT; Sigma) used to measure BLT-esterase (tryptase) activity; Boc-Ala-Ala-Asp-SBenzyl (Bzl) (Enzyme Systems Products (ESP), Dublin, CA) used to measure Asp-ase activity; and Boc-Ala-Ala-Met-SBzl (ESP) used to measure Met-ase activity; were all purchased. Additional substrates including Suc-Phe-Leu-Phe-SBzl (Chymase activity), Boc-Ala-Ala-Nle-SBzl (Nle-ase) and Boc-Ala-Ala-Leu-SBzl (Leu-ase) were a kind gift from J. Powers (Georgia Institute of Technology, Atlanta, GA). All protease activities in purified cytoplasmic extracts from transiently transfected COS-7 cells were estimated using a microtiter assay (Smyth et al. 1993). Fifty microliters of dilutions of cytoplasmic extracts [1 to 10×10^6/ml lysis buffer (0.3 to 3.0 mg protein/ml), 50 mM Tris HCl, 0.15 M NaCl, 0.5% (v/v) NP40, 0.5 mM ethylenediaminetetraacetate, pH 7.2] were added to 100 μl of 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid; DTNB), made up in 100 mM Hepes, 50 mM $CaCl_2$, pH 7.5. The reaction was initiated by the addition of 50 μl of substrate (BLT – final concentration of 150 μM and other thiobenzyl ester substrates (Boc-

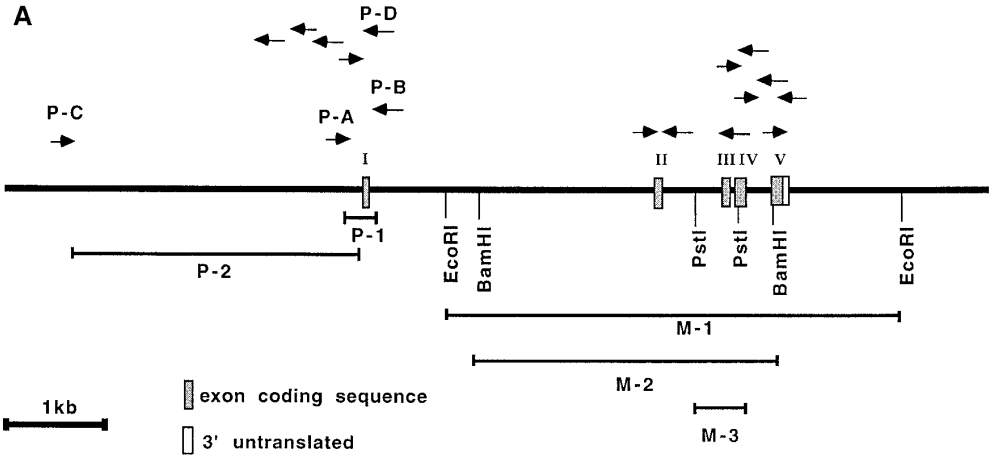

Fig. 1 (For continuation and legend see p. 344)

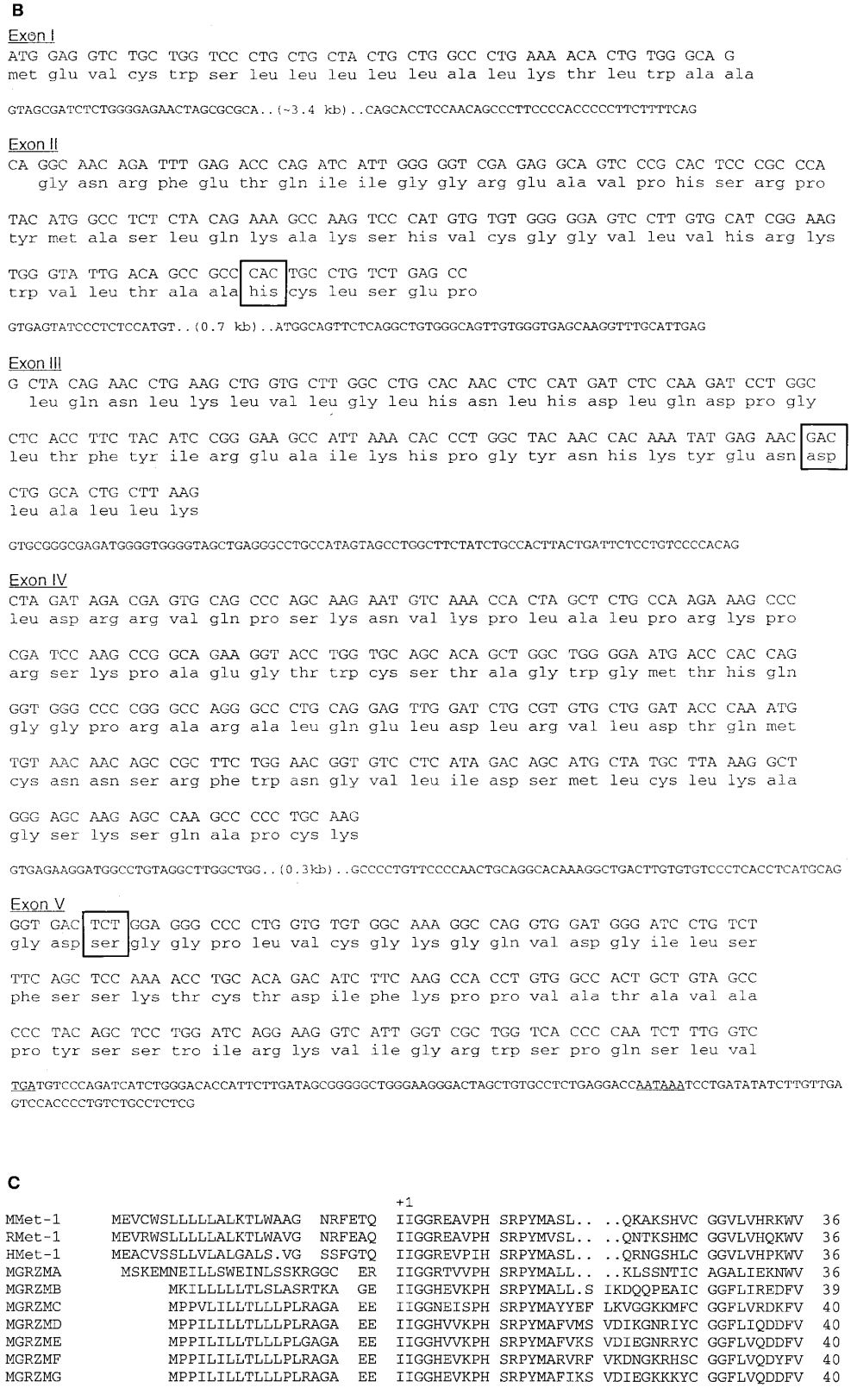

Fig. 1 (For continuation and legend see p. 344)

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MMet-1      LTAAHCLSE. PLQNLKLVLG LHNL-HDLQD PGLTFYIREA IKHPGY..NH KYENDLALLK          92
RMet-1      LTAAHCLSE. PLQQLKLVFG LHSL.HDPQD PGLTFYIKQA IKHPGY..NL KYENDLALLK          92
HMet-1      LTAAHCLAQ. RMAQLRLVLG LHTL..DS.. PGLTFHIKAA IQHPRYKPVP ALENDLALLQ          91
MGRZMA      LTAAHCNVG. ..KRSKFILG AHSINKEPEQ QILTV.KKAF PY.PCY.DEY TREGDLQLVR          90
MGRZMB      LTAAHCEGS. ...IINVTLG AHNI.KEQEK TQQVIPMVKC IPHPDYNP.K TFSNDIMLLK          93
MGRZMC      LTAAHCKGR. ...SMTVTLG AHNI.KAKEE TQQIIPVAKA IPHPDYNP.D DRSNDIMLLK          94
MGRZMD      LTAAHCKNSS VQSSMTVTLG AHNI.TAKEE TQQIIPVAKD IPHPDYNA.T IFYSDIMLLK          98
MGRZME      LTAAHCANA. ...TMTVTLG AHNI.KAKEE TQQIIPVAKA IPHPDYNA.T RFTNDIMLLK          94
MGRZMF      LTAAHCTGS. ...SMRVILG AHNI.KAKEE TQQIIPVAKA IPHPAYDD.K DNTSDIMLLK          94
MGRZMG      LTAAHCRNR. ...SMTVTLG AHNI.KAKEE TQQIIPVAKA IPHPAFNR.K HGTNDIMLLK          94

MMet-1      LDRRVQPS.. KNVKPLALPR KPRSKPAEGT WCSTAGWGMT HQGG.PRARA LQELDLRVLD          149
RMet-1      LDGRVKPS.. KNVKPLALPR KPRDKPAEGS RCSTAGWGIT HQRG.QLAKS LQELDLRLLD          149
HMet-1      LDGKVKPS.. RTVRPLALPS K.RQVVAAGT RCSMAGWGLT HQGG.RLSRV LRELDLQVLD          147
MGRZMA      LKKKATVN.. RNVAILHLPK KGDDVK.PGT RCRVAGWGRF GNKS.APSET LREVNITVID          146
MGRZMB      LKSKAKRT.. RAVRPLNLPR RNVNVK.PGD VCYVAGWGRM APMG.KYSNT LQEVELTVQK          149
MGRZMC      LVANAKAT.. RAVRPLNLPR RNAHVK.PGD ECYVAGWGKV TPDG.EFPKT LHEVKLTVQK          150
MGRZMD      LESKAKAT.. KAVRPLKLPR SNARVK.PGD VCSVAGWGSR SINDTKASAR LREVQLVIQE          155
MGRZME      LESKAKAT.. KAVRPLKLPR PNARVK.PGD VCSVAGWGPR SINDTKASAR LREAQLVIQE          151
MGRZMF      LESKAKAT.. KAVRPLKLPR PNARVK.PGH VCSVAGWGRT SINATQRSSC LREAQLIIQK          151
MGRZMG      LESKAKRT.. KAVRPLKLPR PNARVK.PGD VCSVAGWGKT SINATKASAR LREAQLIIQE          151

MMet-1      TQMCNNSRFW NGV..LIDSM LCLKAGSKSQ APCKGDSGGP LVCGKGQVD. GILSFSSKTC          206
RMet-1      TRMCNNSRFW NGV..LTDSM LCLKAGAKGQ APCKGDSGGP LVCGKGKVD. GILSFSSKNC          206
HMet-1      TRMCNNSRFW NGS..LSPSM VCLAADSKDQ APCKGDSGGP LVCGKGRVLA GVLSFSSRVC          205
MGRZMA      RKICNDEKHY NFHPVIGLNM ICAGDLRGGK DSCNGDSGSP LLCDG..ILR GITSFGGEKC          204
MGRZMB      DRECES.YF. .KNRYNKTNQ ICAGDPKTKR ASFRGDSGGP LVCKK..VAA GIVSYGYKD.          203
MGRZMC      DQVCES.QF. .QSSYNRANE ICVGDSKIKG ASFEEDSGGP LVCKR..AAA GIVSYGQTD.          204
MGRZMD      DEECKK.RF. ..RYYTETTE ICAGDLKKIK TPFKGDSGGP LVCHN..QAY GLFAYAKNG.          208
MGRZME      DEECKK.RF. ..RHYTETTE ICAGDLKKIK TPFKGDSGGP LVCDN..KAV GLLAYAKNA.          204
MGRZMF      DKECKK.YF. ..YKYFKTMQ ICAGDPKKIQ STYSGDSGGP LVCNN..KAV GVLTYGLNR.          204
MGRZMG      DEECKK LW. ..YTYSKTTQ ICAGDPKKVQ APYEGESGGP LVCDN..LAY GVVSYGINR.          204

MMet-1      TDIFKPPVAT AVA.PYSSWI RKVIGRWSPQ SLV                                       238
RMet-1      TDIFKPTVAT AVA.PYSSWI RKVIGRWSPQ PLT                                       238
HMet-1      TDIFKPPVAT AVA.PYVSWI RKVTGRSA                                             232
MGRZMA      GDRRWPGVYT FLSDKHLNWI KKIMKGSV                                             232
MGRZMB      ..GSPPRAFT KVS.SFLSWI KKTMKSS                                              227
MGRZMC      ..GSAPQVFT RVL.SFVSWI KKTMKHS                                              228
MGRZMD      ..TISSGIFT KVV.HFLPWI SWNMKLL                                              232
MGRZME      ..TISSGVFT KIV.HFLPWI SRNMKLL                                              228
MGRZMF      ..TIGPGVFT KVV.HYLPWI SRNMKLL                                              228
MGRZMG      ..TITPGVFT KVV.HFLPWI STNMKLL                                              228
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Fig. 1 **A** Genomic organization of the mouse *MMet-1* gene. The exon-intron structure of the *MMet-1* gene is indicated (*top*). The entire gene is contained within one genomic clone (λMGM#4) elucidated from three overlapping [*Eco* RI (M-1), *Bam* HI (M-2), and *Pst* I (M-3)] subclones and two PCR products (P-1 and P-2) derived from this genomic clone. A simple restriction map of these is shown, including *Bam* HI, *Eco* RI, and *Pst* I. *Bam* HI, *Eco* RI, or *Pst* I fragments were subcloned into pBluescript KS^+ and PCR products derived P-1 (using primers P-A and P-B) and P-2 (primers P-C and P-D). Sequence strategy involved oligonucleotide primers as represented by the *arrows*. **B** Sequence encompassing the *MMet-1* exon-intron boundaries. The locations of the active site residues His-, Asp-, and Ser- are *boxed*. DNA sequences of the *MMet-1* gene that includes all of the exons, and some of the intron sequences. Introns 1 and 2 have not been completely sequenced, their approximate size (in brackets) was determined by restriction enzyme mapping and PCR (Materials and methods). The inferred amino acid sequences are indicated *below* their corresponding DNA sequences. The stop codon (TGA) and the polyadenylation signal (AATAAA) are *underlined*. **C** Amino acid comparison between *MMet-1*, other *Met-1* species, and other known mouse granzymes. Source of the sequences is as follows: *RMet-1* (Smyth et al. 1992), *HMet-1* (Pilat et al. 1994), mouse granzyme *A* (MGRZMA; Hershberger et al. 1992), mouse granzyme *B* (MGRZMB; Lobe et al. 1986a), mouse granzyme *C* (MGRZMC; Jenne et al. 1988b), mouse granzymes *D, E, F* (MGRZMD/E/F; Jenne et al. 1989c), and mouse granzyme *G* (Jenne et al. 1989). Numbering from the first amino acid of the mature protein Ile^{+1}

Ala-Ala-Met/Asp/Leu-; Suc-Phe-Leu-Phe-) – final concentration 150 μM]. Results were calculated as the mean O.D. units ± standard error for duplicate samples. The duration of the assay was 0 to 2 h and color development was measured at 0.D. 405 nm on a Dynatech MR 5000 microplate reader. Controls of sample and DTNB alone or DTNB and substrate alone were also performed.

Results and Discussion

Isolation of the MMet-1 gene

In order to isolate genomic clones encoding the *MMet-1* gene, a genomic library was screened with a full-length *RMet-1* cDNA (Smyth et al. 1992). DNA derived from a single reactive phage clone, designated MGM#4, was restricted with *Bam* HI, *Eco* RI, or *Pst* I (Fig. 1A). To ascertain that our cloned genomic DNA comprised entirely genuine sequences, we compared its restriction pattern with that of the endogenous *MMet-1* gene locus in genomic DNA. In Southern blotting experiments (Thia et al. 1995; data not shown), each hybridizing band in mouse genomic DNA corresponded to fragments of the λ MGM#4 *MMet-1* clone. No polymorphism was detected when restriction fragments generated from genomic DNA of C57BL/6, BALB/c, SJL, or DBA/2 mice were compared (data not shown). These data also suggested that *MMet-1* was a single copy gene.

Genomic organization and comparison with other granzyme genes

We sequenced genomic DNA encompassing the *MMet-1* gene, including 1.4 kb of the 5′ UT region, the complete coding region, and a portion of the 3′ UT region. Nucleotide sequence analysis of clones M-1, M-2 and M-3 and PCR products P-1 and P-2 confirmed the presence of the entire *MMet-1* gene, which was approximately 5.2 kb (ATG start site to polyadenylation signal) in length (Fig. 1B). By comparison with the cDNA sequence, the gene was found to encode the 238 amino acid protein on five exons, with four intervening introns of approximately 3.5 kb, 0.75 kb, 88 bp, and 375 bp (Fig. 1B). *MMet-1* along with orthologous genes *RMet-1* (Smyth et al. 1995b) and *HMet-1* (GZMM) (Pilat et al. 1994) and a potential evolutionary partner, human neutrophil elastase (Takahashi et al. 1988), share a four intron/five exon gene organization. Seven of eight exon/intron transitions of the *MMet-1* gene fulfill the GT-AG rule (Mount 1982). The substrate specificity site (in the case of the *MMet-1* gene, an alanine (residue 178; Fig. 1B), is encoded within the fourth exon, as it is in *HMet-1* (*GZMM*), *RMet-1*, and other chymotrypsin-like serine proteases (Bell et al. 1984). On the basis of the number and position of introns, the *MMet-1* serine protease resembles the recently described new sixth class of serine proteases (Irwin et al. 1988). However, intron 1 falls at the codon for Gly (–7), which is the last residue prior to the putative MMet-1 propeptide Asn (–6) – Gln (–1). This unusual position of intron 1 at residue (–7) in *MMet-1* is shared with *HMet-1* (*GZMM*; Pilat et al. 1994), *RMet-1* (Smyth et al. 1995b), and the neutrophil elastase-like genes, azurocidin (*AZU1*), proteinase-3 (*PRTN3*), and neutrophil elastase (*ELA2*; Zimmer et al. 1992). This gene organization is a distinguishing feature of this group of serine proteases colocalized at human chromosome 19p13.3 and syntenic mouse chromosome 10C (Pilat et al. 1994; Thia et al. 1995), again indicating their close evolutionary relationship and further suggesting that they may have arisen by gene duplication and divergence.

Isolation of the MMet-1 cDNA

MMet-1 cDNA was isolated by RT-PCR of total RNA from mouse BALB/c spleen cells cultured in IL-2 for 7 days. The *MMet-1* cDNA sequence was verified from the MGM#4 *MMet-1* clone. The *MMet-1* cDNA encodes a putative mature protein with 238 amino acids (Fig. 1C). The predicted N-terminus of the mature MMet-1 protein (+1) is based upon the conserved sequence Ile-Ile-Gly-Gly found in all active granzymes. The amino acid sequence of the predicted MMet-1 protein has been aligned with *RMet-1*, *HMet-1*, and other previously isolated mouse granzymes A to G (Hershberger et al. 1992; Lobe et al. 1986b; Jenne et al. 1988b, c; Fig. 1C). Not surprisingly, *MMet-1* is most similar to *RMet-1* (86.4% identity) and *HMet-1* (69.6% identity) and demonstrated less than 45% identity with any other mouse granzyme. This lineup indicates that the *Met-1* granzyme is quite distinct from any other family member. While all *Met-1* granzymes contain the catalytic triad (His^{41}, Asp^{87} and Ser^{184}) and several stretches of amino acids (VLTAA around His^{41}; GDSGGP around Ser^{184}; etc.) conserved in all granzymes, several features distinguish this granzyme from all others. All mature *Met-1* granzymes contain eight cysteine residues, six of which are conserved in all granzymes and expected to form three disulfide bonds (26–42, 122–153, 169–190), while the other two cysteines of *MMet-1* (position 180 and 206) may be analogous to the

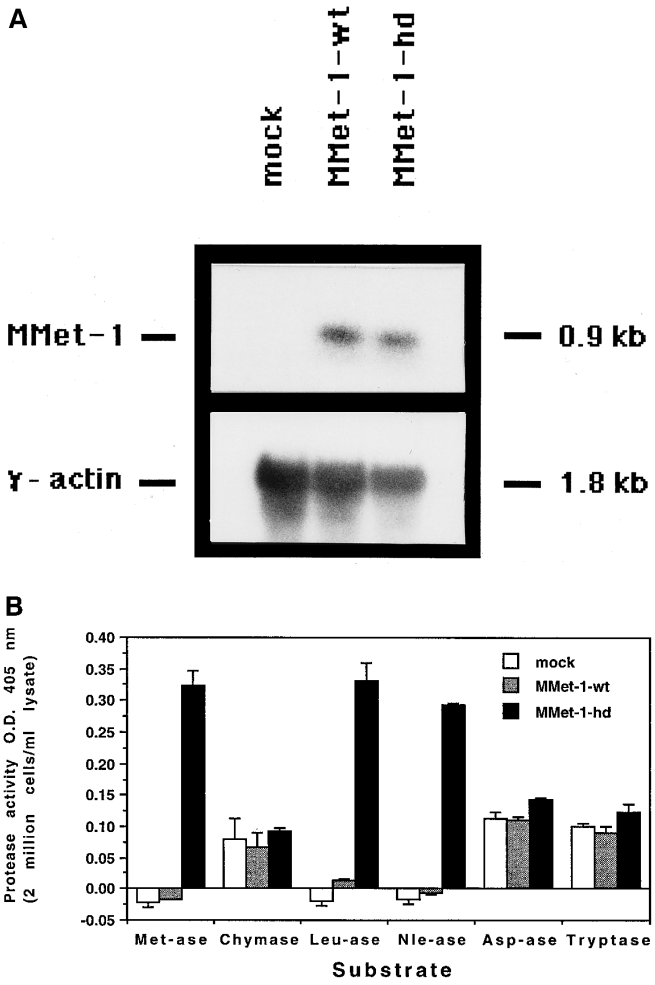


Fig. 2 **A** Northern blot analysis of COS-7 performed three days after transfection with various *MMet-1* cDNAs. Lane 1, mock transfected; lane 2, *MMet-1*-wt; and lane 3, *MMet-1*-hd. Northern blot analysis was performed on 20 μg of RNA and the filter was sequentially hybridized with ^{32}P-labeled cDNA probes for *MMet-1* (exposure, 1 day) or human γ-actin (4 h). **B** Protease activities of COS-7 cell lysates following harvest 72 h after transfection with mock vector alone, *MMet-1*-wt, or *MMet-1*-hd. Activity was estimated using the Boc-Ala-Ala-Met-SBzl (150 μM; *Met-ase*); Boc-Ala-Ala-Asp-SBzl (150 μM; Asp-ase); BLT (150 μM; tryptase); Suc-Phe-Leu-Phe-SBzl (150 μM; chymase); Boc-Ala-Ala-Leu-SBzl (150 μM; Leu-ase); and Boc-Ala-Ala-Nle-SBzl (150 μM; Nle-ase) substrates. These substrates were used to measure the protease activity of doubling dilutions of cell lysates with a modified microtiter assay (see Materials and methods). Mean units of activity ± standard error were defined by the O.D. at 405 nm for 2 million cells/(~600 μg protein)/ml lysate and this experiment is representative of two performed. The duration of the assay was 2 h

disulfide bond linking residues (position 191 and 220) in α-chymotrypsin which bridge the active-site serine. The QAPCKGD sequence immediately preceding the active site Ser^{184} and the Ser^{202} to Tyr^{221} sequence further downstream is essentially identical in all *Met-1* granzymes and quite distinct from any other granzyme family member. Only one potential N-glycosylation site (Asn^{154}-Asn-Ser) is conserved amongst all *Met-1* granzymes, with both *RMet-1* and *HMet-1* having one or two other sites, respectively.

Expression of recombinant MMet-1

The processing and activation of granzymes is normally a two-step pathway that involves cleavage of a signal prepeptide, followed by removal of an amino-terminal activation propeptide (Caputo et al. 1993; Masson and Tschopp 1987; Bleackley et al. 1988; Smyth et al. 1995c). By inference, the granzyme *MMet-1* may be synthesized as a preproenzyme with an amino-terminal signal peptide, followed by an activation propeptide immediately N-terminal to the Ile^{+1} residue of the mature protein. While granzyme B, H, and several other granule serine proteases, including cathepsin G, share activation dipeptide sequences (Jenne and Tschopp 1988a), no such sequence was predicted in *MMet-1* or other *Met-1* for that matter. The typical acidic granzyme prodipeptide at the amino terminus is not present in any *Met-1*, but rather the leader peptide ends with a Gln residue, and the von Heijne (1986) consensus algorithm suggests that a longer activation hexapeptide might regulate the protease activity of *MMet-1*. In order to determine the serine protease specificity of *MMet-1*, we devised a PCR strategy that deleted the potential activation hexapeptide within the leader sequence and produced active recombinant *MMet-1* in COS-7 cells. COS-7 cells were first transfected with expression plasmids containing either unmodified *MMet-1*-wild-type (wt) or modified *MMet-1*-hexapeptide deleted (hd) cDNAs. While no monoclonal antibody specific for *MMet-1* was available to determine whether similar levels of MMet-1 protein were produced, transiently transfected COS-7 cells expressed similar levels of *MMet-1* mRNA 72 h after transfection with both *MMet-1* cDNAs (Fig. 2A).

Substrate specificity

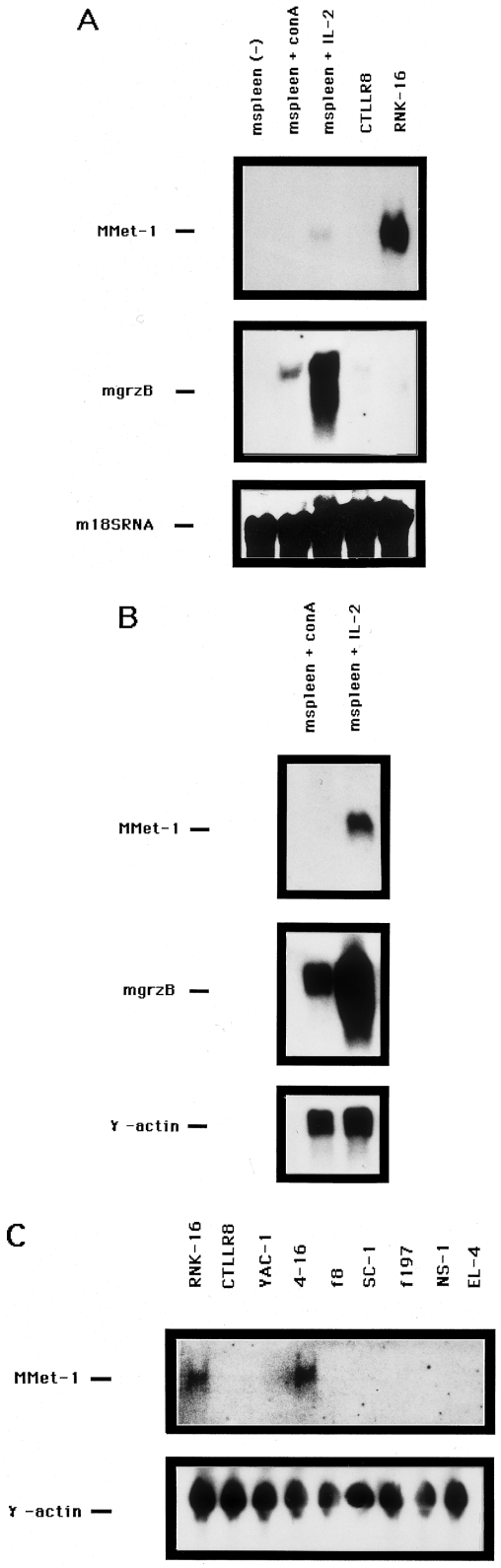
The protease activities of lysates of COS-7 cells transfected with *MMet-1*-wt or *MMet-1*-hd cDNAs were first estimated using the preferred Met-ase substrate, Boc-Ala-Ala-Met-SBzl thiobenzyl ester (Fig. 2B). *Met-ase* activity was not detected in cells transfected with the *MMet-1*-wt construct; however, the transfection of the *MMet-1*-hd cDNA resulted in enzymatically active MMet-1 protein (Fig. 2B). We concluded that transfection of the hexapeptide deleted form of the *MMet-1* cDNA results in enzymatically active *MMet-1* and that removal of the activation hexapeptide is critical to this activity. The results presented here are consistent with a model of granzyme processing in which *MMet-1* is normally synthesized as a preproenzyme. Further analysis of these COS-7 lysates indicated that cells transfected with the *MMet-1*-hd cDNA did hydrolyze substrates with Leu- and Nle- at P_1 (Fig. 2B), but not substrates with Asp-, Lys-, or Phe- at P_1 (Fig. 2B). This data formally demonstrates that the *MMet-1* gene encodes a granzyme with *Met-ase* activity and that recombinant *MMet-1* shares a substrate profile very similar to recombinant *HMet-1* (Smyth et al. 1996) and native *RMet-1* (Smyth et al. 1992). This finding is consistent with the high level of amino acid conservation between *RMet-1* and *MMet-1*, particularly in regions of the substrate binding pocket that influence protease activity. The combination of amino acid residues that have been thought to determine the specificity of all granzyme substrate binding pockets (–6, +15, +16, +17, +28 relative to the active-site Ser^{184}) are conserved. Furthermore, molecular modelling against crystal α-chymotrypsin (CHA) and mutational analysis of *HMet-1* have suggested that Lys^{179} (CHA192) and Ser^{201} (CHA216) may play a critical role in the specificity of this granzyme (Smyth et al. 1996), as these too are conserved in *MMet-1*.

Expression of MMet-1 in CD3⁻ LGL

Northern analysis using a full-length *MMet-1* cDNA detected a single 0.9 kb *MMet-1* mRNA in the total cellular RNA and poly A^+ mRNA from IL-2 stimulated spleen $CD3^-$ LGL of BALB/c mice (Fig. 3A, B). *MMet-1* mRNA was also observed in total cellular RNA from IL-2-stimulated spleen $CD3^-NK1.1^+$ LGL of C57BL/6 mice (data not shown). By comparison, MMet-1 mRNA could not be detected in total cellular RNA or poly A^+ mRNA from ConA-treated spleen cells (Fig. 3A, B), *MMet-1* mRNA was also detected in total cellular RNA from the mouse $CD3^-$ $NK1.1^+$ LGL leukemia cell line, 4–16 (Fig. 3C). Detection of *MMet-1* mRNA was not possible in the pre-NK cell lines f8, SC-1, and f197 (Fig. 3C). Furthermore, a number of mouse CTL, T, and other cell lines did not express *MMet-1* mRNA (Fig. 3C, data not shown). This data was consistent with previous reports that have suggested that both *RMet-1* and *HMet-1* have only been detected in mature $CD3^-$ LGL and not pre-NK, T or other leukocyte and non-leukocyte cell populations (Smyth et al. 1992; Smyth et al. 1993; Smyth et al. 1995a).

5′ flanking DNA sequence motifs

Over a total of 1047 nucleotides of *MMet-1* 5′ flanking sequence there was an approximate 82% identity when aligned with the *RMet-1* 5′ flanking region using BESTFIT (Fig. 4A). A large number of potential regulatory motifs occurred throughout the first 1 kb of the 5′ flanking region and perhaps most interesting are those also conserved in the *RMet-1* 5′-flanking region. These elements included AP-2 [$^{-29}$CCCCCAGGG^{-21}; $^{-797}$GGCCTGGG-$^{-790}$], AP-3 [^{136}AAACCACA^{-129}], and glucagon-G3A [$^{-418}$TCAGGCG^{-412}] sequences (Fig. 4A). These and other



highly conserved upstream sequences shared between *MMet-1* and *RMet-1* are obvious candidates for conferring NK-specific expression, particularly those within the first 300 bp which appear essential for the activity of the *RMet-1* promoter (Smyth et al. 1995b). There was only some limited sequence similarity when compared with the putative 5′ promoters of other serine protease genes, including *HMet-1* (*GZMM*; Pilat et al. 1994), which has a 76.9% identity with the *MMet-1* 5′ flanking region, but only between nucleotides $^{-88}$G to $^{-37}$A. The comparatively limited degree of similarity between the *HMet-1* and mouse *Met-1* 5′ flanking regions was surprising, in light of the high degree of between *RMet-1* and *MMet-1* 5′ flanking regions (even as far upstream as –3.3 kb). It should be noted that sequences further 5′ in *HMet-1* (i.e., beyond –800) remain to be elucidated and these may be found to share more similarity with their mouse counterparts.

Function of the MMet-1 5′ flanking Region

The *MMet-1* 5′ flanking and promoter region was analyzed for its ability to control a promoterless CAT reporter gene to determine whether this DNA could confer CD3$^-$ LGL-restricted expression of *MMet-1*. Approximately 3.3 kb of *MMet-1* 5′ flanking sequence was generated by PCR (see Materials and methods). This 3.3 kb of the *MMet-1* 5′ flanking region was incorporated into a CAT construct and transfected into an *MMet-1*$^+$ LGL leukemia cell line (4–16); intro pre-NK cell lines (SC-1, f8, and f197); into MMet-1$^-$ cytotoxic T (CTLLR8), mouse T lymphoma (YAC-1 and EL-4) and mouse myeloma (NS-1) cell lines. All nine cell lines tested were able to produce CAT in significant quantities in response to the RSV promoter (18 ± 1 to 61 ± 2 pg CAT/200 μg protein) and none produced significant CAT activity following transfection with the pCAT basic gene (-1 ± 1 to 1 ± 1 pg; Fig. 4B). Transfection of the 5′ *MMet-1*-CAT construct yielded 37 ± 10 pg of CAT activity in the mouse LGL cell line 4–16, and slightly less (31 ± 11 pg) in the rat LGL cell line RNK-16. These levels of CAT activity were comparable or less than those previously observed in RNK-16 cells transfected with an equivalent 5′ *RMet-1*-CAT construct (Smyth et al. 1995b). Weak expression of CAT activity was also observed in two of three pre-NK cell lines (SC-1 and f-197) transfected with the 5′ *MMet-1*-CAT construct (4 ± 2 to 6 ± 2 pg; however, MMet-1 mRNA expression was undetectable in these cell lines by Northern analysis. By contrast, no CAT activity was observed following transfection

Fig. 3 Northern blot analysis of *MMet-1* mRNA expression in various mouse and rat cell lines and primary cells/tissues. Total cellular RNA and poly A$^+$ mRNA was isolated as described in Materials and methods. Panels A and C, total cellular RNA; and panel B, poly A$^+$ mRNA. BALB/c spleen was either immediately harvested or cultured for 7 days in 100 units/ml IL-2 or 1 μg/ml con A. Northern blot analysis was performed on 1–2 μg of poly A$^+$ mRNA or 20 μg of total cellular RNA and the filters were sequentially hybridized with ^{32}P-labeled cDNA probes for *MMet-1* (exposure, 5 days), mouse granzyme *B* (grzB; 5 days), mouse 18S rRNA (2 h) or human γ-actin (1 day)

Fig. 4 **A** Nucleotide alignment of the 5′ flanking regions of *MMet-1* (M) and *RMet-1* (R). Comparing nucleotides –1502 to –1 of *MMet-1* with –1175 to –1 of *RMet-1* using BESTFIT (GCG Sequence Analysis Software Package), a gap weight of 5.0 and length weight of 0.3. Gaps are denoted by *dots*, identities by *vertical linkers*, and mismatches by *spaces*. **B** Functional analysis of the *MMet-1* 5′ flanking region. Mouse cell lines, 4–16, SC-1, f197, f8, CTLLR8, and YAC-1, and the rat NK leukemia cell line, RNK-16, were transfected with a construct (–3340 to +1) of the *MMet-1* 5′ flanking region driving the CAT gene. Transfections were corrected for protein and 200 μg of extract were assayed for their CAT activity by an ELISA (as described in Materials and methods). The CAT activities shown are expressed as pg/200 μg of protein. Their mean ± SE from three independent transfections are shown. RSVCAT is a positive control plasmid for equivalent transfection into all seven cell lines. The negative control was the enhancerless and promoterless pCAT basic parental construct

of the 5′ *MMet-1*-CAT construct into the mouse cytotoxic T cell line, CTLLR8, the T cell lymphomas, YAC-1 or EL-4 and myeloma cell line NS-1. These data suggest that the region ∼ –3.3 kb to –1 possibly contains most, if not all, the sequence elements necessary for constitutive LGL-specific expression in vitro.

The 5′ flanking region of the *MMet-1* gene shares large regions of identity with the 5′ flanking region of the *RMet-1* gene, and thus future fine mapping by DNAse footprinting and gel retardation analysis should reveal transcription factor binding elements common to both genes. *MMet-1* mRNA was detected in mouse $CD3^-$ $GM1^+$ LGL derived from splenocytes stimulated with IL-2 and the mouse $NK1.1^+$ cell line 4–16. The absence of detectable *MMet-1* mRNA in some pre-NK cell lines that originate from granzyme B 5′ promoter-tax (HTLV-I) transgenic mice (Grossman et al. 1995), and the failure to detect *HMet-1* mRNA in immature human NK cells (Vaz et al. 1995), suggests that *Met-1* is optimally expressed in fully mature LGL. While granzymes *A*, *B*, and *C* are transcribed early in hematopoiesis (Ebnet et al. 1995), transcription of granzyme *D*–*G* genes has only been detectable after short-term in vitro culture (Garcia-Sanz et al. 1990). Mouse granzyme *F* appears to be predominantly expressed by $CD8^+$ T cells (Garcia Sanz et al. 1990), but none other than *Met-1* appear to have a restricted expression in NK cell subsets.

The isolation of *MMet-1* represents the eighth distinct granzyme described in the mouse. Elucidation of the amino acid sequence encoded by *MMet-1* and the identities revealed between *MMet-1* and its species homologues clearly support the evolution of three distinct lymphocyte granzyme subfamilies in the mouse. Each of these subfamilies, *Met-1*, granzyme *A*, and granzymes *B*–*G*, are found at different chromosomal loci (Thia et al. 1995; Jenne et al. 1991; Mattei et al. 1987; Crosby et al. 1990) and are distinguished by their serine protease activities. That NE-like serine proteases and *Met-1* probably evolved from a common ancestor in the same region of the genome (Pilat et al. 1994; Zimmer et al. 1992) and that their products are located in the same type of cytoplasmic granule supports the hypothesis that these proteases may have multiple, partially overlapping biological functions that operate against invading pathogens. Alternatively, *Met-1* may have evolved for a specialized (NK) cytotoxic lymphocyte effector function. Mutation of the *MMet-1* gene in mice by homologous recombination and examination of the biological activities of *MMet-1* purified from COS-7 cell transfections should reveal the function of this unique granzyme activity in LGL.

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