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

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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 domain, Northern blot analysis of expression, and Western blotting and activity analysis in vitro against synthetic peptides used for analysis of vertebrate MMPs. This activity is inhibited by tissue inhibitors of metalloproteinases and by synthetic MMP inhibitors. Recombinant Dm1-MMP in embryonic neural cells, we propose that this enzyme may be involved in the extracellular matrix remodeling during neural development.

This article has been withdrawn by the authors upon request from the Journal. The Journal raised questions regarding Figs. 3 and 5A. The authors were able to locate some, but not all, of the original data generated 20 years ago. Fig. 3 was assembled from three different gels, and an empty area was duplicated. In Fig. 5A, the lane corresponding to Type II collagen, which is not cleaved by the enzyme, was duplicated. The authors state that new experiments were performed. The authors assert that all of the results reported in this article are valid, some of which have been independently confirmed in the literature (Zhang et al. (2006) Genes Dev. 20, 1899; LaFever et al. (2017) Sci. Rep. 7, 44560).

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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 metalloproteinases 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 nervous system in D. melanogaster. MMPs 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 (Cambridge, UK). cDNA libraries constructed in agt11 were from CLONTECH (Palo Alto, CA). Restriction endonucleases and other agents used for molecular cloning were from Roche Molecular Biochemicals. Synthetic oligonucleotides were prepared with the Biometra-1 thermocycler (Cambridge, MA). DNA fragments were radiolabeled with [32P]dCTP (3000 Ci/mmol) purchased from the Radiochemical Centre of Amersham Pharmacia Biotech using a commercial random priming procedure. The procedure used for molecular cloning was from PerkinElmer Life Sciences for 30 cycles of denaturation (94 °C, 1 min), annealing (57 °C, 1 min), and extension (72 °C, 2 min), as described in the GCG software package of the University of Wisconsin Genetics Computer Group. DNA probes were radiolabeled with [32P]dCTP (3000 Ci/mmol) purchased from Amersham Pharmacia Biotech using a commercial random priming procedure.

Probe Preparation and Screening of a Drosophila cDNA Library—Total RNA (30 μg) from polytene chromosome preparations was reverse transcribed into first-strand cDNA using the avian myeloblastosis virus reverse transcriptase (PerkinElmer Life Sciences). The cDNA analysis of DNA and protein sequences was performed with the GCG software package designed by 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. Enzyme Immunoassay—Total RNA (30 μg) from diverse developmental stages of Drosophila was electrophoresed and blotted to Hybond N+ (Amersham Pharmacia Biotech). The blot was hybridized with a ribosomal labeled 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).

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 clytactic domain found in the different vertebrate MMPs, and then we isolated a full-length cDNA clone from a fly larva agt-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 (CAA
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 PRCGVX (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 (RXXR) 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 HEXGHXXGXSXXG (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*.
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 fibronec-
tin-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 ex-
tension 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).

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. 2. Amino acid sequence alignment of Dm1-MMP with different human MMPs showing the highest degree of sequence similarity with Dm1-MMP were extracted from the PILEUP program of the GCG package. Common residues to all sequences correspond to the sequence of Dm1-MMP.
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/Cl, 5 mM CaCl₂, 150 mM NaCl, and 0.05% (v/v) Brij 35. After 12 h at 37 °C, the fluorometric measurements were made at λex = 328 nm and λem = 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.

Ablation of 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 set of basement membrane and extracellular matrix components. For this purpose, a variety of proteins including type IV collagen, laminin, fibronectin, fibrinogen, gelatin, and elastin were incubated with purified Dm1-MMP (35 and 19 kDa, respectively) (Fig. 5B). Lytic bands co-migrating with the proform and active recombinant proteins were observed. An additional band of 21 kDa was also detected. For this purpose, a variety of proteins including type IV collagen, laminin, and elastin were incubated with purified Dm1-MMP. Lytic bands co-migrating with the proform and active recombinant proteins were observed. An additional band of 21 kDa was also detected.

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.
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 spatio-temporal 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. 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.
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 nervous 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.

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