THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 275, No. 46, Issue of November 17, pp. 35978–35985, 2000
Printed in U.S.A.

Dm1-MMP, a Matrix Metalloproteinase from *Drosophila* with a Potential Role in Extracellular Matrix Remodeling during Neural Development*

Received for publication, July 10, 2000, and in revised form, August 15, 2000
Published, JBC Papers in Press, August 29, 2000, DOI 10.1074/jbc.M006045200

Elena Llano, Alberto M. Pendás, Pedro Aza-Blanc‡, Thomas B. Kornberg‡, and Carlos López-Otín§

From the Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Instituto Universitario de Oncología, Universidad de Oviedo, 33006-Oviedo, Spain and the ‡Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

We have cloned and characterized a cDNA encoding *Dm1*-MMP, the first matrix metalloproteinase (MMP) identified in *Drosophila melanogaster*. The isolated cDNA encodes a protein of 541 residues that has a domain organization identical to that of most vertebrate MMPs including a signal sequence, a prodomain with the activation locus, a catalytic domain with a zinc-binding site, and a COOH-terminal hemopexin domain. Northern blot analysis of *Dm1*-MMP expression in embryonic and larval adult tissues revealed a strong expression level in the developing embryo at 10–22 h, declining thereafter and being undetectable in adults. Western blot analysis confirmed the presence of pro- and active forms of *Dm1*-MMP *in vivo* during larval development. *In situ* hybridization experiments demonstrated that *Dm1*-MMP is expressed in a segmented pattern in cell clusters at the midline during embryonic stage 12–13, when neurons of the central nervous system start to arise. Recombinant *Dm1*-MMP produced in *Escherichia coli* exhibits a potent proteolytic activity against synthetic peptides used for analysis of vertebrate MMPs. This activity is inhibited by tissue inhibitors of metalloproteinases and by synthetic MMP inhibitors such as BB-94. Furthermore, *Dm1*-MMP is able to degrade the extracellular matrix and basement membrane proteins fibronectin and type IV collagen. On the basis of these data, together with the predominant expression of *Dm1*-MMP in embryonic neural cells, we propose that this enzyme may be involved in the extracellular matrix remodeling taking place during the development of the central nervous system in *Drosophila*.

In 1962, Gross and Lapière (1) reported the discovery of a collagenolytic enzyme involved in resorbing amphibian tadpole tails during metamorphosis. This report initiated the field of matrix metalloproteinases (MMPs),[1] a family of zinc-dependent endopeptidases. These proteinases play an essential role in the connective tissue remodeling occurring in normal processes in vertebrates, such as embryonic development, bone growth, angiogenesis, wound healing, and limb regeneration (2, 3). In addition, abnormal expression of these enzymes may contribute to a variety of pathological processes including atherosclerosis (4), rheumatoid arthritis (5), neurological diseases (6), and tumor invasion and metastasis (7). To date, the human MMP family consists of 20 distinct proteinases that can be classified into five major subfamilies according to their primary structures, substrate specificity, and cellular localization. These are collagenases, stromelysins, gelatinases, membrane-type MMPs, and other MMPs (1, 8, 9). Structural analysis of MMPs reveals that most are organized into three distinctive and well defined domains as follows: a propeptide with a conserved Cys residue involved in maintaining the enzyme latency, a catalytic domain with a zinc-binding site, and a hemopexin-like domain that plays a role in substrate binding as well as in mediating interactions with the tissue inhibitors of metalloproteinases (TIMPs), a family of endogenous inhibitors of MMPs (10). Additional domains such as fibronectin-like repeats or COOH-terminal hydrophobic extensions are present in other family members like gelatinases or membrane type (MT)-MMPs, thus contributing to an increase in their structural complexity. Interestingly, recent biochemical characterization of diverse mammalian MMPs has shown that these enzymes are not exclusively involved in the degradation of extracellular matrix or basement membrane protein components. These MMPs play direct roles in essential cellular processes such as proliferation, differentiation, angiogenesis, or apoptosis through their ability to catalyze the hydrolysis of a variety of substrates including membrane-bound precursors of cytokines, growth factors, or hormone receptors (11–15).

The finding that MMPs may be involved in a wide variety of biological processes has prompted the search for members of this family in model organisms, in which the functional roles of these enzymes could be recognized and extensively analyzed by using additional experimental approaches. Thus, in addition to the diverse MMPs identified in mammalian tissues, MMPs have also been cloned from *Xenopus laevis* (16, 17), embryonic sea urchin (18), green alga (19), *Caenorhabditis elegans* (20), *Hydra vulgaris* (21), and *Arabidopsis thaliana* (22). However, no member of the MMP family has been cloned in *Drosophila*

* This work was supported by Comisión Interministerial de Ciencia y Tecnología Spain Grant SAF97-0258, and Plan Fondos Europeos para el Desarrollo Regional 1FD97-0214. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF271666.

§ To whom correspondence should be addressed: Dept. de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Spain. Tel.: 34985104201; Fax: 34985103564; E-mail: CLO@correo.uniovi.es.

[1] The abbreviations used are: MMP, matrix metalloproteinase; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; STS, sequence tagged site; TIMP, tissue inhibitor of metalloproteinases; DTT, dithiothreitol; kb, kilobase pair; Mca, (7-methoxycoumarin-4-yl)-acetyl; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Nva, norvalyl.

This paper is available on line at http://www.jbc.org

melanogaster. This is particularly intriguing if we consider that MMPs are assumed to play a decisive role in tissue remodeling during embryogenesis, a process that has been extensively studied in *Drosophila* and that has many other features that are conserved. Furthermore, a number of recent reports provide evidence that *Drosophila* metalloproteases belonging to other families, including those encoded by the *kuzbanian*, *tolloid*, and *tolkin* genes, are key components in many signaling pathways in *Drosophila* and mediate essential processes such as neurogenesis or embryonic patterning (23–27).

Because of the potential importance of MMPs in developmental processes, identification and characterization of members of this family in *Drosophila* are likely to help resolve the functions of these enzymes. In this study, we report the identification and characterization of *Dm1*-MMP, the first MMP family member identified in *D. melanogaster*. We show that it is expressed in larval tissues, with a distinct, reiterated expression pattern in the midline, coinciding with the beginning of neural and glial differentiation during embryogenesis. Furthermore, recombinant *Dm1*-MMP produced in *Escherichia coli* has proteolytic activity against extracellular matrix and basement membrane proteins. On the basis of these data, we propose that *Dm1*-MMP may play a role during the development of the central nervous system in *Drosophila*.

EXPERIMENTAL PROCEDURES

Materials—-Fly cosmid genomic clones in Lorist 6 vector (28) were obtained from the Human Genome Mapping Resource Center (Cambridgeshire, UK). cDNA libraries constructed in λgt11 were from CLONTECH (Palo Alto, CA). Restriction endonucleases and other reagents used for molecular cloning were from Roche Molecular Biochemicals. Synthetic oligonucleotides were prepared with an Applied Biosystems (Foster City, CA) model 392A DNA synthesizer. Double-stranded DNA probes were radiolabeled with $[^{32}P]$dCTP (3000 Ci/mmol) purchased from Amersham Pharmacia Biotech using a commercial random-priming kit from the same company.

Probe Preparation and Screening of a Drosophila cDNA Library—A computer search of the GenBank™ data base STSs for entries with similarity to MMPs previously described identified a sequence (Z31945) contributed by the *D. melanogaster* STS European Mapping Project. This 309-bp sequence revealed significant similarity with the catalytic domain of MMPs. Cosmid clones containing this STS (18a7, 23 g10, 57 g3, 162d9) were used to verify this sequence and to extend it by direct sequencing. To obtain the corresponding cDNA sequence, two specific primers, 5′-CGGCTATCTACCCGCCTCTG (primer 1) and 5′-AGATCTTGTAGGTGAGGTT (primer 2), were used for PCR amplification to prepare a probe to screen a panel of cDNAs from different developmental stages. The PCR was carried out in a GeneAmp 2400 PCR system from PerkinElmer Life Sciences for 30 cycles of denaturation (94 °C, 15 s), annealing (57 °C, 15 s), and extension (72 °C, 20 s). A 271-bp PCR product amplified from larva cDNA was radiolabeled and used to screen a larva cDNA library according to standard procedures (29). Cloned DNA fragments were sequenced with an ABI 337 automatic sequencer (PerkinElmer Life Sciences). Computer analysis of DNA and protein sequences was performed with the GCG software package of the University of Wisconsin Genetics Computer Group.

Chromosomal Mapping—Hybridization to polytene chromosomes squashes using the alkaline phosphatase-based DNA detection system was performed as described (30). cDNA was biotin-labeled by nick translation (Roche Molecular Biochemicals) and used as probe.

RNA Analysis—Total RNA (30 μg) from diverse developmental stages of *Drosophila* was electrophoresed and blotted to Hybdond N+ (Amersham Pharmacia Biotech). The blot was hybridized with a radiolabeled *Dm1*-MMP cDNA and washed according to standard procedures (21). Blots were subsequently hybridized with a ribosomal DNA probe to control for RNA loading. *In situ* hybridization to whole mount embryos was performed using sense and antisense RNA probes synthesized by using the DIG-RNA labeling kit (Roche Molecular Biochemicals). Detection was with anti-DIG-alkaline phosphatase reaction (31).

Expression, Refolding, and Purification of Dm1-MMP—A 735-bp fragment of the *Dm1*-MMP cDNA containing the pro- and catalytic domains was generated by PCR amplification with primers 5′-CGGGATCCGCAATCGGCACCCGTTTCCACC (*Bam*HI-proDm1) and 5′-CGGAATTCATACAGTGACTGGATGGCCGC (*Eco*RI-proDm1) using the full-length *Dm1*-MMP cDNA as template. PCR amplification was performed for 30 cycles using the Expand™ High Fidelity PCR system. Due to the design of the oligonucleotides, the amplified fragment could be cleaved at both ends with *Eco*RI and *Bam*HI and ligated in frame into the pRSETB *E. coli* expression vector (Invitrogen) thereby adding an NH_2-terminal His_6 tag to the protein. The resulting pRSET-proDm1 vector was transformed into BL21(DE3)pLysS *E. coli* cells, and expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (0.5 mM final concentration) followed by further incubation for 3–20 h at 30 °C. Recombinant protein obtained in inclusion bodies was solubilized using 20 mM Tris buffer, pH 7.6, containing 6 M GdnHCl, and 5 mM DTT, and purified in a Superdex-75 column (Amersham Pharmacia Biotech) equilibrated with 20 mM Tris buffer, pH 7.6, containing 3 M GdnHCl, and 5 mM DTT. After SDS-PAGE analysis, peak fractions with the recombinant protein were pooled, and the GdnHCl concentration was adjusted to 6 M. Refolding was achieved by dialysis, first against a 50 mM Tris buffer, pH 7.6, containing 5 mM $CaCl_2$, 200 mM NaCl, 50 μM $ZnSO_4$, 0.05% Brij 35, 20% glycerol, and 2 M GdnHCl, and then against the same buffer with 2 mM DTT, without GdnHCl.

Enzymatic Assays—Enzymatic activity of purified recombinant *Dm1*-MMP was detected using the synthetic fluorescent substrates Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (QF-24), Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2 (QF-35), and Mca-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH_2 (QF-41) (provided by C. G. Knight, University of Cambridge, UK). Routine assays were performed at 37 °C at substrate concentrations of 1 μM in an assay buffer of 50 mM Tris/HCl, 5 mM $CaCl_2$, 150 mM NaCl, 0.05% (v/v) Brij 35, pH 7.6, with a final concentration of Me_2SO of 1% (32). The fluorometric measurements were made in an MPF-44A PerkinElmer Life Sciences spectrofluorometer (λ_{ex} = 328 nm, λ_{em} = 393 nm). For inhibition assays, *Dm1*-MMP (20 nM) and inhibitors were preincubated for 30 min at 20 °C, with BB-94 (British Biotech Pharmaceuticals, Oxford, UK) at concentrations ranging from 0 to 100 nM. Inhibition assays with TIMPs (kindly provided by Drs. V. Knäuper and G. Murphy) were performed at the same conditions with 20 nM concentration of the different inhibitors. Cleavage of type I, type II, and type IV collagens, type I gelatin, type I laminin, fibronectin, and fibrinogen (purchased from Sigma) by recombinant *Dm1*-MMP was followed by SDS-PAGE. All assays were performed in the above described assay buffer for 16 h at 37 °C. The enzyme/substrate ratio (w/w) used in these experiments was 1/10.

Substrate Gel Zymography—Casein zymography was done using a 13% SDS-polyacrylamide gel containing 1 mg/ml casein. Electrophoresis was performed at room temperature, under nonreducing conditions. Following electrophoresis, the gel was washed twice for 1 h each in 100 ml of 2.5% Triton X-100 (v/v) to remove SDS and incubated for 24 h at 37 °C in 50 mM Tris/HCl, 5 mM $CaCl_2$, 150 mM NaCl, 0.05% (v/v) Brij 35, pH 7.6, to allow proteolysis. After that, the gel was stained with Coomassie Blue to visualize the lytic bands.

Antibody Production and Western Blot Analysis—Purified *Dm1*-MMP was injected into rabbits using the multiple injection method developed by Vaitukaitis (33). The rabbits were bled 6 weeks after the injection, and the serum was dialyzed for 24 h at 4 °C against 20 mM phosphate buffer, pH 7.2. The material was then chromatographed in a column of DEAE-cellulose equilibrated and eluted in the same buffer. IgG-containing fractions were collected and stored at −20 °C until used. Western blots were blocked in 5% milk in PBT (PBS containing 0.1% Tween 20) and then incubated for 1 h with rabbit antiserum diluted 1:5000 in PBT. After three washes in PBT, blots were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG at 1:20,000 and developed with the Renaissance chemiluminescence kit (PerkinElmer Life Sciences).

RESULTS

Identification and Characterization of a Drosophila Larva cDNA-encoding a Member of the Matrix Metalloproteinase Family—By analyzing the GenBank™ data base of *Drosophila* expressed sequence tags and STSs, we identified a sequence with similarity to vertebrate MMPs. We used this sequence to isolate a short genomic fragment (Z31945) with significant sequence similarity to a region of the catalytic domain found in the different vertebrate MMPs, and then we isolated a full-length cDNA clone from a a fly larva λgt-11 library. The corresponding 2.2-kb mRNA has an open reading frame with two potential translation start sites. The most likely start site is the second methionine residue since the sequence immediately upstream of the AUG codon corresponding to this residue (*CAAA*

```
   1  CTCAGTCTAGCGCACTAGCGAGCCTCAAAAACGCACTCGAACTTGCAGTTACGGCAGCTTGCTGCGGAAGGCAAATCTCC    80

  81  ATAAGATATAGTATAGCTAAGCAAATTAGCGAGTGTTAAACAAATTAGTTTGTGCTTCCGCTGCGTGAAAAGCTTTCCAC   160

 161  CCAGAGTGAAAAACCCAATTTAGACTGAGAGCGAGAGAAAGCTGCGCCCTCAGGTGAATCATCAGTGGATCTAACAGCTA   240

 241  GCCAAAAGCTTGCTAACTTCTGGCTTACAGTGCGTTAACAGTGAAGTGATATAAAGTTCAAAGTGCCAGCGGCAAGTGGA   320

 321  ACCACATGACAAATAGACGAGCCTCGGGAGCAACCCACTGCAAAACCACCAACAACTGCAATATAAGCAACAACAGCAAC   400
          M  T  N  R  R  A  S  G  A  T  H  C  K  T  T  N  N  C  N  I  S  N  N  S  N

 401  AAAATGACAAACTGCCAAAGTTCAGTGTTCATAGTCGTAGGCACCCTGTTTTCCATCCTGGCAGCGGCTCAATCGGCACC   480
      K  M  T  N  C  Q  S  S  V  F  I  V  V  G  T  L  F  S  I  L  A  A  A  Q  S  A  P

 481  CGTTTCCACCACCACACAGGCAGAGATCTACCTGTCCCAGTTCGGCTATCTACCCGCCTCTGCCCGCAATCCCGCCAGCA   560
       V  S  T  T  T  Q  A  E  I  Y  L  S  Q  F  G  Y  L  P  A  S  A  R  N  P  A  S  S

 561  GTGGGCTACACGACCAGCGGACCTGGGTGAGTGCCATCGAGGAGTTCCAAAGCTTCGCGGGACTGAACATCACCGGCGAG   640
        G  L  H  D  Q  R  T  W  V  S  A  I  E  E  F  Q  S  F  A  G  L  N  I  T  G  E

 641  CTGGATGCGGAGACAATGAAGCTGATGTCCCTGCCCCGCTGCGGAGTTCGGGATCGCGTCGGCACCGGTGACAGCCGCTC   720
      L  D  A  E  T  M  K  L  M  S  L  P  R  C  G  V  R  D  R  V  G  T  G  D  S  R  S

 721  CAAGCGGTACGCCCTGCAGGGCAGTCGCTGGCGTGTGAAGAACCTCACCTACAAGATCTCCAAGTACCCCAAGCGGCTGA   800
       K  R  Y  A  L  Q  G  S  R  W  R  V  K  N  L  T  Y  K  I  S  K  Y  P  K  R  L  K

 801  AGCGAGTCGATGTCGACGCGGAGATCGGTCGCGCATTTGCCGTGTGGTCCGAGGACACCGACCTGACCTTCACCAGGAAG   880
        R  V  D  V  D  A  E  I  G  R  A  F  A  V  W  S  E  D  T  D  L  T  F  T  R  K

 881  ACCTCCGGCCCCGTGCACATCGAGATCAAGTTCGTGGAGAGCGAGCATGGAGACGGCGATGCCTTTGACGGTCAAGGCGG   960
      T  S  G  P  V  H  I  E  I  K  F  V  E  S  E  H  G  D  G  D  A  F  D  G  Q  G  G

 961  CACCCTGGCGCATGCCTTCTTCCCTGTCTTTGGCGGAGATGCCCACTTTGACGACGCGGAACTGTGGACCATTGGCTCGC  1040
       T  L  A  H  A  F  F  P  V  F  G  G  D  A  H  F  D  D  A  E  L  W  T  I  G  S  P

1041  CTCGAGGCACCAATCTGTTCCAGGTGGCCGCTCACGAGTTCGGTCACTCCTTGGGACTCTCCCACTCAGATCAGAGCTCC  1120
       R  G  T  N  L  F  Q  V  A  A  H  E  F  G  H  S  L  G  L  S  H  S  D  Q  S  S

1121  GCTCTGATGGCGCCCTTCTATCGCGGCTTTGAACCCGTCTTCAAGTTGGACGAGGACGACAAGGCGGCCATCCAGTCACT  1200
      A  L  M  A  P  F  Y  R  G  F  E  P  V  F  K  L  D  E  D  D  K  A  A  I  Q  S  L

1201  GTATGGGCGCAAAACAAACCAGCTGAGGCCCACCAACGTGTACCCGGCCACCACCCAGCGGCCATACTCTCCTCCAAAGG  1280
       Y  G  R  K  T  N  Q  L  R  P  T  N  V  Y  P  A  T  T  Q  R  P  Y  S  P  P  K  V

1281  TGCCACTAGACGACTCCATCTGCAAGGACTCCAAGGTAGACACACTCTTCAACTCGGCGCAGGGCGAGACCTACGCCTTC  1360
       P  L  D  D  S  I  C  K  D  S  K  V  D  T  L  F  N  S  A  Q  G  E  T  Y  A  F

1361  AAGGGCGACAAGTACTACAAGCTGACCACGGACTCCGTGGAGGAGGGCTACCCACAGCTCATCTCCAAGGGCTGGCCGGG  1440
      K  G  D  K  Y  Y  K  L  T  T  D  S  V  E  E  G  Y  P  Q  L  I  S  K  G  W  P  G

1441  ATTGCCGGGCAACATCGATGCGGCGTTCACCTACAAGAACGGCAAGACGTACTTCTTCAAGGGTACTCAGTACTGGCGCT  1520
       L  P  G  N  I  D  A  A  F  T  Y  K  N  G  K  T  Y  F  F  K  G  T  Q  Y  W  R  Y

1521  ACCAGGGCCGTCAGATGGACGGCGTCTACCCTAAGGAAATCAGCGAGGGCTTCACGGGCATTCCCGACCATCTGGACGCC  1600
       Q  G  R  Q  M  D  G  V  Y  P  K  E  I  S  E  G  F  T  G  I  P  D  H  L  D  A

1601  GCAATGGTTTGGGGGGGCAATGGCAAGATATACTTCTTCAAGGGCAGCAAGTTCTGGCGCTTCGATCCGGCCAAGAGGCC  1680
      A  M  V  W  G  G  N  G  K  I  Y  F  F  K  G  S  K  F  W  R  F  D  P  A  K  R  P

1681  GCCGGTCAAGGCTAGCTATCCCAAGCCCATCTCCAACTGGGAGGGTGTTCCCAACAACCTGGACGCAGCGCTGAAGTACA  1760
       P  V  K  A  S  Y  P  K  P  I  S  N  W  E  G  V  P  N  N  L  D  A  A  L  K  Y  T

1761  CCAATGGCTACACGTACTTCTTCAAGGGCGACAAGTACTACAGATTCCATGACGCCCGATTTGCTGTTGACTCGGCCACG  1840
        N  G  Y  T  Y  F  F  K  G  D  K  Y  Y  R  F  H  D  A  R  F  A  V  D  S  A  T

1841  CCCCCCTTCCCCAGACCCACCGCCCACTGGTGGTTCGGCTGCAAGAACACGCCCTCGTCCACAGGTAATATCGTCGAAGG  1920
      P  P  F  P  R  P  T  A  H  W  W  F  G  C  K  N  T  P  S  S  T  G  N  I  V  E  G

1921  CTCGGACAACGAGTTCGAACAGCACTCCATGATCCCGCATGCCGACGATGGCAACGGTGATGACTTTGACGCAGGTGAGT  2000
       S  D  N  E  F  E  Q  H  S  M  I  P  H  A  D  D  G  N  G  D  D  F  D  A  G  E  W

2001  GGGACCGACTCAGCGGTAGTTTTGTTTGAGCTCGAATCGATCTACTGGATCCACGAATATCGAATCCCGAACTCTGAAAG  2080
        D  R  L  S  G  S  F  V  *

2081  TCAAAAAGATCTCATAGAAAGAAGTCTTGTATGAGTTGTTACTTAGCGAGAAGCAGTTACAAGTAATGAAATGATAATAT  2160

2161  TAATAAATCCTATCTTTATGGTATGTTTAATTAAGTTAATGTCTATGTAGTGAAATTCTAGTACTCAAGTCGAATGTGTT  2240

2241  TGTCTTTGTTGCCTATGGAAGAAATACATTTATAATACCTATTGAAAAACTAAAAAAAAAAAAAAAAAAAAAAAA  2315
```

FIG. 1. **Nucleotide sequence and deduced amino acid sequence of *Dm1*-MMP.** The amino acid sequence is shown in *single-letter code below* the nucleotide sequence. The methionine residue predicted as translation start site is *underlined*. The cysteine-switch residues and those corresponding to the zinc-binding site are *shaded*.

AUG) perfectly matches the *Drosophila* translation start site consensus sequence ((*C*/*A*)*AA*(*A*/*C*) AUG) (34). Furthermore, this methionine residue immediately precedes a hydrophobic sequence that could direct the protein to the secretory pathway. Assuming that translation starts at this residue, the identified open reading frame encodes a protein of 541 residues with a calculated molecular mass of 60.3 kDa (Fig. 1). Localization of the *Dm1*-MMP gene to polytene chromosomes revealed that it was located to region 60D13 (data not shown).

Comparison of the predicted *Dm1*-MMP sequence with those of the vertebrate MMPs demonstrated that the *Drosophila* MMP has all the structural features typical of members of this family. The stretch of hydrophobic residues close to the proposed initiator methionine strongly suggests the presence of the signal peptide, which is characteristic of most MMPs. *Dm1*-MMP also has a sequence PRCGV*X*D (at positions 91–97), which is a conserved motif in the prodomain of MMPs that is involved in maintaining latency. Seven residues COOH-terminal to this motif, the deduced amino acid sequence contains a furin consensus sequence (R*X*KR) that mediates the intracellular activation of several family members including MT-MMPs and stromelysin-3 (35, 36). In addition, *Dm1*-MMP also contains a putative catalytic domain of about 160 residues, including the consensus motif HE*X*GH*XX*G*XX*HS (at positions 224–234) containing the three His residues involved in the coordination of the zinc atom at the active site and the Ser residue that distinguishes MMPs from other metalloproteinases. This catalytic domain also has a Met residue seven residues COOH-terminal to the zinc-binding site, conserved in all MMPs and proposed to play an essential role in the structure of the active sites of these enzymes (37). Finally, the deduced sequence contains a COOH-terminal fragment of about 200 residues with sequence similarity to hemopexin and found in most family members. On the basis of these structural features, we propose that this nucleotide sequence codes for a new member of the MMP family that we suggest to call *Dm1*-MMP, because it is the first MMP cloned and characterized in *D. melanogaster*.

Sequence analysis has subdivided the collagenases, stromelysins, membrane-type MMPs, and gelatinases to distinct MMP subgroups. However, detailed analysis of the deduced amino acid sequence of *Dm1*-MMP did not allow us to assign it

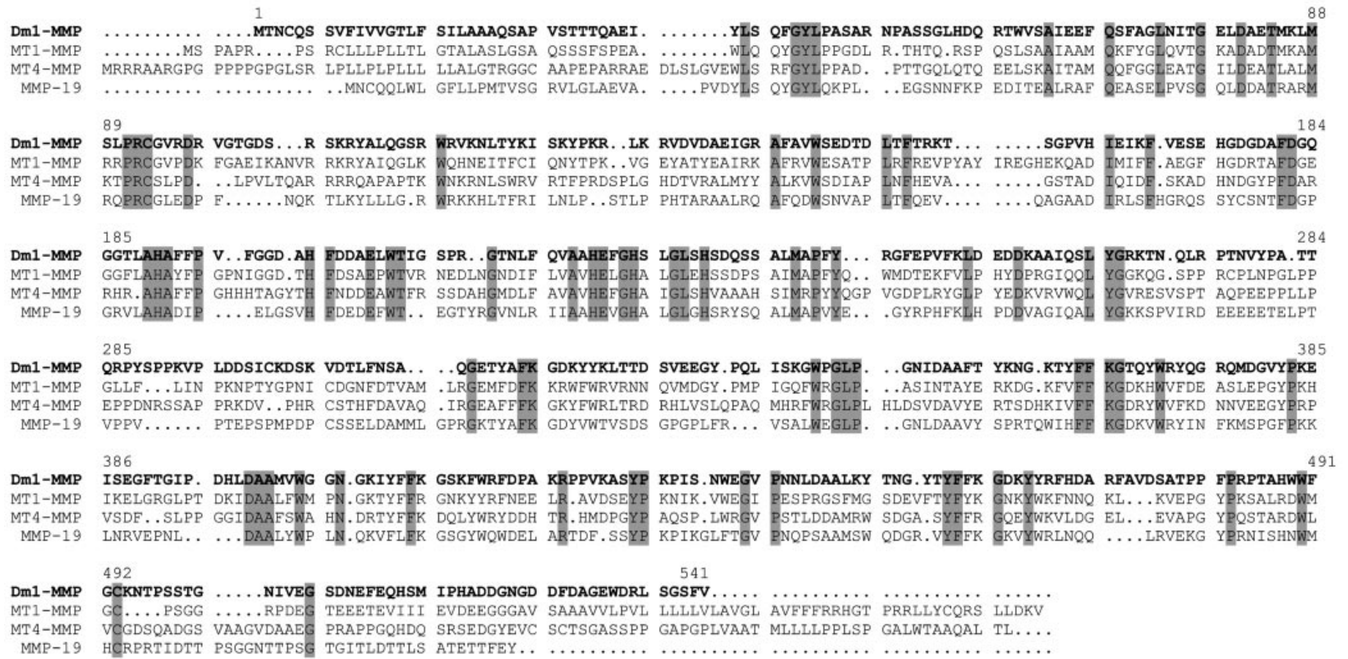


FIG. 2. **Amino acid sequence alignment of *Dm1*-MMP with different human MMPs.** The amino acid sequences of human MMPs showing the highest degree of sequence similarity with *Dm1*-MMP were extracted from the SwissProt data base, and the multiple alignment was performed with the PILEUP program of the GCG package. Common residues to all sequences are *shaded*. Gaps are indicated by *hyphens*. *Numbering* corresponds to the sequence of *Dm1*-MMP.

to any of the main subfamilies (Fig. 2). *Dm1*-MMP lacks the three residues (Tyr, Asp, and Gly) that are conserved in all collagenases and that have been proposed as essential determinants of collagenase specificity (38, 39). The equivalent residues in *Dm1*-MMP are Thr-216, Gln-237, and Ser-239. Stromelysins are characterized by the presence of an insertion of 9 mostly hydrophobic residues in the COOH terminus of their catalytic domain. The sequence of *Dm1*-MMP shows a longer insertion (15 residues) in the homologous region that has marked differences in amino acid sequence when compared with stromelysins. Furthermore, *Dm1*-MMP lacks the fibronectin-like domain present in gelatinases and the hydrophobic transmembrane domain in the COOH terminus characteristic of the MT-MMPs, although it possesses a COOH-terminal extension rich in acidic residues whose functional significance is presently unclear (Fig. 2). There is a growing category of "other MMPs," and we suggest that *Dm1*-MMP should be included with them. Finally, it should be mentioned that during preparation of this manuscript, the genomic sequence of *Drosophila* was reported (40). One of the annotated genes in this sequence (AAF47255) appears to correspond to *Dm1*-MMP although there are some differences in the predicted exons. The first exon of *Dm1*-MMP, which encodes the initiator Met and signal sequence, is not identified in AAF47255, whereas an additional exon is predicted at the 3′-end of AAF47255 which is missing in the corresponding cDNA. The finding of an expressed sequence tag covering the region present in clone AAF47255 together with data derived from sequencing several other cDNA clones are fully compatible with the sequence of *Dm1*-MMP reported in Fig. 2.

Enzymatic Activity of Dm1-MMP Produced in Bacterial Cells—To investigate the enzymatic properties of *Dm1*-MMP, a cDNA construct coding for its pro- and catalytic domains was expressed in *E. coli* as a His fusion protein (Fig. 3). After purification and refolding, a fraction of the proenzyme was autoactivated, resulting in the generation of a protein with a molecular mass of about 19 kDa (Fig. 3). This behavior has been observed previously with some vertebrate pro-MMPs (41).

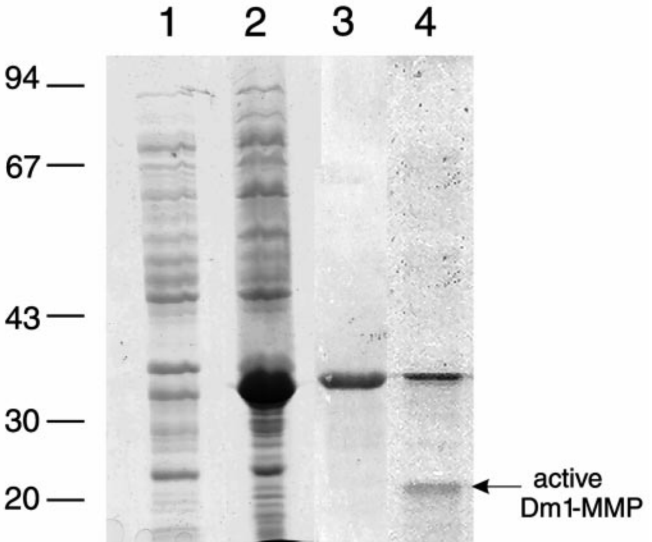


FIG. 3. **Production of recombinant *Dm1*-MMP in *E. coli* BL21(DE3)pLysS.** SDS-PAGE analysis of recombinant *Dm1*-MMP, 5 μl of bacterial extracts transformed with pRSETB (*lane 1*), pRSETB-Dm1-MMP (*lane 2*), and purified *Dm1*-MMP (*lane 3*). The processed form of the enzyme generated after dialysis of the purified pro*Dm1*-MMP (*lane 4*) indicated as active *Dm1*-MMP. The sizes of the molecular weight markers (*MWM*) are shown to the *left*.

In order to assess the substrate specificity of the recombinant protease, a series of synthetic quenched fluorescent peptides commonly used for assaying vertebrate MMPs were employed. As shown in Fig. 4, the general MMP substrate QF-24, the collagenase/gelatinase substrate QF-41, and the stromelysin substrate QF-35 were hydrolyzed by *Dm1*-MMP. Next, we examined the potential inhibition of active *Dm1*-MMP by different available TIMPs and the hydroxamic acid-based inhibitor BB-94 (Fig. 4). For this purpose, we used a constant enzyme concentration of 20 nM in the quenched fluorescent assay, employing QF-41 as substrate. As shown in Fig. 4, TIMP-4 com-

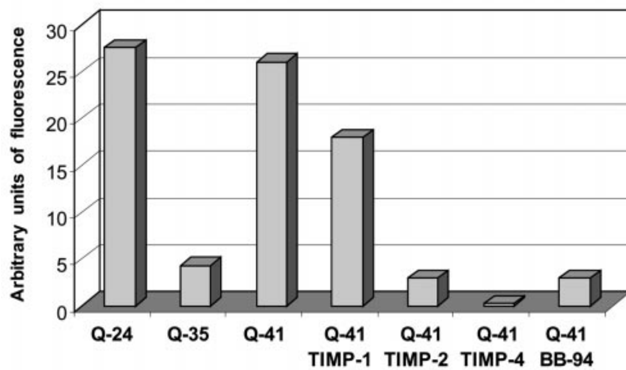


FIG. 4. **Analysis of enzymatic activity of *Dm1*-MMP.** Synthetic fluorescent peptides QF-24, QF-35, and QF-41 (1 μM) were incubated with active *Dm1*-MMP (20 nM) at 50 mM Tris/HCl, 5 mM $CaCl_2$, 150 mM NaCl, and 0.05% (v/v) Brij 35, pH 7.6, with a final concentration of Me_2SO of 1%, for 12 h at 37 °C. The fluorometric measurements were made at λ_{ex} = 328 nm and λ_{em} = 393 nm. Synthetic peptide QF-41 was incubated with active *Dm1*-MMP in the presence or absence of 20 nM of the indicated TIMPs and of the MMP inhibitor BB-94 (100 nM), and fluorescence was monitored as above.

pletely abolished the hydrolyzing activity of *Dm1*-MMP, whereas TIMP-2 and BB-94 extensively blocked this activity. By contrast, the inhibitory effect of TIMP-1 was significantly lower.

We next tested whether *Dm1*-MMP could hydrolyze a series of basement membrane and extracellular matrix components. For this purpose, a variety of proteins including type IV collagen, laminin, fibronectin, fibrinogen, gelatin, and fibrillar collagens were incubated with purified *Dm1*-MMP and the reactions followed by SDS-PAGE. As shown in Fig. 5*A*, the active *Dm1*-MMP was able to degrade mammalian fibronectin and type IV collagen. In both cases, the degrading activity was completely blocked by MMP inhibitors including EDTA, synthetic hydroxamic acid-based compounds like BB-94, and TIMP-4 (data not shown). Fig. 5*A* also shows that no proteolysis was obtained with laminin, fibrinogen, and gelatin. Similarly, type I and type II fibrillar collagens were resistant to hydrolysis, which is consistent with the fact that *Dm1*-MMP lacks the structural determinants to act as a triple helical fibrillar collagenase. Zymogram analysis using casein provided additional evidence on the enzymatic activity of *Dm1*-MMP (Fig. 5*B*). Lytic bands co-migrating with the proform and active *Dm1*-MMP recombinant proteins (35 and 19 kDa, respectively) were observed. An additional band of 21 kDa was also detected in the zymogram. This band is absent in the control extracts and likely corresponds to an intermediate form generated during the activation process (Fig. 5*B*). Taken together, these results provide evidence that *Dm1*-MMP is an active enzyme on extracellular matrix and basement membrane substrates and with the inhibitory profile characteristic of members of the MMP family of endopeptidases.

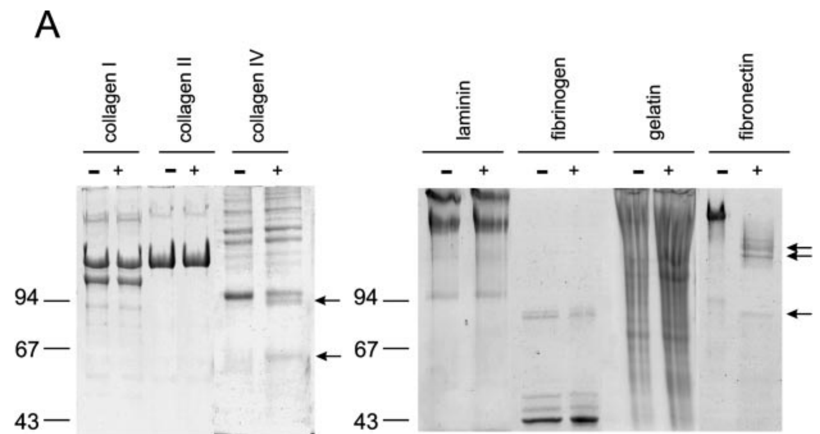
Spatio-temporal Expression Pattern of Dm1-MMP—To determine the temporal expression pattern of *Dm1*-MMP during *Drosophila* development, a Northern blot containing total RNA prepared from different developmental stages was hybridized with the *Dm1*-MMP cDNA. As can be seen in Fig. 6*A*, the *Dm1*-MMP mRNA migrated as a major band of 3.5 kb, although a second transcript of 7 kb was also detected. These *Dm1*-MMP transcripts were first observed in the embryo at 10–22 h. *Dm1*-MMP expression declined to much lower levels throughout all larval stages and was virtually undetectable in adults (Fig. 6*A*). To characterize the abundance of *Dm1*-MMP protein, we performed Western blot analysis of protein extracts from larva, using polyclonal antibodies against the purified recombinant protein. As can be seen in Fig. 6*B*, a major band of about 49 kDa and a minor one of 60 kDa were observed in larva. These bands likely correspond to the active and latent forms of the enzyme, respectively. By contrast, no signal was obtained with the preimmune antiserum (Fig. 6*B*). Finally, the spatial expression pattern of *Dm1*-MMP in *Drosophila* embryos was analyzed by whole mount *in situ* hybridization. In agreement with the results obtained by Northern blot analysis, *Dm1*-MMP mRNA was only detected in stage 12–13 embryos (Fig. 7). At this point, *Dm1*-MMP RNA was mainly in a single cluster of cells present in each segment along the ventral midline. At this developmental stage such pattern resembles the distribution of midline glial cells associated with the developing commissures of the ventral nerve cord (42).

DISCUSSION

This work provides the first characterization of a *Drosophila* MMP. The approach to identify *Dm1*-MMP involved the search of *Drosophila* genomic STSs for sequences conserved in vertebrate MMPs, followed by screening of a *Drosophila* larva cDNA library using the identified STSs as hybridization probes. The isolated full-length cDNA codes for a protein that contains all protein domains characteristic of vertebrate MMPs, including a signal sequence, a propeptide with a conserved Cys residue involved in maintaining enzyme latency, a catalytic domain with the corresponding zinc-binding site, a hinge region, and a COOH-terminal hemopexin domain organized in four recognizable repeats. *Dm1*-MMP also contains a furin-like cleavage site at the end of the propeptide domain that could be involved in the activation of this enzyme by some of the furin-like proteases described in *Drosophila* (43, 44). On the basis of these data, we conclude that the identified protein is a member of the MMP family that has conserved all structural features defined in its vertebrate counterparts as essential determinants for secretion, latency, activation, and catalytic activity of these enzymes.

In addition to all these structural properties, we have also provided evidence that *Dm1*-MMP is a functionally active member of this family of proteolytic enzymes as assessed by its ability to degrade several peptides and proteins widely used as substrates for vertebrate MMPs. Recombinant *Dm1*-MMP exhibited a broad specificity against synthetic substrates, efficiently degrading a general MMP peptide substrate as well as collagenase-gelatinase, and stromelysin-specific substrates. The recombinant *Dm1*-MMP was also able to cleave proteins such as fibronectin and type IV collagen, which are present in extracellular matrix and basement membranes and have been previously documented in *Drosophila* (45–47). Interestingly, all these proteolytic activities mediated by *Dm1*-MMP are inhibited by specific MMP inhibitors including TIMPs, providing additional support for the idea that *Dm1*-MMP behaves as its vertebrate counterparts in terms of enzymatic properties, substrate specificity, and sensitivity to inhibitors.

The finding of a *Drosophila* MMP exhibiting striking structural and functional similarities with MMPs described in other organisms, together with the observation that at least a member of the TIMP gene family is also present in flies (48), strongly suggests that a conserved proteolytic system of tissue remodeling can be fully reconstituted in invertebrates. However, compared with other organisms, the *Drosophila* MMP system is significantly simpler. In fact, 20 different MMPs and 4 TIMPs have been described in human tissues to date, whereas only two MMPs and a single TIMP have been identified in the *Drosophila* genome (40, 48). These results suggest that this protease family has undergone extensive gene duplication events following divergence of invertebrates and vertebrates, perhaps as a consequence of the increasing complexity



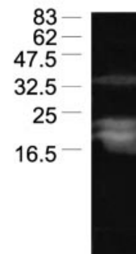
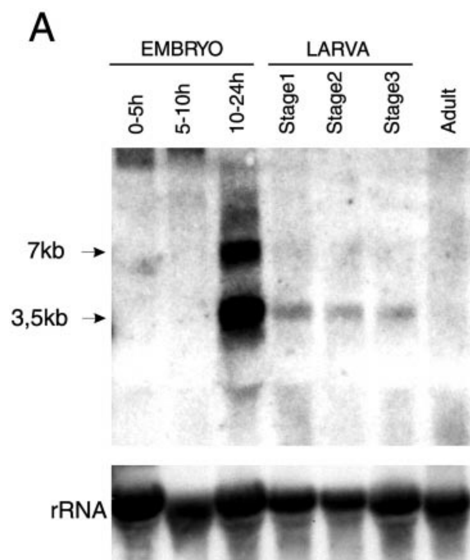


FIG. 5. **Degradation of extracellular matrix compounds by recombinant *Dm1*-MMP.** *A*, type I, II, and IV collagens, laminin, fibronectin, fibrinogen, and gelatin were incubated with buffer alone (− *lanes*) or with 1 μg of *Dm1*-MMP (+ *lanes*). The digestion products were analyzed by SDS-PAGE (8% acrylamide) under reducing conditions and stained with Coomassie Blue after electrophoresis. The sizes of the molecular weight markers (*MWM*) are shown to the *left*. *B*, zymogram analysis of *Dm1*-MMP. *Dm1*-MMP was analyzed by casein zymography under nonreducing conditions. The sizes of the molecular weight markers (*MWM*) are shown to the *left*.



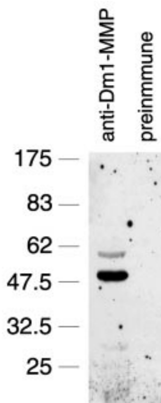


FIG. 6. **Expression analysis of *Dm1*-MMP in diverse *Drosophila* development stages.** *A*, developmental pattern of the *Dm1*-MMP transcripts determined by Northern blot analysis. The filter was hybridized to a *Dm1*-MMP cDNA probe and then to a ribosomal DNA probe to control for RNA loading. *B,* Western blot analysis of larval extracts incubated with polyclonal antibody against *Dm1*-MMP diluted 1/5000 in PBT**.**

of substrates that must be hydrolyzed by mammalian MMPs. However, the possibility that *Drosophila* MMPs may have a broader substrate specificity cannot be ruled out. Nevertheless, the apparently simplified MMP-TIMP system in *Drosophila* may represent a very useful and interesting model for studying the functional role of protease-mediated events during development processes. This aspect is of special interest considering that over many years *Drosophila* has proven to be ideally suited for the analysis of this type of biological questions. In addition, it is remarkable that other experimental systems including *C. elegans* or *A. thaliana* are somewhat incomplete as compared with *Drosophila* if we consider that to date no evidence of presence of TIMPs in these organisms has been reported (20, 22).

As a prelude to analyzing the functional importance of *Dm1*-MMP in development processes, we have examined the spatiotemporal pattern of expression of this enzyme in the *Drosophila* embryo. Interestingly, in the course of embryogenesis, *Dm1*-MMP was detected predominantly in what appear to be midline glial cells, suggesting that this enzyme may have a role in the

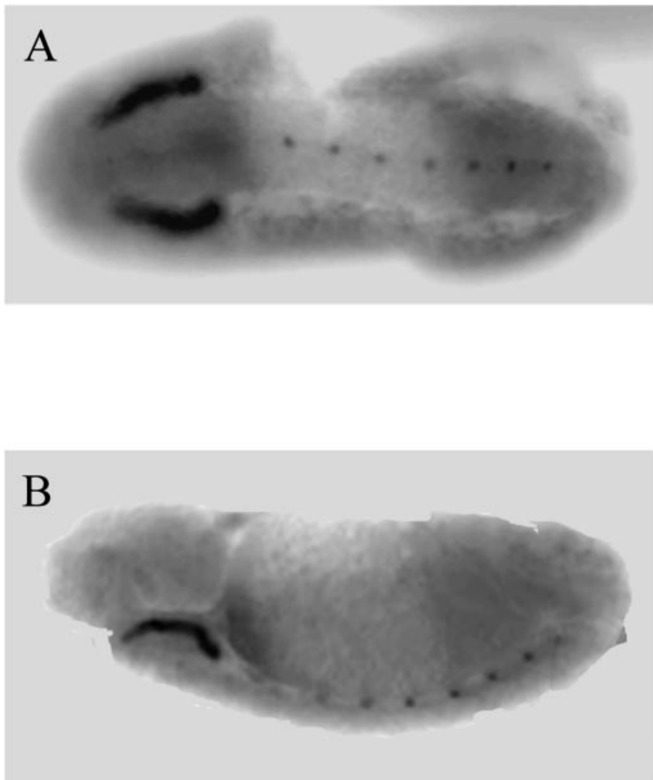


FIG. 7. **Embryonic pattern of *Dm1*-MMP gene expression.** *In situ* hybridization to stage 12–13 embryos using a *Dm1*-MMP antisense RNA probe. *A,* ventral view; *B,* lateral view. Hybridization signal is detected in midline glial cells. Signal at the salivary glands was detected with the sense and antisense probe.

development of the *Drosophila* neural system. The observed pattern of expression has interesting parallels to the expression of previously described genes such as *buttonless* (49), and this similarity may provide clues to the putative function of *Dm1*-MMP in development of *Drosophila* neural system. The midline glia are specialized non-neuronal cells that play a major role in growth cone guidance (50–52). Thus, during neural development, these cells are thought to provide guidance cues for extending axons and at the same time to migrate and contribute to separate the two axon commissures. *Dm1*-MMP synthesized by these glial cells could be directly involved in these processes. In this way, its proteolytic activity on extracellular matrix proteins may facilitate growth cone penetration through the complex cellular environment of the nervous system. Consistent with this possibility, previous work has demonstrated that MMPs are associated with extending neurites in mammals (53, 54). Likewise, the ADAM metalloprotease encoded by *kuzbanian* is required for axonal extension in the embryonic central nervous system of *Drosophila* (23–27, 55). Alternatively, *Dm1*-MMP might play more specific and subtle roles than providing space for axonal growth by degrading extracellular matrix proteins. Instead, this protease could help regulate the availability of proteins sequestered as inactive molecules in the extracellular matrix or help produce guidance signals encrypted in cell surface molecules located in the environment of midline glial cells. In this regard, it is of interest that a number of midline glia or growth cone guidance proteins such as Fasciclin II, Neuroglian, Wrapper, Frazzled, and Klingon, contain several fibronectin-like domains in their extracellular region (56–60). Our finding that *Dm1*-MMP can degrade fibronectin suggests that these proteins could be potential targets of a regulated action of this protease. The advantages of *D. melanogaster* as an experimental model will make it possible to combine genetic and biochemical approaches to understand the biological meaning of the presence of *Dm1*-MMP during neural development and to identify functionally relevant targets of this protease.

In conclusion, we have cloned *Dm1*-MMP the first member of the MMP family identified and characterized in *Drosophila*. This enzyme exhibits extensive structural similarities with its vertebrate counterparts in terms of similar domain organization and the presence of critical residues for enzymatic activity. Likewise, functional analysis has confirmed that *Dm1*-MMP is able to degrade synthetic substrates and extracellular matrix remodeling and basement membrane protein components that are targets of the proteolytic action of vertebrate MMPs. Expression analysis has revealed an unexpected specificity to its synthesis and suggests interesting roles of this protease in development of the neural system. Further studies, including analysis with mutant *Drosophila* deficient in *Dm1*-MMP, will be required to elucidate the precise role of this protease in any of the extensive extracellular matrix remodeling processes taking place during *Drosophila* development.

Acknowledgments—We thank all members from our group for support and helpful comments and S. Alvarez, F. Rodríguez, C. Garabaya, and M. Fernández for excellent technical assistance. We are also grateful to Drs. V. Knäuper and Jan O. Stracke (School of Biological Sciences, University of East Anglia, UK) for reagents and technical discussions. The Instituto Universitario de Oncologia is supported by Obra Social Cajastur-Asturias.

REFERENCES

1. Gross, J., and Lapière, C. M. (1962) *Proc. Natl. Acad. Sci. U. S. A.* **54,** 1197–1204
2. Nagase, H., and Woessner, F., Jr. (1999) *J. Biol. Chem.* **274,** 21491–21494
3. Lund, R. L., Romer, J., Bugge, T. H., Nielsen, B. S., Frandsen, T. L., Degen, J. L., Stephens, R. W., and Dano, K. (1999) *EMBO J.* **18,** 4645–4656
4. Halpert, I., Sires, U. I., Roby, J. D., Potter-Perigo, S., Wight, T., Shapiro, S. D., Welgus, H. G., Wickline, S. A., and Parks, W. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 9748–9753
5. Konttinen, Y., Ainola, M., Valleala, H., Ma, J., Ida, H., Mandelin, J., Kinne, R. W., Santavirta, S., Sorsa, T., López-Otín, C., and Takagi, M. (1999) *Ann. Rheum. Dis.* **58,** 691–697
6. Yong, V. W., Krekoski, C. A., Forsyth, P. A., Bell, R., and Edwards, D. R. (1998) *Trends Neurosci.* **21,** 75–80
7. MacDougall, J. R., and Matrisian, L. M. (1995) *Cancer Metastasis Rev.* **14,** 351–362
8. Park, H. I., Ni, J., Gerkema, F. E., Liu, D., Belozerov, V. E., and Sang, Q.-X. A. (2000) *J. Biol. Chem.* **275,** 20540–20544
9. Uría, J. A., and López-Otín, C. (2000) *Cancer Res.* **60,** 4745–4751
10. Brew, K., Dinakarpandian, D., and Nagase, H. (2000) *Biochim. Biophys. Acta* **1477,** 267–283
11. Werb, Z. (1997) *Cell* **91,** 439–442
12. Murphy, G., and Gavrilovic, J. (1999) *Curr. Opin. Cell Biol.* **11,** 614–621
13. Yu, Q., and Stamenkovic, I. (2000) *Genes Dev.* **14,** 163–176
14. Couet, J., Sar, S., Jolivet, A., Hai, M. T. V., Milgrom, E., and Misrahi, M. (1996) *J. Biol. Chem.* **271,** 4545–4552
15. Koshikawa, N., Giannelli, G., Cirulli, V., Miyazaki, K., and Quaranta, V. (2000) *J. Cell Biol.* **148,** 615–624
16. Stolow, M. A., Bauzon, D. D., Li, J., Sedgwick, T., Liang, V. C. T., Sang, Q. A., and Shi, Y. B. (1996) *Mol. Biol. Cell* **7,** 1471–1483
17. Yang, M., Murray, M. T., and Kurkinen, M. (1997) *J. Biol. Chem.* **272,** 13527–13533
18. Lepage, T., and Gache, C. (1990) *EMBO J.* **9,** 3003–3012
19. Kinoshita, T., Fuzukawa, H., Shimada, T., Saito, T., and Matsuda, Y. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4693–4697
20. Wada, K., Sato, H., Kinoh, H., Kajita, M., Yamamoto, H., and Seiki, M. (1998) *Gene* (*Amst.*) **21,** 57–62
21. Leontovich, A. A., Zhang, J., Shimokawa, K., Nagase, H., and Sarras, M. P., Jr. (2000) *Development* **127,** 907–922
22. Maidment, J. M., Moore, D., Murphy, G. P., Murphy, G., and Clark, I. M. (1999) *J. Biol. Chem.* **274,** 34706–34710
23. Rooke, J., Pan, D., Xu, T., and Rubin, G. M. (1996) *Nature* **273***,* 1227–1231
24. Pan, D., and Rubin, G. M. (1997) *Cell* **90,** 271–280
25. Qi, H., Rand, M. D., Wu, X., Sestan, N., Wang, W., Rakic, P., Xu, T., and Artavanis-Tsakonas, S. (1999) *Science* **283,** 91–94
26. Marqués, G., Musacchio, M., Shimell, M. J., Wunnenberg-Stapleton, K., Cho, K. W., and O'Connor, M. B. (1997) *Cell* **91,** 417–426
27. Sotillos, S., Roch, F., and Campuzano, S. (1997) *Development* **124,** 4769–4779
28. Siden-Kiamos, I., Saunders, R. D., Spanos, L., Majerus, T., Treanear, J., Savakis, C., Louis, C., Glover, D. M., Ashburner, M., and Kafatos, F. C. (1990) *Nucleic Acids Res.* **18,** 6261–6270
29. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. Langer-Safer, P. R., Levine, M., and Ward, D. C. (1982) *Proc. Natl. Acad. Sci.*

U. S. A. **79,** 4381–4385
31. Tautz, D., and Pfeifle, C. (1989) *Chromosoma* **89,** 81–85
32. Willembrock, F., Crabbe, T., Slocombe, P. M., Sutton, C. W., Docherty, A. J. P., Cockett, M. I., O'Shea, M. I., Brocklehurst, K., Phillips, I. R., and Murphy, G. (1993) *Biochemistry* **32,** 4330–4337
33. Vaitukaitis, J. L. (1981) *Methods Enzymol.* **73,** 46–52
34. Cavener, D. R. (1987) *Nucleic Acids Res.* **15,** 1353–1361
35. Pei, D., and Weiss, S. J. (1995) *Nature* **375,** 244–247
36. Sato, H., Kinoshita, T., Takino, T., Nakayama, K., and Seiki, M. (1996) *FEBS Lett.* **393,** 101–104
37. Bode, W., Gomis-Rüth, F. X., and Stöcker, W. (1993) *FEBS Lett.* **331,** 134–140
38. Sánchez-López, R., Alexander, C. M., Behrendtsen, O., Breathnach, R., and Werb, Z. (1993) *J. Biol. Chem.* **268,** 7238–7247
39. Freije, J. P., Díez-Itza, I., Balbín, M., Sánchez, L. M., Blasco, R., Tolivia, J., and López-Otín, C. (1994) *J. Biol. Chem.* **269,** 16766–16773
40. Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., *et al.* (2000) *Science* **287,** 2185–2195
41. Stracke, J. O., Hutton, M., Stewart, M., Pendás, A. M., Smith, B., López-Otín, C., Murphy, G., and Knäuper, V. (2000) *J. Biol. Chem.* **275,** 14809–14816
42. Spana, E. P., Kopczynski, C., Goodman, C. S., and Doe, C. Q. (1995) *Development* **121,** 3489–3494
43. Roebroek, A. J., Creemers, J. W., Pauli, I. G., Kurzik-Dumke, U., Rentrop, M., Gateff, E. A., Leunissen, J. A., and Van de Ven, W. J. (1992) *J. Biol. Chem.* **267,** 17208–17215
44. Roebroek, A. J., Creemers, J. W., Pauli, I. G., Bogaert, T., and Van de Ven, W. J. (1993) *EMBO J.* **12,** 1853–1870
45. Fessler, J. H., and Fessler, L. I. (1989) *Annu. Rev. Cell Biol.* **5,** 309–339
46. Cecchini, J. P., Knibiehler, B., Mirre, C., and Le Parco, Y. (1987) *Eur. J. Biochem.* **165,** 587–593
47. Gratecos, D., Naidet, C., Astier, M., Thiery, J. P., and Semeriva, M. (1988) *EMBO J.* **7,** 215–223
48. Pohar, N., Godenschwege, T. A., and Buchner, E. (1999) *Genomics* **57,** 293–296
49. Chiang, C., Patel, N. H., Young, K. E., and Beachy, P. A. (1994) *Development* **120,** 3581–3593
50. Klambt, C., Jacobs, J. R., and Goodman, C. S. (1991) *Cell* **64,** 801–815
51. Goodman, C. S. (1996) *Annu. Rev. Neurosci.* **19,** 341–377
52. Menne, T. V., Luer, K., Technau, G. M., and Klambt, C. (1997) *Development* **124,** 4949–4958
53. Nordstrom, L. A., Lochner, J., Yeung, W., and Ciment, G. (1995) *Mol. Cell. Neurosci.* **6,** 56–68
54. Muir, D. (1994) *Exp. Cell Res.* **210,** 243–252
55. Fambrough, D., Pan, D., Rubin, G. M., and Goodman, C. S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13233–13238
56. Grenningloh, G., Rehm, E. J., and Goodman, C. S. (1991) *Cell* **67,** 45–57
57. Bieber, A. J., Snow, P. M., Hortsch, M., Patel, N. H., Jacobs, J. R., Traquina, Z. R., Schilling, J., and Goodman, C. S. (1989) *Cell* **59,** 447–460
58. Noordermeer, J. N., Kopczynski, C. C., Fetter, R. D., Bland, K. S., Chen, W. Y., and Goodman, C. S. (1998) *Neuron* **21,** 991–1001
59. Kolodziej, P. A., Timpe, L. C., Mitchell, K. J., Fried, S. R., Goodman, C. S., Jan, L. Y., and Jan, Y. N. (1996) *Cell* **87,** 197–204
60. Butler, S. J., Ray, S., and Hiromi, Y. (1997) *Development* **124,** 781–792