A Novel Matrix Metalloproteinase Gene (XMMP) Encoding Vitronectin-like Motifs Is Transiently Expressed in Xenopus laevis Early Embryo Development*

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To study the role of matrix metalloproteinases (MMPs) in early vertebrate development, we cloned cDNAs for six different MMPs from the frog Xenopus laevis embryos at different stages of development and describe here a novel MMP called XMMP. Xenopus XMMP has 604 amino acids including a putative signal peptide of 22 residues. At the carboxy-terminal end of the propeptide, XMMP has a 37-amino-acid-long insertion domain containing a segment that is 38% identical with a rat vitronectin sequence between residues 108–135. Following this domain is an RRKR motif, a putative cleavage site for intracellular activation by furin proteases. XMMP lacks a proline-rich linker peptide, or hinge region, typically found in other MMPs between the catalytic domain and carboxy-terminal “hemopexin/vitronectin-like” domain. In XMMP, the carboxy-terminal domain is composed of four tandem repeats that are 21–33% identical to a sequence (residues 213–264) encoded by vitronectin exon-5. Interestingly, XMMP gene is transiently expressed during Xenopus embryo development. XMMP mRNA of 3.0 kilobase pairs was undetected in the blastula stage embryo, induced in gastrula embryo, expressed in neurula embryo, and then down-regulated in pretailbud embryo. In comparison, other Xenopus MMP genes that we have cloned show a different developmental regulation. In blastula embryo, the only MMP gene expressed was found to be 92-kDa type IV collagenase, which was also expressed in the gastrula, neurula, and pretailbud embryos. Expression of stromelysin-1, stromelysin-3, and two different membrane type-MMPs was first detected in the neurula and pretailbud embryos. These results suggest that MMPs and the novel XMMP reported here play a role in Xenopus early development.

Proteolytic degradation of the extracellular matrix (ECM) of cells and tissues is largely controlled by a superfamily of enzymes called matrix metalloproteinases (MMPs;Refs. 1–7). Many MMPs are expressed widely during embryogenesis but not in adult life, suggesting that MMPs and the degradation and remodeling of ECM play an important role in growth and development. Thus far, 14 genetically distinct MMPs have been cloned and characterized from humans. They include three different collagenases, which are the only enzymes that can cleave fibril-forming collagens such as collagen types I, II, and III, the most abundant proteins in the body. Gelatinases A and B, also known as 72- and 92-kDa type IV collagenases, digest denatured collagen (gelatin) and basement membranes, a special type of ECM of epithelial, endothelial, fat, muscle, and peripheral nerve cells (8). Stromelysins are three different MMPs with wide substrate specificity, and stromelysin-1 degrades almost any ECM component including cartilage proteoglycans (9).

All MMPs are produced as inactive proenzymes, which are activated by proteolytic removal of their NH2-terminal domain (10–13). Therefore, it is significant that stromelysin-1 (MMP3) proteolytically activates other MMPs such as interstitial collagenase-1 (MMP1), matrixilysin (MMP7), and 92 kDa type IV collagenase (MMP9), suggesting that stromelysin-1 plays a special “upstream” role in ECM degradation and remodeling (14–16).

Recently, five groups have described four new members of the MMP family (17–22). These new MMPs are not secreted but remain anchored to the cell surface through their COOH-terminal transmembrane domain, and accordingly, they were named membrane type-MMPs (MT-MMPs). Studies on the human MT1-MMP indicate that it activates gelatinase A (MMP2), suggesting that MT-MMPs may facilitate pericellular matrix degradation at the cell surface and promote tumor cell invasion and metastasis (12, 17). Our in situ mRNA hybridization studies on the developmental expression of the chicken MT3-MMP gene show that it is expressed at high levels in several embryonic tissues such as neural tube, dorsal root ganglia, respiratory epithelium, and developing cartilage and muscle (22).

To gain insight on the role of MMPs in early vertebrate development, we used two “universal” MMP primers (22) and RT-PCR to clone cDNAs for six different MMPs from Xenopus laevis embryos of different developmental stages. Here we describe the cloning and developmental expression of a novel MMP called XMMP. Xenopus XMMP contains 604 amino acids including a putative 22-residue signal peptide, suggesting that XMMP is a secretory protein. XMMP contains an RRKR motif at the carboxy-terminal end of the propeptide, suggesting that it is proteolytically activated intracellularly by furin proteases. In the propeptide, XMMP also has a 37-amino-acid-long insertion domain (ID) not found in other MMPs. XMMP lacks a proline-rich hinge region typically found in other MMPs between the catalytic domain and carboxy-terminal hemopexin/vitronectin-like domain. Interestingly, the XMMP gene is transiently expressed in gastrula and neurula stage embryos. In
comparison, other Xenopus MMPs that we have cloned show a different pattern of gene expression. The 92-kDa type collagenase (MMP9) mRNA is inherited as a maternal mRNA in the blastula stage embryo and expressed throughout the later developmental stages. Stromelysin-1, stromelysin-3, and two different MT-MMP genes (MT2-MMP and MT3-MMP) are first abundantly expressed in pretailed bud embryo.

MATERIALS AND METHODS

RT-PCR—Xenopus embryo cDNA ends strategy was used. For the 5′ end cDNA ends strategy, the novel Xenopus MMP gene expression (a 3.0-kilobase pair mRNA). The specific PCR products were cloned and sequenced using the Anchor primer and a nested specific primer derived from the XMMP, the following rapid amplification of 5′-CCAGTAGTGAGCATGGGATTAGTC-3′

5′-GCAAGATTTGATCAGCCACACC-3′

5′-CCAGTAGTGAGCATGGGATTAGTC-3′. We confirmed the XMMP sequence (Fig. 2) by independent cloning and sequencing of full-length cDNA. The PCR product was amplified using the Anchor primer and a nested specific primer derived from the Xenopus XMMP sequence: 5′-GCTGCACTGCGGTCTCAGCTG-3′. The specific PCR products were cloned and sequenced to obtain the 5′ end sequence of XMMP. For the 3′ end of the mRNA, the "lock-docking" NN, oligo(T) primer was used as described in the CLONTECH 3′ AmpliFinder rapid amplification of cDNA ends kit. The first PCR was carried out with the primers from the kit and the degenerate Y35 primer to construct a 3′ end cDNA sublibrary. The specific PCR products were amplified using the 3′ Anchor primer and a nested specific primer derived from the Xenopus XMMP sequence: 5′-CCAGTAGTGAGCATGGGATTAGTC-3′. We confirmed the XMMP sequence (Fig. 2) by independent cloning and sequencing of full-length clones generated by PCR and specific primers for XMMP. Sequences were analyzed using the MacVector program (IBI/Kodak).

RESULTS AND DISCUSSION

All MMPs, from a variety of species including a collagenase-like hatching enzyme from sea urchin (33), have two highly conserved sequence domains. The first domain is at the carboxyl-terminal end of the propeptide: PRGC(N/V/D), and the second is in the zinc binding site: VA/A/N/H/E/F/L/GH. We syn-

3. putative signal peptides (30) were predicted using on-line server. We note that the 5′-untranslated nucleotide sequence of XMMP (Fig. 2) contains three methionine codons upstream of the putative initiator methionine. However, the indicated methionine is likely to be the initiator because it translates to a typical signal peptide; the residues upstream of this methionine would form a hydrophilic peptide, suggesting that it is not synthesized (30). Multiple sequence alignments were performed using the Wisconsin GCG program PILEUP. Data shown in Fig. 3 were generated using a gap weight of 4.0 and gap length weight of 0.10, which were found to be the most stringent setting that introduced the fewest number of "artificial" gaps flanking 1–3 amino acids, to align cysteines and to highlight the hinge region and other conserved features of MMPs. To find similar sequences in the public data base, both blastn and blastx searches were performed against the nonredundant and dbEST data bases in the National Center for Biotechnology Information (31). Also, in a BLAST search, the Xenopus XMMP amino acid sequence was compared with 215,495 protein sequences.

Northern Hybridization—Total RNA (10 μg) isolated from different stage Xenopus embryos by the modified acid-phenol procedure (24, 25) was run on a 1% agarose-formaldehyde gel in 1 × MOPS buffer. The RNA from the agarose gel was blotted in 10 × SSC (1.5 M NaCl, 0.15 M sodium citrate) to Nytran membrane. The membrane was photographed under UV light to visualize the 28 and 18 S ribosomal RNA bands that served as a control for the amount of RNA loaded and transferred. A 0.55-kilobase pair Xenopus cDNA for XMMP was 32P-labeled by PCR (32) and hybridized overnight at 65 °C in 5 × SSC, 5 × Denhardt, 0.1 mg/ml denatured salmon sperm DNA, 0.1% SDS. The membrane was washed at 65 °C in 0.1 × SSC, 0.1% SDS and exposed to Kodak x-ray film.

2The address of the server is http://www.cbs.dtu.dk/services/chsnpal.html.
thesized two universal MMP primers for these domains (22) and used RT-PCR to clone cDNAs for six different MMPs from *Xenopus* embryos at different stages of development. From the blastula embryos (stage 8), only one major PCR product of about 900-bp was detected (Fig. 1A, top). This was cloned, sequenced, and identified as the *Xenopus* homologue of 92-kDa type IV collagenase (data not shown). Using the universal MMP primers, the 900-bp product was barely detectable (yet visible in the original gel) in later developmental stages of gastrula, neurula, and pretailbud embryos. However, using RT-PCR and specific primers for the *Xenopus* 92-kDa type IV collagenase, it was detected at similar levels in all developmental stages (Fig. 1A, bottom). Apparently, in later stages other more abundant MMP mRNA templates compete for the same

![Fig. 2. Xenopus XMMP.](http://www.jbc.org/)

The cDNA-derived amino acid sequence of 604 residues of *Xenopus* XMMP, the first bar indicates a putative signal peptide cleavage site, and the second bar shows the N-linked glycosylation site. The boxed sequences mark two conserved MMP sequences that were used in primer design to clone *Xenopus* MMPs (see Fig. 1), and an RRKR motif, a putative cleavage site for intracellular furin-proteinases. The underlined sequence is a 37-residue-long ID. The circled GS residues refer to a missing hinge region in XMMP (see Fig. 5). The large box is a domain of four tandem repeats of a motif similar to that encoded by vitronectin exon 5 (see Fig. 4). The nine Cys residues are shown in bold, and a “signature” Cys residue is circled. Two polyadenylation signals are underlined.
universal MMP primers in PCR. In a control experiment, we also found throughout the development comparable levels of expression of rpL8 mRNA encoding a *Xenopus* ribosomal protein (Fig. 1A, middle).

From the *Xenopus* embryos of gastrula (stage 13), neurula (stage 15), and pre-tailbud (stage 24) stages, a prominent PCR product of about 550 bp was detected (Fig. 1A, top). Cloning and sequencing of this product showed that it contained several different MMP-related sequences. From gastrula and neurula embryos, this product contained a sequence derived from a novel member of the MMP family (called XMMP) that was further characterized as described below. From pretailbud embryos, this product contained two different sequences derived from two different *Xenopus* MT-MMPs that are, respectively, similar to MT2-MMP and MT3-MMP from human and chicken (Refs. 19, 20, and 22; data not shown).

A second prominent PCR product of 350 bp from pretailbud embryos, visible also from neurula embryos (Fig. 1, top), was found to contain two additional MMP-related sequences. One sequence represents a *Xenopus* homologue for stromelysin-1 (data not shown), and the other is identical to the *Xenopus* stromelysin-3 sequence (data not shown) as described previously by Patterton *et al.* (28).

In Northern blotting, the novel *Xenopus* XMMP gene was found to encode a single 3.0-kilobase pair mRNA. It is particularly striking that this transcript, which was undetected in blastula stage embryo, is first induced at gastrula and continues to be expressed at high level at neurula stage. In comparison, this transcript almost completely disappears in pretailbud embryo, suggesting that XMMP gene is down-regulated in later *Xenopus* development (Fig. 1B).

Using rapid amplification of cDNA ends, a 5' and 3' end RT-PCR strategy of cDNA cloning (see “Materials and Methods”), allowed us to determine 3217 nucleotides and the complete 604-amino acid sequence for the novel *Xenopus* XMMP (Fig. 2, deposited as GenBank™ accession number U82541). XMMP contains a putative signal peptide of 22 amino acids with a characteristic hydropathy profile (30), and no other significant hydrophobic segments, suggesting that it is a secretory protein. The sequence contains five potential sites for N-linked sugars (Asn173, Asn267, Asn419, Asn422, and Asn588) and nine Cys residues, four of them in the putative signal peptide. Of note is that Cys273 (circled in Fig. 2), which is an insertion, is not found in any other MMPs except NMMP, another novel MMP that we recently cloned from cultured...
primary chicken embryo fibroblasts. Excluding the signal peptide and any post-translational modifications, Xenopus XMMP has a calculated molecular weight of 70,115.

In comparison with the bullfrog Rana catesbeiana tadpole collagenase (34), Xenopus collagenase-3 (MPM3, GenBank accession number L49412), and Xenopus stromelysin-3 (28), XMMP is most related to stromelysin-3 (20% identical amino acids). As shown in Fig. 3, a sequence alignment reveals 21% (131/635) identical residues between all four frog MMPs. One conspicuous feature of the Xenopus XMMP that makes it different from all other MMPs is a 37-amino acid-long ID, related to vitronectin (see below), that is situated at the end of the propeptide after the conserved PRCGVPD domain. In stromelysin-3 and MT-MMPs, a similarly located insertion is 8–13 residues long (19–22, 28). After the insertion domain there is an RRKR motif (Fig. 3), which is a putative cleavage site for intracellular furin, a Golgi-associated proteinase family. This and a related motif are also found in stromelysin-3 (RQKR) and MT-MMPs (RRKR or RRRR). Pei and Weiss (11, 12) recently showed that this motif leads to an unusual intracellular activation of the human stromelysin-3 and MT1-MMP by a furin-dependent mechanism. Accordingly, Xenopus XMMP may be proteolytically activated by a similar intracellular mechanism.

Another conspicuous feature of XMMP is that it lacks a proline-rich linker peptide or hinge region typically found in all other MMPs between the catalytic domain and COOH-terminal hemopexin/vitronectin-like domain. Representative MMP hinge sequences shown are flanked by consensus residues in bold. As shown in Fig. 4, we suggest that in other MMPs, a homologously placed hinge region is 17–72 residues long and most pronounced in the 92-kDa type IV collagenase. We also note that a common feature of the hinge region is that it begins with glycine and ends with proline three residues upstream of the conserved cysteine residue. The hinge region, which appears to be the most variable region of MMPs, plays a role in MMP activity and substrate selection (for references, see Ref. 35).

Similar to other MMPs, the COOH-terminal domain of XMMP contains four tandem repeats of a motif similar to that in vitronectin, an abundant cell adhesion protein in plasma and tissues (36). The COOH-terminal domain plays a role in the substrate specificity and possibly other protein-protein interactions of MMPs (6, 7). In the crystal structure of porcine collagenase this domain is folded as a four-bladed b-propeller (35). As shown in Fig. 5, we have discovered that XMMP repeats 2, 3, and 4 are 21–33% identical to a sequence encoded by vitronectin exon-5 (residues 213–264), whereas repeat 1 is more related to a sequence encoded by vitronectin exon-4 (residues 166–212). The similarity of the four tandem repeats is also obvious in the consensus MMP sequence (49); they are 24–39% identical with the sequence encoded by vitronectin exon-5. We note that vitronectin exons 4 and 5 code for related sequences (30% identical), suggesting that they evolved from a common ancestral exon (50). Furthermore, we find that the ID sequence of XMMP (residues 146–173) is 38% identical and 62% chemically similar with a rat vitronectin sequence between residues 108–135. The biological significance, if any, of the ID of XMMP is not known at present. In comparison with the sequences encoded by vitronectin exons 4 and 5, which are highly conserved in evolution, the vitronectin sequence of residues 108–135 is not conserved between rat, rabbit, mouse, and human (36). It is also of interest that the COOH-terminal hemopexin/vitronectin-like domain of MMPs is highly conserved in the COOH-terminal end of nectinepsin from quail. Nectinepsin is a novel ECM protein with an RGD cell binding motif and an aspartyl proteinase domain (37).

When we searched the 3217-nucleotide sequence of XMMP for human and mouse ESTs in data bases, we could not find any significant matches by these analyses. This is interesting, especially because there are already 465,858 ESTs from human

Fig. 4. MMP hinge. All MMPs, with the exception of XMMP, have a proline-rich hinge region (linker peptide) between the catalytic domain and COOH-terminal hemopexin/vitronectin-like domain. Representative MMP hinge sequences shown are flanked by consensus residues in bold. The sequences are from Tc1, bullfrog Rana tadpole collagenase (34); XCL3, Xenopus collagenase-3 (GenBank accession number L49412); PCL1, porcine interstitial collagenase (GenBank accession number X54472); XST3, Xenopus stromelysin-3 (28); and Sue, sea urching hatching enzyme (33). The other sequences are from human MAT, matrilysin (42), which lacks the hinge region and COOH-terminal domain; 72k, 72-kDa type IV collagenase (43); ST3, stromelysin-3 (44); MT1, MT2, MT3, and MT4, four different MT-MMPs (17–21); and 92k, 92-kDa type IV collagenase (45).
and 63,404 ESTs from mouse (August, 1996). Because these ESTs are derived from a variety of tissues, mainly from adult tissues, it is reasonable to propose that the MMP gene is expressed specifically during early vertebrate development. This is in line with our recent results by Northern blotting and RT-PCR indicating only low levels of XMMP transcript in select adult Xenopus tissues such as heart, liver, and ovaries.\(^4\)

Thirty-five years ago Jerry Gross described the first evidence for MMPs as “a diffusible collagenolytic factor” from bullfrog tadpole tissues in culture (38), and then asked how tadpoles lose their tails (39). Part of the answer may involve collagenase-1 (MMPI) that Oofusa et al. (34) recently cloned from metamorphosing bullfrog tadpoles. During frog metamorphosis, also the small intestine of tadpoles goes through a rapid tissue remodeling, epithelial cell death and renewal by adult tissue remodeling, epithelial cell death and renewal by adult

In conclusion, we have studied here only a few early stages of Xenopus embryo development for the expression of MMP genes (blastula, gastrula, neurula, and pre-tailbud) and cloned cDNAs for six different MMPs, including a novel MMP called XMMP. Using the universal MMP primers (22) and RT-PCR we expect to clone other members of the MMP family from other stages of Xenopus development, morphogenesis, and tissue remodeling. Our results make it clear that MMPs are an evolutionary conserved family of ECM degrading enzymes in Xenopus. Future experiments will explore in more detail the role of MMPs in early vertebrate development.

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Addendum—Three novel MMPs were recently cloned, Xenopus collagenase-4 by Stolow et al. (46), human MMP18 by Cossins et al. (47) and porcine enamelysin by Bartlett et al. (48). The XMMP described here is distinct from these MMPs.

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**Fig. 5.** Evolutionary conservation of XMMP and vitronectin. A, alignment of ID sequence with a rat vitronectin sequence between residues 108–135. Identical residues are bold. B, four tandem XMMP repeats (see Fig. 2) are aligned with sequences encoded by rat vitronectin exons 4 and 5. Bold residues are identical between XMMP and vitronectin. C, rat vitronectin exons 4 and 5 encode sequences with 30% (16 of 53) identical amino acids shown in bold.
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