

## Structural and Enzymatic Characterization of *Drosophila* Dm2-MMP, a Membrane-bound Matrix Metalloproteinase with Tissue-specific Expression\*

Received for publication, January 4, 2002, and in revised form, April 11, 2002  
Published, JBC Papers in Press, April 19, 2002, DOI 10.1074/jbc.M200121200

Elena Llano‡§, Geza Adam§¶, Alberto M. Pendás‡, Víctor Quesada‡, Luis M. Sánchez‡, Iñigo Santamaría‡, Stéphane Noselli¶, 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 ¶Institut de Recherches Signalisation, Développement et Cancer, Centre de Biochimie-UMR 6543-CNRS, Parc Valrose, 06108 Nice Cedex 2, France

We report the isolation and characterization of a cDNA encoding Dm2-MMP, the second matrix metalloproteinase (MMP) identified in the *Drosophila melanogaster* genome. The cloned cDNA codes for a polypeptide of 758 residues that displays a domain organization similar to that of other MMPs, including signal peptide, propeptide, catalytic, and hemopexin domains. However, the structure of Dm2-MMP is unique because of the presence of an insertion of 214 amino acids between the catalytic and hemopexin domains that is not present in any of the previously described MMPs. Dm2-MMP also contains a C-terminal extension predicted to form a cleavable glycosylphosphatidylinositol anchor site. Western blot and immunofluorescence analysis of S2 cells transfected with the isolated cDNA confirmed that Dm2-MMP is localized at the cell surface. Production of the catalytic domain of Dm2-MMP in *Escherichia coli* and analysis of its enzymatic activity revealed that this proteinase cleaves several synthetic peptides used for analysis of vertebrate MMPs. This proteolytic activity was abolished by MMP inhibitors such as BB-94, confirming that the isolated cDNA codes for an enzymatically active metalloproteinase. Reverse transcription-PCR analysis showed that Dm2-MMP is expressed at low levels in all of the developmental stages of *Drosophila* as well as in adult flies. However, detailed *in situ* hybridization at the larval stage revealed a strong tissue-specific expression in discrete regions of the brain and eye imaginal discs. According to these results, we propose that Dm2-MMP plays both general proteolytic functions during *Drosophila* development and in adult tissues and specific roles in eye development and neural tissues through the degradation and remodeling of the extracellular matrix.

The matrix metalloproteinases (MMPs)<sup>1</sup> are a family of structurally related enzymes that play major roles in the connective tissue remodeling occurring in a variety of physiological conditions, such as embryonic growth and development, angiogenesis, wound healing, or reproductive processes (1–3). In addition, deregulated production of these endopeptidases is associated with a number of pathological conditions including rheumatoid arthritis (4), atherosclerosis (5), and tumor invasion and metastasis (6). To date, more than 20 distinct MMPs have been identified in human tissues (7–9). These MMPs have been classified into six major subfamilies according to their primary structures, domain organization, cellular localization, and substrate specificity. These subfamilies are collagenases, stromelysins, gelatinases, matrilysins, membrane-type MMPs, and other MMPs (9). The structure of most of these enzymes is organized into several characteristic domains: a signal peptide to direct secretion from the cell, a prodomain 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). Several members of the human MMP family lack some of these well defined domains. Thus, MMP-23, the most distantly related family member, does not contain a cleavable signal peptide (11, 12), whereas matrilysins have lost the hemopexin domain (9). In contrast, additional domains such as fibronectin-like repeats or C-terminal hydrophobic extensions have been incorporated into the structure of other family members like gelatinases or membrane-type MMPs, thereby contributing to generate a considerable diversity in the structural organization of these enzymes.

In close parallelism with the structural complexity of MMPs, recent functional studies have revealed that these proteinases are also involved in processes distinct from those derived from their ability to degrade the different protein components of the extracellular matrix and basement membranes. Thus, MMPs play direct roles in essential cellular processes such as proliferation, differentiation, angiogenesis, apoptosis, or defense responses through their ability to target other substrates including proteinase inhibitors, chemokines, antimicrobial peptides, and membrane-bound precursors of growth factors, cytokines, and hormone receptors (13–18).

\* This work was supported by Comisión Interministerial de Ciencia y Tecnología Grant SAF00-0217, Plan Fondos Europeos para el Desarrollo Regional Grant 1FD97-0214, a CNRS grant (ATIPE), l'Association pour la Recherche sur le Cancer Grants 5550 and 7594, and a Fondation pour la Recherche Médicale grant. The Instituto Universitario de Oncología is supported by Obra Social Cajastur-Asturias. 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) AJ289232.

§ These authors contributed equally to this work.

¶ 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.

<sup>1</sup> The abbreviations used are: MMP, matrix metalloproteinase; GdnHCl, guanidinium hydrochloride; HA, hemagglutinin; RT, reverse transcription; TIMP, tissue inhibitors of metalloproteinase; PBS, phosphate-buffered saline.

An important aspect in understanding the increased functional diversity of this growing family of proteolytic enzymes is to identify and characterize MMPs in model organisms, in which the functional relevance of these enzymes can be extensively analyzed by using distinct experimental strategies. Thus, in addition to the diverse MMPs identified in vertebrates, proteases belonging to this family have also been described in a variety of organisms from plants such as *Arabidopsis thaliana* (19), soybean (20), and cucumber (21), to invertebrates like *Caenorhabditis elegans* (22), sea urchin (23), *Hydra vulgaris* (24), and more recently *Drosophila melanogaster* (25). In this regard, we have described the finding and characterization of *Dm1*-MMP, the first member of this proteinase family identified in *Drosophila* (25), a model organism that is central to the study of developmental biology. Because of the relevance of MMPs in developmental processes, the finding of additional members of this family in *Drosophila* may contribute to uncover new substrates and new functions for these enzymes, potentially extrapolable to vertebrate MMPs. In this study, we report the cloning and the structural and enzymatic characterization of *Dm2*-MMP, the second member of the MMP family identified in *D. melanogaster*. Interestingly, extensive searches of the *Drosophila* genome revealed the presence of only two MMPs in this organism, thus offering a simplified and genetically tractable system to study MMP function in development. We have also performed an expression analysis of *Dm2*-MMP during *Drosophila* development, showing that the gene is expressed at all stages. Detailed *in situ* hybridization analysis at the larval stage revealed a strong expression in discrete regions of the brain and eye imaginal discs. Taken together, our data suggest that this novel MMP member may play both general and specific degradative roles in the extracellular matrix remodeling processes occurring in *Drosophila* during development and in adult tissues.

#### EXPERIMENTAL PROCEDURES

**Materials**—Synthetic oligonucleotides were prepared with an Applied Biosystems (Foster City, CA) model 392A DNA synthesizer. Restriction endonucleases and reagents used for molecular cloning were from Roche Molecular Biochemicals. Double-stranded DNA probes were radiolabeled with [<sup>32</sup>P]dCTP (3000 Ci/mmol) from Amersham Biosciences, using a commercial random priming kit from the same company.

**Probe Preparation and Hybridization of a *Drosophila* cDNA Library**—Screening of the GenBank™ data base for entries with similarity to previously described MMPs allowed us to identify a sequence (AC005894) contributed by the Berkeley *Drosophila* Genome Project. This sequence revealed regions with significant similarity to the catalytic and hemopexin domains of MMPs. To obtain the corresponding cDNA sequence, two specific primers 5'-TGCAGACCGCCCTGGACGT (primer 1, catalytic domain) and 5'-ATGTAGGTGCGGTTGTTGTGG (primer 2, hemopexin domain) were used for PCR amplification to prepare a probe for screening of cDNAs from different developmental stages. The PCR reaction was carried out in a GeneAmp 2400 PCR system from PerkinElmer Life Sciences for 30 cycles of denaturation (94 °C for 15 s), annealing (60 °C for 15 s), and extension (72 °C for 45 s). A 1473-bp PCR product amplified from pupa cDNA was radiolabeled and used to screen a pupa cDNA library according to standard procedures (26). In addition, we obtained a 4120-bp full-length cDNA (SD03462) identified by the Berkeley *Drosophila* Genome Project (Research Genetics). Cloned DNA fragments were sequenced with an Applied Biosystems 310A 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.

**Construction of an Eukaryotic Expression Vector for *Dm2*-MMP and Immunolocalization**—Full-length *Dm2*-MMP cDNA was subcloned into pRmHA-3 expression vector (27). In addition, a 24-bp linker coding for the hemagglutinin (HA) epitope of the human influenza virus was inserted immediately downstream of the putative furin cleavage motif. S2 cells were transfected with 1 µg of plasmid DNA, using the FuGENE 6 reagent (Roche Molecular Biochemicals) according to the manufactur-

er's instructions. Forty-eight hours after transfection, CuSO<sub>4</sub> was added to a final concentration of 0.7 mM to induce the cells and incubated for 6–18 h to produce the protein. The cells were then fixed for 10 min in cold 4% paraformaldehyde in PBS, washed in PBS, and incubated for 10 min in the presence or absence of 0.2% Triton X-100 in PBS. Fluorescent detection was performed by incubating the slides with monoclonal antibody 12CA5 (Roche Applied Science) against HA (diluted 1:2500), followed by another incubation with goat anti-mouse fluoresceinated antibody (diluted 1:50). After washing in PBS, the slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and observed in a Bio-Rad confocal laser microscope. S2 extracts were also obtained for Western blot analysis of the *Dm2*-MMP-HA protein.

**Preparation of Cell Membrane Fractions and Western Blot Analysis**—S2 cells were transiently transfected with the pRmHA-3-*Dm2*-MMP-HA plasmid as described previously. The cells were scraped from the plates, and the membrane fractions were prepared essentially following the procedure described by Strongin *et al.* (28). The extracts were separated by SDS-PAGE, analyzed by Western blotting with an anti-HA monoclonal antibody, and detected with an enhanced chemiluminescence kit (Amersham Biosciences).

**Production of Recombinant *Dm2*-MMP in *Escherichia coli***—A 462-bp fragment of the *Dm2*-MMP cDNA containing the catalytic domain was generated by PCR amplification with primers 5'-GGAATTCATATGTTCGCCCTGCAGGGACCCAAG (*NdeI*-proDm2) and 5'-CGGGATCCTTAGTACAACCTGCTGAATGCCATA (*BamHI*-proDm2) using the full-length *Dm2*-MMP cDNA as a template. PCR amplification was performed for 30 cycles using the Expand™ high fidelity PCR system. Because of the design of the oligonucleotides, the amplified fragment could be cleaved at both ends with *NdeI* and *BamHI* and ligated in frame into the pET *E. coli* expression vector (Novagen). The resulting pET-Dm2 vector was transformed into BL21(DE3) *E. coli* cells, and expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (final concentration, 0.5 mM) followed by further incubation for 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 dithiothreitol and purified in a Superdex-75 column (Amersham Biosciences) equilibrated with 20 mM Tris buffer, pH 7.6, containing 3 M GdnHCl and 5 mM dithiothreitol. 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<sub>2</sub>, 200 mM NaCl, 50 µM ZnSO<sub>4</sub>, 0.05% Brij 35, 20% glycerol, and 2 M GdnHCl and then against the same buffer with 2 mM dithiothreitol and without GdnHCl.

**Enzymatic Assays**—Enzymatic activity of purified recombinant *Dm2*-MMP was analyzed by using the synthetic fluorescent substrates Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH<sub>2</sub> (QF-24), Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH<sub>2</sub> (QF-35), and Mca-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH<sub>2</sub> (QF-41) (provided by C. G. Knight, University of Cambridge, Cambridge, UK). The assays were performed at 37 °C at substrate concentrations of 1 µM in a buffer containing 50 mM Tris/HCl, 5 mM CaCl<sub>2</sub>, 200 mM NaCl, 0.05% (v/v) Brij 35, pH 7.6, and 1% Me<sub>2</sub>SO (29). For inhibition assays, *Dm2*-MMP (20 nM) and inhibitors were preincubated for 30 min at 20 °C, with 1 mM EDTA or with BB-94 (British Biotech Pharmaceuticals, Oxford, UK) at concentrations ranging from 0 to 100 nM. The fluorometric measurements were made in an MPF-44A PerkinElmer Life Sciences spectrofluorometer (λ<sub>exc</sub> = 328 nm, λ<sub>em</sub> = 393 nm).

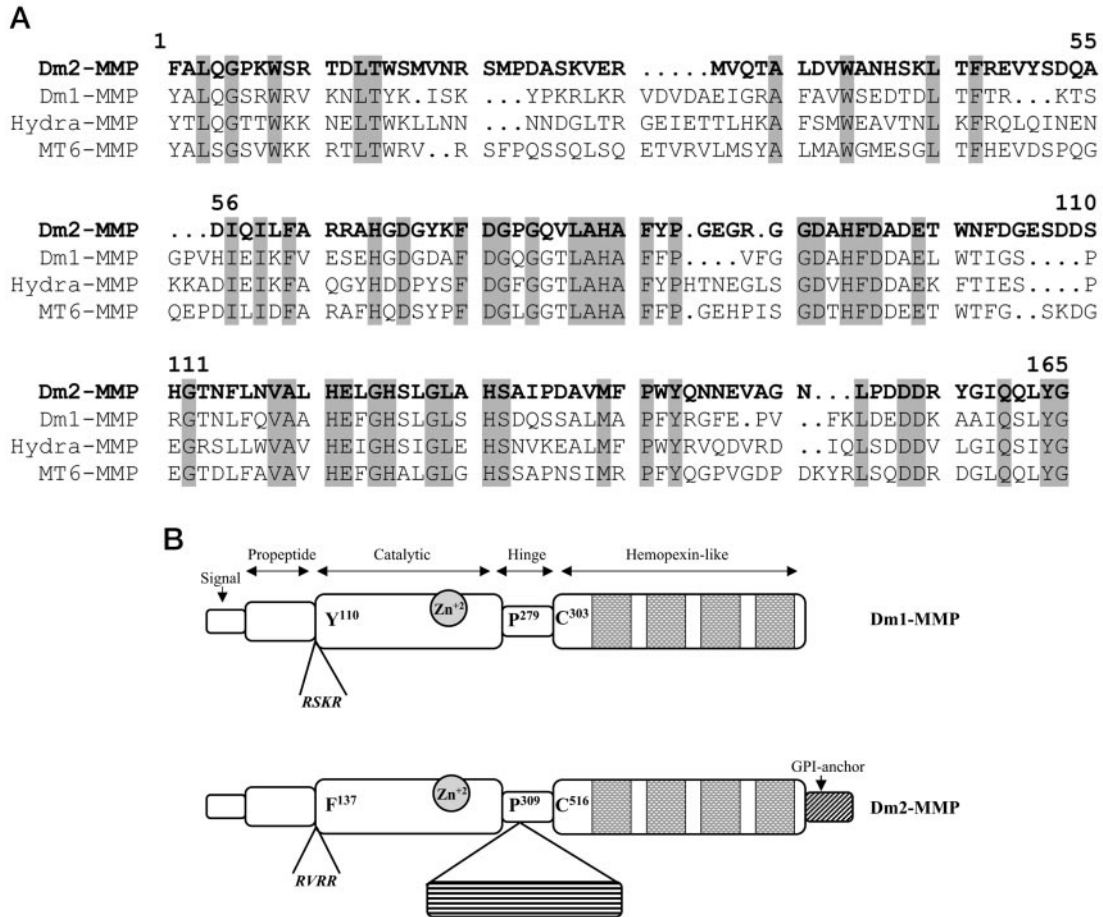
**Homology Modeling**—A three-dimensional model of the catalytic domain of *Dm2*-MMP was calculated using Swiss-Model, a semiautomated modeling server (30), and analyzed with the Swiss Protein Data Bank Viewer. The quality of the resulting models was verified automatically with WhatCheck and manually with the Swiss Protein Data Bank Viewer. Electrostatic analyses of the model were performed with MolMol (31).

**RT-PCR Analysis**—RT-PCR was performed for analysis of *Dm2*-MMP expression during *Drosophila* development or in adult flies. Total RNA was extracted from diverse developmental stages of *Drosophila* or from adult tissues, and 1 µg was used to perform the reverse transcription. The PCR reaction was carried out for 35 cycles of denaturation (94 °C for 15 s), annealing (60 °C for 15 s), and extension (72 °C for 25 s) using two specific oligonucleotides 5'-TTACTCATGCAGTTTGAT-TATCT (primer 1), and 5'-CTCCACGTAAGATCCGTTCTG (primer 2). The integrity of RNA was verified by similar RT-PCR experiments using specific primers for the *Drosophila* gene encoding ribosomal protein 49 (rp-49).

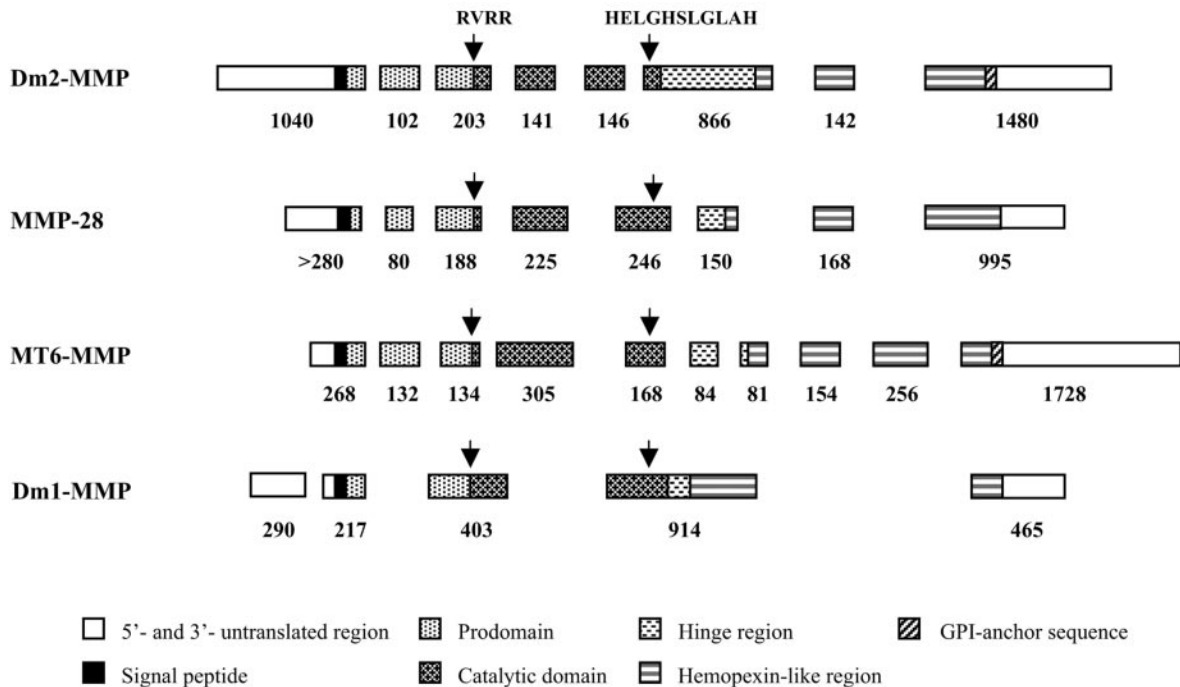
**In Situ Hybridization**—RNA *in situ* analysis was performed as described previously (32) with the following modifications. The hybridiza-

1	ttaagcctcgaaacatagaataaagataaacacagttttgttctactgatcgaggaatagtttcgtgtccaagtgttttcgttgcgcg	90
91	agcgaaagagagcggcgcaaatgtttccaaaatgtttctccaccctgctcgcactctttgcgcaatccaatgtgcatccaggagctgtc	180
	M F S K Y V L A T L L A L F A Q S M C I Q E L S	
181	cctccccaccggaagatctcactccaccgctgagcagggtccaagaagcaaaaaaccgctatctccgagatataatgtacaattacct	270
	L P P E G S H S T A A T R S K K A K T A I S E D I M Y N Y L	
271	catgcagtttgattatctgcccaagtcggacctggaacgggtgctctgagcaccgaggaccagctgaaagaagcaatccggagctcca	360
	M Q F D Y L P K S D L E T G A L R T E D Q L K E A I R S L Q	
361	gtcttttgcaacattacagttacggggcaaatcgactcggcaacggccggcctaatacagaaacctcggcggcggtggggcagacaag	450
	S F G N I T V T G E I D S A T A R L I Q K P R C G V G D R R	
451	gtccggcagcagcttctcgcagataacctgtatcacgaaatcggtcccaatgtgctgggtgagggccttcgacctgacgggaccacaagtg	540
	S A D S F T S P D N L Y H E I G S N V R V R R F A L Q G P K W	
541	gtccagaacggatcttacgtggagcatgggtcaacaggtcgtgcccgatgctccaaagtcgagagaatgggtgcagaccgacctggagct	630
	S R T D L T W S M V N R S M P D A S K V E R M V Q T A L D V	
631	ttggggcaaccactcgaagctgacattccggcaggtctacagcagaccaggccgacattcagatactctttgagggcgcgcgacggcga	720
	W A N H S K L T F R E V Y S D Q A D I Q I L F A R R A H G D	
721	tggtataaattcagatggacctgggtcaggtgctggcccagcctctatcccggcagggggcgtggcggagatgccatttcgatgcgga	810
	G Y K F D G P G Q V L A H A F Y P G E G R G G D A H F D A D	
811	cgagacgtggaacttgacggcgaaatccgatgatagccatggaaacaaatttctgaacgtggccctgcacgaaactgggtcattcgctggg	900
	E T W N F D G E S D D S H G T N F L N V A L H E L G H S L G	
901	actggcccactcccgatccccggatgctggctcagttttccatgggtaccagaataacgaggtggcgggcaacctgcccagcagcatcgcta	990
	L A H S A I P D A V M F P W Y Q N N E V A G N L P D D R Y	
991	tggtcattcagcagttgtacggcactaaggagaagacctggggggcacaatacaaccacaacaacgacaacaaccacgacgacccat	1080
	G I Q Q L Y G T K E K T W G P Y K P Q T T T T T T T T T M	
1081	gcgcgcatgatttaccgggcagataaaaccggcctactggccatggaacaaatccaagcaacaatcccaacaatgatcgaaatcggtccag	1170
	R A M I Y R A D K P A Y W P W N N P S N N P N N D R N R A R	
1171	ggagcggcaggaggaggagcggcgccggc	1260
	E R Q E E E R R R Q E K E R R R Q E E E R R R H Q E E E R R R	
1261	ccaggtggaggcggcagcggc	1350
	Q V E E R Q R Q E E E R W R Q E Q E R Q E E E N R R R E I E	
1351	gcataaaagccagtgaggaggaatcctagcaaggaacggaaatcgctccaggagcggcggcggcggcggcggcggcggcggcggcggcggc	1440
	H K S Q W E R N P S K E R N R P R E R Q E M E R R R Q E Q E	
1441	gcgacaagcagcaggagcagcaggagcaggagatcgctggcggcggcggcggcggcggcggcggcggcggcggcggcggcggcggcggcggc	1530
	R Q E Q E Q E Q E Q E D R R R E R E R D R Q L E W E R R N R N	
1531	tggtgcccgggaaccagtcactcccacggcacaataaccaccccggagaccacaacaagccataatcccacgggtcaccggcagcaccacca	1620
	G A R E P V T P T A N T T P R P T N K P Y P T V H R Q H H H	
1621	ccataacaagccgggaaaccacggc	1710
	H N K P R K P K P D S C M T Y Y D A I S I I R G E L F I F R	
1711	gggaccgtactgtggcagcttggaaacttctggtctgtataatggctatcccacggagatcaggagacattggtccgctctgcccggaaa	1800
	G P Y L W R I G T S G L Y N G Y P T E I R R R H W S A L P E N	
1801	tctccaaggtgagctgtatatacgaaaacaagcagcagcaaaatgtgttctcataggtcggcagatattatgtattcaactcgggtgat	1890
	L T K V D A V Y E N K Q R Q I V F F I G R E Y Y V F N S V M	
1891	gctagctcctggctcccgaaccacttggcagctggtgctgcccaccacttggaccacatcgatgcctcctcgtgtggggccacaa	1980
	L A P G F P K P L A S L G L P P T L T H I D A S F V W G H N	
1981	caaccgacacatgaccagcggcacactgtactggcgcacgcagcactacagggcaggtggaattggattaccggcggcagatgag	2070
	N R T Y M T S G T L Y W R I D D Y T G Q V E L D Y P R D M S	
2071	catctggtcgggagtggtggtacaatatagatggcgcattccagctacttggatggcaagacgtacttctttaagaacctgggactgggga	2160
	I W S G V G Y N I D A A F Q Y L D G K T Y F F K N L G Y W E	
2161	gttcaacgacgaccgatgaaggtggccatgcccaggcctaagctctcggcagcaaggtggatgagctgcccggcagtgctaatgaggt	2250
	F N D D R M K V A H A R A K L S A R R W M Q C A R S A N E V	
2251	ggagcagcagcagcggc	2340
	D D E Q R W T A S L V S E G E E T G R S G S R E L R I N H F	
2341	cattcttctgactctttgtgtagcactgcgaactggcggagttaaagattgaatgtggagcgtgacctagaagaatgtctaaactaagag	2430
	<u>I L S I L L L A I A N W R S</u> *	
2431	agatggtttttgtaacaaaaatgaaagaagcatattggtctgttatgatccaattcttcaaggagctttgcaagtaaaaaaccggtaga	2520
2521	tctagtattcaactactcaaaaactgcaaaagtgcataatctacttaggtacaagaagggaacttaaatgtaagcttggccaattttaaat	2610
2611	tggaaatcaaaaagtgtttaagattgagttgtaaatgttagcggcctgagcagatttaatgcattttaactgcagagcgcagctgtgtg	2700
2701	acagctctggcgtctcaatgcccccaagacgacaccgagcggccttctctagatttaaggaaaaataatcaacacaccagtgaaag	2787

FIG. 1. Nucleotide sequence and deduced amino acid sequence of *Dm2-MMP*. The amino acid sequence is shown in single-letter codes below the nucleotide sequence. The cysteine-switch residues and those corresponding to the zinc-binding site are shaded. The putative glycosylation sites and glycosylphosphatidylinositol anchor are singly and doubly underlined, respectively.



**FIG. 2. Structural comparisons between Dm2-MMP and other vertebrate and invertebrate MMPs.** A, amino acid sequence alignment of the catalytic domains of Dm2-MMP and those of different vertebrate and invertebrate MMPs. The amino acid sequences of the different MMPs were extracted from the SwissProt data base, and the multiple alignment was performed with the PILEUP program of the GCG package. Residues common to all sequences are shaded. Gaps are indicated by dots. The numbering corresponds to the sequence of the catalytic domain of Dm2-MMP. B, domain organization of Dm2-MMP and comparison with that of Dm1-MMP. The characteristic insertion of the Dm2-MMP is indicated by the horizontally striped box.



**FIG. 3. Structural organization of the Dm2-MMP gene and comparison with that of other MMPs genes.** Exons in Dm2-MMP, MT6-MMP, MMP-28, and Dm1-MMP genes are indicated by boxes with their respective sizes in bp. Exon regions defining conserved protein domains in MMPs are aligned.

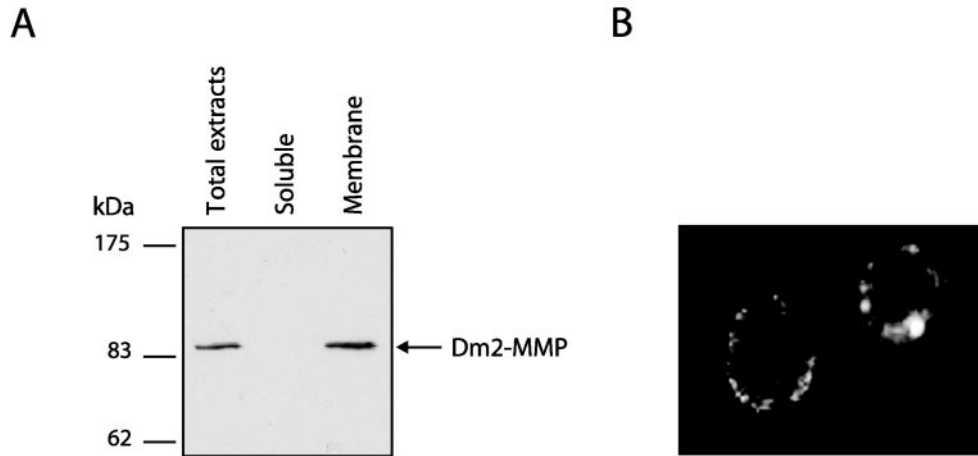


FIG. 4. **Membrane localization of *Dm2-MMP*.** *A*, Western blot analysis from S2 cells transiently transfected with the same *Dm2-MMP*-HA vector. The *Dm2-MMP* band was detected with a monoclonal anti-HA antibody in the total extracts and in the plasma membrane fractions but not in the soluble fraction. *B*, immunofluorescent detection of *Dm2-MMP*-HA in transiently transfected S2 cells with the anti-HA antibody. Fluorescence was observed under a confocal laser microscope and localized to the surface of the S2 cells.

tions were carried out overnight at 55 °C, and the alkaline phosphatase detection buffer was prepared from Sigma Fast TM 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets instead of Tris/HCl. Digoxigenin-labeled RNA probes made from cDNA SD04362 were prepared according to the protocol described for the Genius system (Roche Molecular Biochemicals).

#### RESULTS

**Molecular Cloning and Structural Characterization of a *Drosophila* cDNA Coding for a Matrix Metalloproteinase**—A computer search of the data base of *Drosophila* genomic sequences allowed us to identify a sequence (AC005894) that contains a region with significant similarity to the catalytic and hemopexin domains found in most vertebrate MMPs. To define the precise structure of this putative novel fly MMP, we undertook the isolation of a cDNA encoding this protein. First, we prepared a specific probe for this enzyme by PCR amplification of total  $\lambda$ -phage DNA obtained from a fly pupa cDNA library. The identity of the PCR-amplified product was confirmed by nucleotide sequencing, and then it was used as a probe to isolate a 3.4-kb cDNA clone from the same fly pupa  $\lambda$ gt-11 library. The sequence of this clone was subsequently confirmed and extended by nucleotide sequence analysis of a 4120-bp cDNA clone (SD03462) obtained from the Berkeley *Drosophila* Genome Project and isolated from a Schneider L2 cell cDNA library. Computer analysis of the 3.4- and 4.1-kb cDNA isolated sequences revealed the presence of a unique open reading frame that encodes a protein of 758 residues with a calculated molecular mass of 89 kDa (Fig. 1). These sequences would be encoded by one of the annotated genes (CG1794, located in region 45F6–46A1) in the *Drosophila* genome sequence recently reported (33). Nevertheless, there are several differences in the number and location of computer-predicted exons for CG1794, when compared with the experimental data derived from the cDNA sequences reported herein (Fig. 1). In addition, pairwise comparisons of the protein sequence derived in this work with those described previously for other MMPs demonstrated that the new sequence contains all the structural features present in members of this protease family (Fig. 2A). From N terminus to C terminus, it includes a predicted N-terminal cleavable signal peptide that targets these proteolytic enzymes to the secretory pathway. The deduced sequence also contains a propeptide region with the conserved motif PRCGVXD (at positions 106–112), which is involved in maintaining the latency of these proteinases. This prodomain ends in a stretch of basic amino acid residues (RVRR, at positions 133–136) that mediates the intracellular activation of several

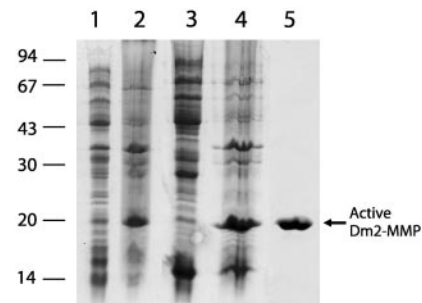


FIG. 5. **Production of recombinant *Dm2-MMP* in *E. coli* BL21(DE3).** 5  $\mu$ l of bacterial extracts transformed with pETB (lane 1), pETB-*Dm2-MMP* (lane 2), soluble fraction (lane 3), insoluble fraction (lane 4), and purified *Dm2-MMP* (lane 5) were analyzed by SDS-PAGE. The sizes of the molecular weight markers are shown to the left.

MMPs including membrane-type MMPs, stromelysin-3, and MMP-28 by furin-like enzymes (7, 8, 34, 35).

The sequence alignment shown in Fig. 2B also confirms the presence in the *Drosophila* MMP sequence of a catalytic domain of about 170 residues, containing the zinc-binding site (HEXGHXXGXXH, at positions 257–267) and the Ser residue (position 268) that distinguishes MMPs from other metalloproteinases. There is also a conserved Met residue located seven residues C-terminal to the zinc-binding site that is proposed to play an important role in the structure of the MMPs active sites (36). Finally, the identified sequence contains a C-terminal fragment of about 200 residues similar to the hemopexin-like domain found in most MMPs. According to these structural features, we suggest that the cloned cDNA encodes a new MMP family member that has been named *Dm2-MMP*, because it is the second MMP identified and characterized in *D. melanogaster*.

A more detailed analysis of the amino acid sequence deduced for *Dm2-MMP* revealed that in addition to common features shared with MMPs, it also shows some specific features unique to this protein (Fig. 2A). Thus, it contains an insertion of about 200 amino acids in the hinge region located between the catalytic and hemopexin domains (at positions 308–509). This insertion is not present in the sequence of *Dm1-MMP* or in those reported for the remaining members of this family. In this extra domain found in *Dm2-MMP*, there is a long stretch of Thr residues and multiple repeats of a seven-amino acid sequence containing Glu, Gln, and Arg residues (RQEEERR, or variants of this sequence). It is also remarkable that *Dm2-MMP* lacks

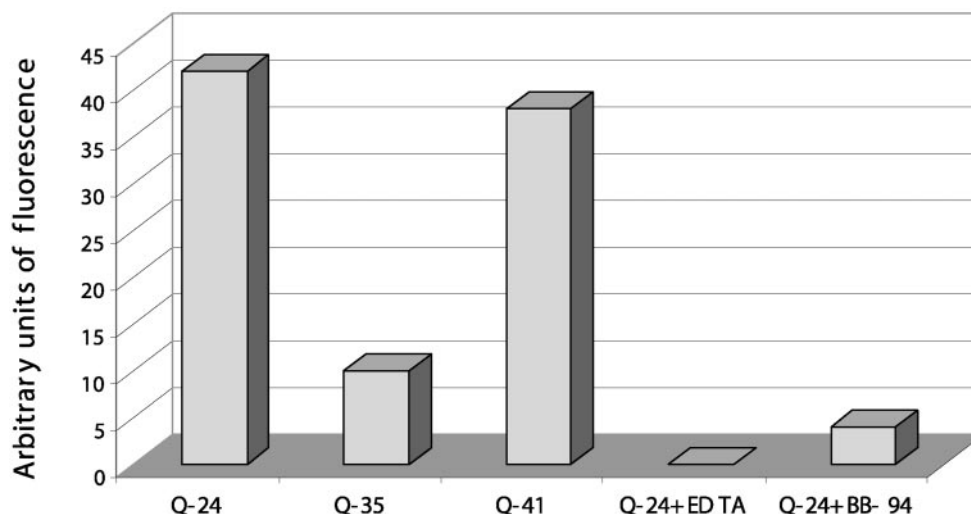


FIG. 6. **Analysis of enzymatic activity of *Dm2*-MMP.** Synthetic fluorescent peptides QF-24, QF-35, and QF-41 (1  $\mu$ M) were incubated with active *Dm2*-MMP (20 nM) in 50 mM Tris/HCl, 5 mM CaCl<sub>2</sub>, 150 mM NaCl, 0.05% (v/v) Brij 35, 1% Me<sub>2</sub>SO, pH 7.6, for 12 h at 37 °C. The fluorometric measurements were made at  $\lambda_{\text{ex}} = 328$  nm and  $\lambda_{\text{em}} = 393$  nm. The synthetic peptide QF-24 was incubated with active *Dm2*-MMP in the presence or absence of 1 mM EDTA and in the presence or absence of the MMP inhibitor BB-94 (100 nM), and fluorescence was measured as above.

conserved residues characteristic of collagenases or stromelysins (37, 38), as well as the fibronectin-like domain present in all gelatinases. Interestingly, *Dm2*-MMP possesses a C-terminal extension rich in acidic residues and ending in a stretch of hydrophobic residues that is predicted to form a cleavable glycosylphosphatidylinositol anchor site (program available at 129.194.186.123/GPI-anchor/index\_en.html). Therefore, it is likely that *Dm2*-MMP belongs to the subfamily of membrane-type MMPs structurally characterized by having a hydrophobic region at the C-terminal end as well as by the presence of the furin-like activation sequence between the propeptide and the catalytic domain (39–42). The intron-exon distribution of the gene encoding *Dm2*-MMP is also somewhat similar to that of some vertebrate MMPs, such as MMP-28 and MT6-MMP, and markedly different from that of *Dm1*-MMP (Fig. 3).

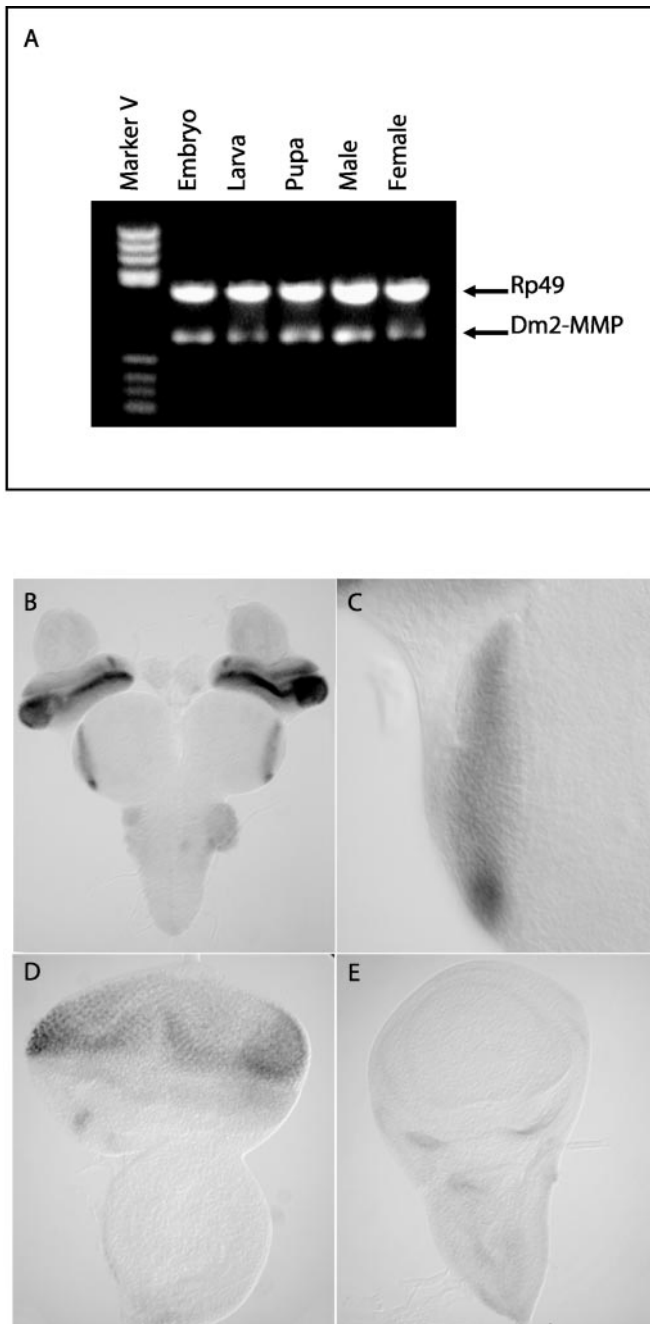
**Membrane Localization of *Dm2*-MMP**—To provide experimental support to the above hypothesis on the putative membrane localization of *Dm2*-MMP, we transfected S2 cells with pRmHa-3-*Dm2*-MMP-HA, a construct containing the HA epitope immediately downstream of the furin-like cleavage motif present in the amino acid sequence deduced for *Dm2*-MMP. Transfected cells were then analyzed by Western blot with a mouse monoclonal antibody (12CA5) specific for this viral epitope. As shown in Fig. 4A, *Dm2*-MMP was detected in the membrane-enriched fractions but not in the soluble fraction. To provide additional information on the subcellular distribution of *Dm2*-MMP, we transfected S2 cells with the pRmHa-3-*Dm2*-MMP-HA construct, and transfected cells were then analyzed by immunofluorescence with the 12CA5 antibody. As shown in Fig. 4B, a fluorescent pattern surrounding the cell was clearly visualized in a serial optical section obtained by the confocal microscope. Taken together, these results provide experimental evidence that *Dm2*-MMP is a member of the membrane-type MMP subfamily of MMPs.

**Analysis of the Enzymatic Activity of Recombinant *Dm2*-MMP**—After the preceding findings showing that *Dm2*-MMP was structurally related to MMPs, we next evaluated the possibility that this protein could be a functionally active member of this proteinase family. For this purpose, a cDNA construct coding for the *Dm2*-MMP catalytic domain was expressed in *E. coli* following the same strategy described previously for *Dm1*-MMP (25) (Fig. 5). To assess the substrate specificity of the recombinant protease, a series of quenched fluorescent

peptides commonly used for assaying vertebrate MMPs were employed. As shown in Fig. 6, the general MMP substrate QF-24, the collagenase/gelatinase substrate QF-41, and the stromelysin substrate QF-35 were hydrolyzed by *Dm2*-MMP. Next, we examined the potential inhibition of active *Dm2*-MMP by EDTA and the hydroxamic acid-based inhibitor BB-94, using QF-24 as substrate. As can be seen in Fig. 6, EDTA completely abolished the hydrolyzing activity of *Dm2*-MMP, whereas BB-94 extensively blocked this proteolytic activity.

**Homology Modeling of the Catalytic Domain of *Dm2*-MMP**—The amino acid sequence determined for *Dm2*-MMP is similar to several vertebrate MMPs of known three-dimensional structure, thus opening the possibility of creating a computer model of the structure of this *Drosophila* enzyme, especially in terms of the size of its S1' pocket, an essential specificity determinant in MMPs. The depth of this pocket is largely determined by the residue located at position 214 (MMP-1 numbering) (43–47). MMPs with small residues at that key position show a channel across the protease structure, allowing the cleavage of substrates with bulky P1' side chains. By contrast, MMPs with large residues at position 214 occlude the S1' channel and leave a cavity that can only accept middle sized substrates. The predicted structure of *Dm2*-MMP with an Asn residue at the equivalent position (Asn-253) reveals a large and open S1' pocket (data not shown), thereby predicting that this enzyme may accommodate bulky residues in this site.

**Expression Pattern of *Dm2*-MMP**—To determine the temporal expression pattern of *Dm2*-MMP during *Drosophila* development, we performed RT-PCR of total RNA prepared from different developmental stages and adult flies. As can be seen in Fig. 7A, *Dm2*-MMP is detected in all of the developmental stages tested as well as in male and female adult flies. However, *Dm2*-MMP transcripts could not be detected by standard Northern blot assays, indicating that it is expressed at low levels or in few specific cells. To get information on the temporal and spatial pattern expression of *Dm2*-MMP, we performed whole mount *in situ* hybridization to third instar larvae. As shown in Fig. 7 (B–E), expression is restricted to specific domains, indicating that *Dm2*-MMP has tissue-specific functions. Hybridization to eye imaginal discs reveals a strong staining behind the morphogenetic furrow, a region where photoreceptors are formed during larval development. In the brain, expression is strong in the optic lobe region, where photoreceptors



**FIG. 7. Expression analysis of *Dm2*-MMP in diverse *Drosophila* development stages.** A, the presence of *Dm2*-MMP transcripts during development was determined by RT-PCR, using total RNA extracts prepared from different stages. Amplification of ribosomal protein 49 was used as a control for RNA integrity. B–E, whole mount *in situ* hybridization to third instar larvae using a *Dm2*-MMP antisense RNA probe. Expression of *Dm2*-MMP is detected in the optic lobe (B and C) in the brain and in imaginal discs (D and E). C corresponds to a close up of the optic lobe, in which expression is detected in the lamina. In eye imaginal discs (B and D), expression is strong behind the morphogenetic furrow where photoreceptors form. In wing imaginal discs (E), expression is weak and diffuse. No signal was detected with a control sense probe.

project their axons. Altogether, these data suggest a role for *Dm2*-MMP in eye and nervous system formation.

#### DISCUSSION

This work describes the cloning and characterization of the second MMP identified in *D. melanogaster*. The strategy used to clone *Dm2*-MMP was based on the search for structural

motifs of vertebrate MMPs in *Drosophila* genomic sequences, followed by hybridization of a *Drosophila* cDNA library with a probe derived from a MMP-related sequence identified in the fly genome. This approach allowed us to isolate a full-length cDNA coding for a protein that contains all structural domains characteristic of MMPs, including the signal peptide, the prodomain, the catalytic domain, the hinge region, and the C-terminal hemopexin domain. In addition, the recombinant protein produced in *E. coli* is able to degrade several peptides widely used as substrates for vertebrate MMPs, and this proteolytic activity is abolished by MMP inhibitors. According to these structural and enzymatic properties, we conclude that the identified cDNA codes for a functionally active member of the MMP family that we called *Dm2*-MMP. Nevertheless, the sequence of *Dm2*-MMP is unique because of the presence of an insertion of 200 amino acids in the hinge region located between the catalytic and hemopexin domains. This sequence is not present in *Dm1*-MMP or in other MMPs from different sources. The functional significance of this domain characteristic of *Dm2*-MMP is presently unknown, although it could serve to mediate interactions with other proteins, including the putative substrates of this enzyme.

The structural differences between the two MMPs identified in *Drosophila* can be also extended to their respective expression patterns. Thus, *Dm2*-MMP is expressed, albeit at low levels, in all of the developmental stages of *Drosophila*, as well as in male and female adult flies. By contrast, *Dm1*-MMP is strongly expressed in the developing embryo at stages 12 and 13, declining thereafter and being almost undetectable in adult flies (25). These differences in the expression patterns of the two MMPs present in *Drosophila* are consistent with the possibility that both enzymes play distinct roles in the tissue remodeling processes occurring during fly development. Interestingly, *Dm2*-MMP is expressed in discrete regions of the nervous system both in embryos (data not shown) and larvae, suggesting a specific role of this enzyme in this tissue. *Dm2*-MMP proteolytic activity on the extracellular matrix may play an important role in photoreceptors growth cone guidance and/or cell rearrangement in the retina and nervous system. In this respect, it is interesting to note that some MMPs are found to be associated with extending neurites and play important roles in growth cones in mammals (48–50). The different substrate specificities of *Dm1*-MMP and *Dm2*-MMP, as assayed using synthetic substrates, together with the nonoverlapping patterns of MMP expression, suggest distinct roles for *Dm1*-MMP and *Dm2*-MMP in development. Further comparative genetic loss- and gain-of-function studies will provide cues on the cellular role of each MMP in *Drosophila*. In any case, it is remarkable that seemingly, only two MMPs are necessary in *Drosophila* to accomplish the extensive matrix remodeling required during embryonic and larval development, metamorphosis, and adult life. In fact, after exhaustive screening of the *Drosophila* genome, we have not found any evidence of the presence of additional MMP genes other than those encoding *Dm1*-MMP, and *Dm2*-MMP. Nevertheless, it is very interesting that one of these MMPs (*Dm1*-MMP) seems to be a secreted protease, whereas *Dm2*-MMP is membrane-bound, thereby providing a further level of versatility in the type of substrates that can be targeted by these two enzymes. On the other hand, it is remarkable that we have not found other *Drosophila* TIMP genes distinct from that recently described by Pohar *et al.* (51). Therefore, we conclude that the *Drosophila* MMP proteolytic system is complete in terms of presence of both proteases and inhibitors with the ability to mediate and modulate tissue remodeling processes in this organism but extremely simple when compared with the MMP system operating in verte-

brates. Thus, to date 24 MMP genes and four TIMP genes have been identified in the human genome (7–10, 52), indicating that these protease and inhibitor families have undergone extensive gene duplication events after the divergence of vertebrates and invertebrates.

At present we can only speculate on why *Drosophila* has only two MMPs in its genome, whereas humans or other organisms, including *C. elegans*, have many more members of this protease family (52). It has been proposed that this fact could reflect the different developmental processes occurring in these organisms (53). Thus, in vertebrates and nematodes, growth and development involve extensive cell migration and rearrangement, whereas *Drosophila* undergoes an early syncytial stage prior to cellularization. The low number of MMPs in *Drosophila* could therefore indicate that cell release from the extracellular matrix is a generic requirement in this organism, whereas the complex matrix-remodeling processes taking place in the other organisms involve more specific and highly regulated functions and, consequently, a higher number of proteolytic enzymes potentially associated with them. On this basis, it has been suggested that *Drosophila* may not be a good model to study specific processes associated with these MMP functions (53). However, we consider that the simple comparison of the number of MMP genes with the fact that *Drosophila* undergoes an early syncytial stage should not be used to get definitive conclusions regarding the functional relevance of the MMP system in this organism or its value as a model for extrapolating MMP functions to other organisms. Thus, the syncytial stage lasts just for the first 2 h of *Drosophila* development, in the absence of zygotic transcription, whereas full *Drosophila* development takes 11 days at 25 °C. Furthermore, *Drosophila*, like any other multicellular organism, undergoes several crucial developmental processes involving cell rearrangements and migration, as well as wound healing (54, 55). On the other hand, behind the apparent simplicity of the MMP system in *Drosophila*, it may be possible to uncover new mechanisms controlling MMP expression or function of potential relevance to vertebrate MMPs. Likewise, the limited number of endogenous enzymes in *Drosophila* as compared with human may represent a unique system to study vertebrate MMPs in gain-of-function studies through expression of heterologous proteases in flies, without interfering with the *Drosophila* endogenous system. Finally, we must consider that the high number of MMPs in vertebrates may also reflect a significant degree of redundancy in this system, a possibility that is consistent with the lack of significant abnormalities in most mutant mice deficient in specific MMPs (56). In summary, and despite some caveats, we believe that the simplified MMP-TIMP system from *Drosophila*, whose description is now completed with the finding of *Dm2*-MMP, may be an advantageous model for studying the role of protease-mediated events during development processes. Consistent with this proposal, recent studies in this organism have established the relevance of diverse proteases, including metalloproteinases like Kuzbanian, Tolloid, and Tolkin, in signaling pathways operating in essential processes such as neurogenesis or embryonic patterning (57–61).

In summary, we have cloned and characterized *Dm2*-MMP, the second member of the MMP family identified in *D. melanogaster*. This enzyme exhibits structural and functional similarities with *Dm1*-MMP as well as with vertebrate MMPs, in terms of similar domain organization, profile of activity against synthetic substrates, and sensitivity to inhibitors. However, *Dm2*-MMP also exhibits unique features including an extremely long hinge region that distinguishes this enzyme from all the remaining MMPs described in any organism. In addition, the predicted architecture of the catalytic domain of *Dm2*-

MMP, with an unusually large and open S1' pocket, supports the proposal that this protease may have a wide substrate specificity. Further biochemical studies, together with genetic analysis of mutant flies deficient in *Dm2*-MMP, will be required to identify the relevant *in vivo* substrates of this protease and to define its precise role in any of the extensive extracellular matrix remodeling processes taking place during *Drosophila* development.

**Acknowledgments**—We thank all members from our groups for support and helpful comments and F. Rodríguez for excellent technical assistance. We especially thank François Agnès, Laurence Bianchini, Olivier Devergne, Conchi Martínez, Begoña Granadino, and Javier Rey for help.

#### REFERENCES

- Nagase, H., and Woessner, F. Jr. (1999) *J. Biol. Chem.* **274**, 21491–21494
- Murphy, G., and Gavrilovic, J. (1999) *Curr. Opin. Cell Biol.* **11**, 614–621
- Vu, T. H., and Werb, Z. (2000) *Genes Dev.* **14**, 2123–2133
- 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
- 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
- Nelson, A. R., Fingleton, B., Rothenberg, M. L., and Matrisian, L. M. (2000) *J. Clin. Oncol.* **18**, 1135–1149
- Lohi, J., Wilson, C. L., Roby, J. D., and Parks, W. C. (2001) *J. Biol. Chem.* **276**, 10134–10144
- Marchenko, G. N., and Strongin, A. Y. (2001) *Gene (Amst.)* **265**, 87–93
- Uriá, J. A., and López-Otín, C. (2000) *Cancer Res.* **60**, 4745–4751
- Brew, K., Dinakarpandian, D., and Nagase, H. (2000) *Biochim. Biophys. Acta.* **1477**, 267–283
- Velasco, G., Pendas, A. M., Fueyo, A., Knauper, V., Murphy, G., and López-Otín, C. (1999) *J. Biol. Chem.* **274**, 4570–4576
- Pei, D., Kang, T., and Qi, H. (2000) *J. Biol. Chem.* **275**, 33988–33997
- Werb, Z. (1997) *Cell* **91**, 439–442
- McQuibban, G. A., Gong, J. H., Tam, E. M., McCulloch, C. A., Clark-Lewis, I., and Overall, C. M. (2000) *Science* **289**, 1202–1206
- Wilson, C. L., Ouellette, A. J., Satchell, D. P., Ayabe, T., Lopez-Boado, Y. S., Stratman, J. L., Hultgren, S. J., Matrisian, L. M., and Parks, W. C. (1999) *Science* **286**, 113–117
- Couet, J., Sar, S., Jolivet, A., Hai, M. T. V., Milgrom, E., and Misrahi, M. (1996) *J. Biol. Chem.* **271**, 4545–4552
- Yu, Q., and Stamenkovic, I. (2000) *Genes Dev.* **14**, 163–176
- Koshikawa, N., Giannelli, G., Cirulli, V., Miyazaki, K., and Quaranta, V. (2000) *J. Cell Biol.* **148**, 615–624
- Maidment, J. M., Moore, D., Murphy, G. P., Murphy, G., and Clark, I. M. (1999) *J. Biol. Chem.* **274**, 34706–34710
- Pak, J. H., Liu, C. Y., Huangpu, J., and Graham, J. S. (1997) *FEBS Lett.* **404**, 283–288
- Delorme, V. G., McCabe, P. F., Kim, D. J., and Leaver, C. J. (2000) *Plant Physiol.* **123**, 917–927
- Wada, K., Sato, H., Kinoh, H., Kajita, M., Yamamoto, H., and Seiki, M. (1998) *Gene (Amst.)* **21**, 57–62
- Lepage, T., and Gache, C. (1990) *EMBO J.* **9**, 3003–3012
- Leontovich, A. A., Zhang, J., Shimokawa, K., Nagase, H., and Sarras, M. P. Jr. (2000) *Development* **127**, 907–922
- Llano, E., Pendas, A. M., Aza-Blanc, P., Kornberg, T. B., and López-Otín, C. (2000) *J. Biol. Chem.* **275**, 35978–35985
- Sambrook, J., and Russell, D. W. (2000) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Bunch, T. A., Graner, M. W., Fessler, L. I., Fessler, J. H., Schneider, K. D., Kerschen, A., Choy, L. P., Burgess, B. W., and Brower, D. L. (1998) *Development* **125**, 1679–1689
- Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995) *J. Biol. Chem.* **270**, 5331–5338
- Willembrock, F., Crabbe, T., Slocum, 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
- Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* **18**, 2714–2723
- Koradi, R., Billeter, M., and Wüthrich, K. (1996) *J. Mol. Graphics* **14**, 51–55
- Gavis, E. R., and Lehmann, R. (1992) *Cell* **71**, 301–313
- 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
- Pei, D., and Weiss, S. J. (1995) *Nature* **375**, 244–247
- Sato, H., Kinoshita, T., Takino, T., Nakayama, K., and Seiki, M. (1996) *FEBS Lett.* **393**, 101–104
- Bode, W., Gomis-Rüth, F. X., and Stöcker, W. (1993) *FEBS Lett.* **331**, 134–140
- Sánchez-López, R., Alexander, C. M., Behrendtsen, O., Breathnach, R., and Werb, Z. (1993) *J. Biol. Chem.* **268**, 7238–7247
- Freije, J. P., Díez-Izta, I., Balbín, M., Sánchez, L. M., Blasco, R., Tolivia, J., and López-Otín, C. (1994) *J. Biol. Chem.* **269**, 16766–16773
- Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994) *Nature* **371**, 61–65
- Puente, X. S., Pendas, A. M., Llano, E., Velasco, G., and López-Otín, C. (1996) *Cancer Res.* **56**, 944–949
- Llano, E., Pendas, A. M., Freije, J. P., Nakano, A., Knauper, V., Murphy, G., and López-Otín, C. (1999) *Cancer Res.* **59**, 2570–2576

42. Velasco, G., Cal, S., Merlos-Suarez, A., Ferrando, A. A., Alvarez, S., Nakano, A., Arribas, J., and López-Otín, C. (2000) *Cancer Res.* **60**, 877–882
43. Welch, A. R., Holman, C. M., Huber, M., Brenner, M. C., Browner, M. F., and Van Wart, H. E. (1996) *Biochemistry* **35**, 10103–10109
44. Stams, T., Spurlino, J. C., Smith, D. L., Wahl, R. C., Ho, T. F., Qoronfleh, M. W., Banks, T. M., and Rubin, B. (1994) *Nat. Struct. Biol.* **1**, 119–123
45. Lovejoy, B., Welch, A. R., Carr, S., Luong, C., Broka, C., Hendricks, R. T., Campbell, J. A., Walker, K. A., Martin, R., Van Wart, H., and Browner, M. F. (1999) *Nat. Struct. Biol.* **6**, 217–221
46. Bode, W., Fernandez-Catalan, C., Tschesche, H., Grams, F., Nagase, H., and Maskos, K. (1999) *Cell. Mol. Life Sci.* **55**, 639–652
47. Lovejoy, B., Cleasby, A., Hassell, A. M., Longley, K., Luther, M. A., Weigl, D., McGeehan, G., McElroy, A. B., Drewry, D., Lambert, M. H., and Jordan, S. R. (1994) *Science* **263**, 375–377
48. Machida, C. M., Rodland K. D., Matrisian, L., Magun, B. E., and Ciment, G. (1989) *Neuron* **2**, 1587–1596
49. Zuo, J., Ferguson, T. A., Hernandez, Y. J., Stetler-Stevenson, W. G., and Muir, D. (1998) *J. Neurosci.* **18**, 5203–5211
50. Oh, L. Y. S., Larsen P. H., Krekoski, C. A., Edwards, D. R., Donovan, F., Werb, Z., and Yong, V. W. (1999) *J. Neurosci.* **19**, 8464–8475
51. Pohar, N., Godenschwege, T. A., and Buchner, E. (1999) *Genomics* **57**, 293–296
52. Brinckerhoff, C. E., and Matrisian, L. M. (2002) *Nat. Rev. Mol. Cell. Biol.* **3**, 207–214
53. Coates, D., Siviter, R., and Isaac, R. E. (2000) *Biochem. Soc. Trans.* **28**, 464–469
54. Montell, D. J. (1999) *Development* **126**, 3035–3046
55. Woolley, K., and Martin, P. (2000) *Bioessays* **22**, 911–919
56. Egeblad, M., and Werb, Z. (2002) *Nat. Rev. Cancer* **2**, 163–176
57. Rooke, J., Pan, D., Xu, T., and Rubin, G. M. (1996) *Nature* **273**, 1227–1231
58. Pan, D., and Rubin, G. M. (1997) *Cell* **90**, 271–280
59. Qi, H., Rand, M. D., Wu, X., Sestan, N., Wang, W., Rakic, P., Xu, T., and Artavanis-Tsakonas, S. (1999) *Science* **283**, 91–94
60. Marqués, G., Musacchio, M., Shimell, M. J., Wunnenberg-Stapleton, K., Cho, K. W., and O'Connor, M. B. (1997) *Cell* **91**, 417–426
61. Sotillos, S., Roch, F., and Campuzano, S. (1997) *Development* **124**, 4769–4779