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A novel hydra matrix metalloproteinase (HMMP) functions in extracellular matrix degradation, morphogenesis and the maintenance of differentiated cells in the foot process

Alexey A. Leontovich[1], Jinsong Zhang[1], Ken-ichi Shimokawa[2], Hideaki Nagase[2] and Michael P. Sarras Jr[1,*]

[1]Department of Anatomy and Cell Biology, and [2]Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, Kansas 66160, USA

*Author for correspondence (e-mail: msarras@kumc.edu)

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SUMMARY

As a member of Cnidaria, the body wall of hydra is structurally reduced to an epithelial bilayer with an intervening extracellular matrix (ECM). Biochemical and cloning studies have shown that the molecular composition of hydra ECM is similar to that seen in vertebrates and functional studies have demonstrated that cell-ECM interactions are important to developmental processes in hydra. Because vertebrate matrix metalloproteinases (MMPs) have been shown to have an important role in cell-ECM interactions, the current study was designed to determine whether hydra has homologues of these proteinases and, if so, what function these enzymes have in morphogenesis and cell differentiation in this simple metazoan. Utilizing a PCR approach, a single hydra matrix metalloproteinase, named HMMP was identified and cloned. The structure of HMMP was similar to that of vertebrate MMPs with an overall identity of about 35%. Detailed structural analysis indicated some unique features in (1) the cysteine-switch region of the prodomain, (2) the hinge region preceding the hemopexin domain, and (3) the hemopexin domain. Using a bacterial system, HMMP protein was expressed and folded to obtain an active enzyme. Substrate analysis studies indicated that recombinant HMMP could digest a number of hydra ECM components such as hydra laminin. Using a fluorogenic MMP substrate assay, it was determined that HMMP was inhibited by peptidyl hydroxamate MMP inhibitors, GM6001 and matlistatin, and by human recombinant TIMP-1. Whole-mount in situ studies indicated that HMMP mRNA was expressed in the endoderm along the entire longitudinal axis of hydra, but at relatively high levels at regions where cell-transdifferentiation occurred (apical and basal poles). Functional studies using GM6001 and TIMP-1 indicated that these MMP inhibitors could reversibly block foot regeneration. Blockage of foot regeneration was also observed using antisense thio-oligo nucleotides to HMMP introduced into the endoderm of the basal pole using a localized electroporation technique. Studies with adult intact hydra found that GM6001 could also cause the reversible de-differentiation or inhibition of transdifferentiation of basal disk cells of the foot process. Basal disk cells are adjacent to those endoderm cells of the foot process that express high levels of HMMP mRNA. In summary, these studies indicate that hydra has at least one MMP that is functionally tied to morphogenesis and cell transdifferentiation in this simple metazoan.

Key words: Matrix metalloproteinase, MMP, ECM, hydra, development, morphogenesis, cell differentiation.

INTRODUCTION

Hydra arose early in metazoan evolution before divergence of protosomes and deuterostomes. Its body plan is organized as a gastric tube with a mouth and ring of tentacles at the apical pole and a foot process at the basal pole. The entire body wall of hydra is structurally reduced to an epithelial bilayer with an intervening extracellular matrix (ECM). The organism has about 20 different cell types that are distributed along the longitudinal axis in a specific manner (Bode and Bode, 1984; Bosch, 1998). For example, battery cells are restricted to the tentacle ectoderm and basal disk cells are restricted to the base of the foot process. Despite this restricted distribution pattern, the cells of hydra are in constant division and turnover. This division occurs by stem cells in the body column that lead to differentiated body column cells that are constantly displaced toward the poles (Campbell, 1967). When body column ectoderm cells are displaced into the tentacles or foot process they trans-differentiate into battery cells and basal disk cells, respectively (Bode et al., 1986). Battery cells and basal disk cells are eventually shed when they reach the tip of the tentacles or central portion of the base of the foot process. As

a consequence of this extensive cell turnover, hydra are highly regenerative (Bode and Bode, 1984; Bosch, 1998). This high regenerative capacity allows one to analyze developmental processes using a variety of cell biological and molecular approaches. Our laboratory has therefore utilized hydra as an epithelial model to study the role of cell-ECM interactions during morphogenesis and during the process of cell transdifferentiation. Previous studies have established that hydra ECM has a similar molecular composition to that seen in vertebrate species (Sarras et al., 1991a, 1994, Zhang et al., 1994) and functional studies have established that cell-ECM interactions are critical to developmental processes in hydra (Sarras et al., 1991b, 1993, 1994, 1997; Zhang et al., 1994). Because matrix metalloproteinases (MMPs) have been shown to be critical to a wide variety of developmental processes in vertebrates involving cell-ECM interactions (see reviews by Werb and Chin, 1998; Werb et al., 1999), we have attempted to determine whether hydra has homologues of MMPs and, if so, what role these enzymes have in the development of this simple metazoan.

MATERIALS AND METHODS

Hydra culture

Hydra vulgaris and the 105 strain of *Hydra magnipapillata* were used in all experiments. The animals were cultured at 18±1°C in glass trays with hydra media (1 mM $CaCl_2$, 1 mM $NaHCO_3$, 0.25 mM $MgCl_2$). Hydra were fed freshly hatched shrimp at least 3 times a week and the media was changed daily.

Library screening and RT-PCR

The initial search for a hydra MMP was conducted using PCR. We utilized the degenerate primers 5P-2 (5′-CCMMGVTGYSGVR-WBCCWGA) and 3P-2 (5′-YTCRTSNTCRTCRAARTGRRHRTC-YCC) [**R**=A or G; **Y**=C or T; **W**=A or T; **S**=C or G; **M**=A or C; **B**=C or G or T; **D**=A or G or T; **H**=A or C or T; **V**=A or C or G; **N**=A or C or G or T] previously reported by Takino et al. (1995). A hydra random-primed c-DNA library in uni-ZAP vector (Stratagene) was used as a template for the initial PCR using Taq polymerase. The PCR product was then used as a probe to screen both hydra random-primed and oligo(dT)-primed cDNA libraries (Stratagene) using standard protocols (Sambrook et al., 1989). Positive colonies were selected for re-screening or for subsequent in vivo excision of the cDNA inserts.

DNA sequencing

The DNA sequence was determined using the T7 sequenase chain-termination DNA sequencing method (Amersham) or using an ABI PRISM XL 377 DNA sequencer in combination with a Big Dye terminator chemical kit (Perkin-Elmer, Applied Biosystems, Foster City, CA) at the Biotechnology Support Facility at the University of Kansas Medical Center. The DNA clones were sequenced in both directions using a primer-walking strategy.

Construct preparation and protein expression

Our expression work was designed to obtain a full-length form of HMMP (signal peptide through the C-terminal hemopexin domain) with a calculated mass of 55.4 kDa. For protein expression, we used the pET expression system (Novagen). The hydra MMP cDNA clone was amplified by PCR with the primers 5′-GAGAATCATATGT-TCATTTCGTTAG and 5′-GTTATGGATCCTTTTCCTAAAAAAG. PFU polymerase (Stratagene) was used for the reaction. The PCR product was ligated into TA vector (Invitrogen), then cut with *Nde*I and *Bam*HI restriction enzymes and ligated into pET-19b vector (Novagen). The obtained construct was then sequenced to assure that the His tag of the pET-19b vector and whole MMP message was in the same reading frame. BL-21DE3 cells were transformed with the construct. For protein expression, we used procedures described earlier by Suzuki et al. (1998) with the modification that host cells were grown at 20°C before and after expression induction with IPTG.

For expression of recombinant human MMP-3, we used the pET-3a instead of the pET-19b vector. We utilized the same expression procedures described above except the host cells were grown at 37°C. Recombinant human MMP-3 catalytic domain was isolated and folded to active form as described by Suzuki et al. (1998). Recombinant human TIMP-1 was expressed in CHO cells as described by Huang et al. (1996) and was purified using anti-human TIMP-1 affinity chromatography. For in vivo blocking studies, recombinant TIMP-1 buffer was exchanged with hydra media.

Isolation, folding and purification of recombinant HMMP

Inclusion bodies were prepared as previously described (Huang et al., 1996) with the following modifications. After treatment with deoxycholate, the lysate was incubated for 16 hours with 1 μg/ml of DNAse to remove DNA. The inclusion bodies were solubilized with 8 M urea and the protein was fractionated on a MacroPrep HighQ (Bio Rad) anion exchange column. The fractions were subjected to SDS-PAGE and those containing the peak amount of recombinant HMMP protein were pooled and diluted to an A_{280} of about 0.1 in folding buffer I [50 mM Tris-HCl (pH 8.5), 6M urea, 0.15 M NaCl, 1 mM DTT, 5 mM $CaCl_2$, and 100 μM $ZnCl_2$]. Cystamine at a final concentration of 20 mM was then added to this solution and the mixture was incubated for 16 hours at 4°C. Following this incubation step the mixture was then dialyzed for 24 hours against one change of 10 volumes of folding buffer I, followed by dialysis against two changes (24 hours each) of folding buffer II (50 mM Tris-HCl (pH 7.5), 0.15M NaCl, 5 mM $CaCl_2$, 50 μM $ZnCl_2$ and 0.02% NaN_3). The dialyzed protein was concentrated 4-fold with an Amicon concentrator, aliquoted and stored at −70ºC.

SDS-PAGE, zymography, ECM preparation and fluorescent substrate assay

SDS-PAGE was performed according to the method of Laemmli (1970). For zymography, gelatin (Fisher) was added to polyacrylamide at a concentration of 0.8 mg/ml and the incubation conditions for visualization of gelatinase activity in the gels were as described by Yan et al. (1995). Hydra extracellular matrix was isolated according to Sarras et al. (1991a). To quantify HMMP activity, the MMP fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (Bachem Bioscience Inc., King of Prussia, PA) was incubated with HMMP at a final concentration 1 μM in TNC buffer [50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 10 mM $CaCl_2$, 0.05% Brij-35 and 0.02% NaN_3]. Following incubation at 22°C, the reaction was stopped by adding 900 μl of 3% acetic acid and the fluorescent product was measured using a Hitachi F-3010 fluorescence spectrophotometer set at an excitation wavelength of 325 nm and an emission wavelength of 393 nm.

Antibodies and MMP inhibitors

Antibodies specific to hydra laminin were previously characterized (Sarras et al., 1994). The peptidyl hydroxamate MMP inhibitor GM6001 and an inactive form of GM6001 (control peptide) were purchased from AMS Scientific (Plesant Hill, CA, USA) while the MMP inhibitor Matlistatin was a gift from Dr K Tazawa, Sankyo Co. These compounds were dissolved in DMSO as 10 mM stock solutions.

In situ hybridization

Whole-mount in situ localization of HMMP mRNA was performed using a digoxigenin-labelled RNA probe that corresponded to nucleotide number 342 through the end of the full-length cDNA clone.

The probe incorporated the catalytic domain and the hemopexin domain of HMMP.

Fixation, processing, hybridization and visualization of the riboprobe in whole-mount preparations was performed as previously described by Grens et al. (1995, 1999) and Martinez et al. (1997). Briefly, hydra were fixed with 4% paraformaldehyde after relaxation of the polyps in 2% urethane. Specimens were subsequently treated with ethanol and proteinase K to facilitate diffusion of the probes into the epithelial bilayer. To stabilize digested tissues, specimens were re-fixed with 4% paraformaldehyde and then prehybridyzed in hybridization solution (50% formamide, 5× SSC, 1× Denhardt's, 200 mg/ml tRNA, 0.1% Tween 20, 0.1% CHAPS, 100 μg/ml heparin) to block non-specific hybridization sites. This was followed by a 48 hour hybridization with the digoxigenin-labelled RNA probe and a subsequent wash in hybridization solution and SSC. Specimens were washed in MAB (100 mM maleic acid, 150 mM NaCl, pH 7.5) and pre-blocked in MAB with 20% of sheep serum and 1% BSA. This was followed by the 16 hour incubation at 4°C in the same solution with anti- digoxigenin antibody (1:2000). Animals were washed eight times with MAB and then briefly in alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween-20). Specimens were then stained with BM purple AP substrate (Boehringer Mannheim), dehydrated with ethanol and mounted in Euparal (Asco Laboratories).

Amino acid sequence analysis

Proteins to be sequenced were separated on SDS-PAGE under reducing or non-reducing conditions and transferred to PVDF membrane (Millipore Corp., Bedford, MA, USA) in 10 mM CAPS buffer with 10% methanol. Sequencing was performed by the Biotechnology Support Facility at the University of Kansas Medical Center and the Molecular Biology Resource Facility at the University of Oklahoma Health Sciences Center.

Statistical analysis and DNA and protein sequence analysis

Statistical tests were performed using the SigmaStat software program (Jandel Corporation). Comparisons of DNA sequences were conducted using the Blastn and Blastx programs and the GenBank database (NCBI, NIH). Protein alignments were obtained using the Blastp, GCG and MacVector 5.0 programs. Molecular masses of proteins were determined using the BioMax 1D software program distributed by the Eastman Kodak. Evolutionary tree analysis was kindly performed by Dr Linda Frisse in the laboratory of Dr Kelly Thomas (University of Missouri Kansas City, Kansas City, Missouri) using a MacVector program and a MEGA program (Kumar et al., 1993).

Inhibitor studies

The effect of the hydroxamate peptides GM6001 and matlistatin on HMMP activity was examined using the fluorescent substrate assay described before. Briefly, 2.5 μg of the HMMP protein fraction was incubated with various concentrations of the inhibitors for 6 hours and the residual activity was measured against Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2. The effect of recombinant human TIMP-1 on HMMP activity was also determined by incubation 2.5 μg of the HMMP fraction for 1 hour with various concentrations of TIMP-1 (60 nM-2.5 μM) in TNC buffer. Following this incubation, fluorescent substrate was added and the activity of the reaction mixture was measured.

In vivo functional studies

(1) Effects of MMP inhibitors on foot regeneration

Animals without buds were used for foot regeneration experiments. Animals were cut in the lower peduncle area at approximately the same level. Immediately after foot excision, hydra were placed into hydra media with MMP inhibitor GM6001, control peptide, or recombinant human TIMP-1 at concentrations described in the Results. For all peptide studies, the final DMSO concentration was 0.1%. DMSO at a final concentration 0.1% was added along with TIMP-1 to improve the penetration of the protein into the basolateral compartment of epithelial cells along the regenerating pole (Zhang and Sarras, 1994). For all recovery experiments, hydra were washed several times in hydra media and observed for an additional 5-7 days. The degree of foot regeneration was monitored by (1) observing the morphology of the foot process under a dissection microscope, (2) determining the ability of basal disk cells to produce mucus by placing a glass probe against the basal pole of the regenerating hydra and monitoring if cells could adhere to the probe, (3) assaying for the basal disk cell marker, peroxidase following the procedures of Hoffmeister (1996), and (4) monitoring the cellular morphology of basal disk cells using Nomarski optics.

(2) Effects of GM6001 on basal disk cells in adult polyps

Adult hydra without buds were incubated for up to 5 days with GM6001 or control peptide under the conditions described for foot regeneration. The differentiated state of the basal disk cells was monitored as described above for the foot regeneration experiments. For all recovery experiments, hydra were washed several times in hydra media and observed for an additional 2-5 days.

(3) Effects of antisense thio-oligo nucleotides on foot regeneration

Because transfection approaches have not been successfully applied to Cnidarians, we developed a procedure to specifically test the effect of antisense oligonucleotides on head or foot regeneration in hydra. This approach was developed based on discussions with Dr Hans Bode (Dev. Biol. Center, University of Irvine, CA) who has attempted to develop a localized electroporation technique (LEP) in order to introduce expression constructs into hydra. Applying the localized electroporation procedure initially outlined by H. Bode (personal communication), we attempted to use the approach to test the hypothesis that HMMP was involved in processes related to foot morphogenesis. To test this hypothesis, we designed antisense thio-oligo nucleotides from the cDNA sequence of HMMP. Based on the work of Dr Richard W. Wagner (Wagner, 1994, 1995; Flanagan et al., 1996), a series of 20-mer oligonucleotides were designed with phosphorothioate linkages. Six oligonucleotides were synthesized that included antisense sequences to portions of the 5′ UTR, initiation site, coding sequence, 3′ UTR, a sense strand of the 5′ UTR and a mismatch (randomized) oligonucleotide. The oligonucleotides (100 μM) were introduced into hydra endodermal cells of the foot pole using electroporation (BioRad Gene Pulser) and a micropipette drawn on a pipette puller as described in detail previously (Yan et al., 2000a,b). In the case of foot regeneration, the degree of normal foot morphogenesis was monitored both morphologically and functionally as described for the MMP inhibitor studies. Controls involved mismatched thio-oligo 20-mer oligonucleotides (randomized sequence) or sense oligonucleotides if a particular antisense oligonucleotide was found to block morphogenesis.

RESULTS

Cloning of hydra MMP

Using a PCR approach, a 370 bp amplification product was obtained from the hydra random-primed cDNA library. The deduced amino acid sequence of this PCR product matched with a variety of mammalian MMPs. The PCR product was used to screen both hydra random-primed and oligo(dT)-primed cDNA libraries and nine cDNA clones were obtained that had 34% identity with human MMP-3 and 32% identity with porcine MMP-1. Analysis of the deduced amino acid

sequence of a full-length clone (1642 bp) indicated that hydra MMP (HMMP) contained a signal peptide, a pro-domain, a catalytic domain, a hinge region, and a C-terminal hemopexin domain as shown in Fig. 1A and B (left side of panel). The GenBank accession number for HMMP is AF162688.

A cDNA fragment (342 through the end of the full-length clone) was used as a probe for northern blot analysis. These studies revealed two HMMP mRNA transcripts that were 1.6 kb and 1.9 kb in length with the larger transcript being more intense (Fig. 1B, right side of panel).

The deduced amino acid sequence of HMMP has some unique features

Comparison of the amino acid sequence of HMMP with other MMPs (Table 1) shows that HMMP has an overall 30-35% identity with known MMPs. The sequence identity is about 10% higher (40-47%) if we compare only catalytic domains (Table 1). A probable furin cleavage site for activation of the pro-enzyme exists at amino acid residues R^{101} R^{102}-Y^{103} (Fig. 1A). The least identical region is the C-terminal hemopexin domain (23-38%). The sequences of two critical regions, the Cys-switch and catalytic Zn^{2+}-binding region, are well conserved in HMMP (Fig. 1C). An unusual feature of HMMP is that the multiple prolines commonly found in the hinge region between the catalytic domain and the hemopexin domain of MMPs are reduced to only two in HMMP (Table 1; Fig. 2, upper panel). The hinge region of HMMP is somewhat hydrophilic in nature suggesting that this region is exposed as shown by the crystal structure of the full-length porcine MMP-1 (Li et al., 1995). In addition, the hemopexin domain of HMMP lacks the two cysteines commonly found in other MMPs (Fig. 2, lower panel). This is of particular interest in regard to the highly conserved cysteine that is normally found at the N terminus of the hemopexin domain of vertebrate MMPs. Computation of an evolutionary tree of MMPs indicated that HMMP grouped separately from all other mammalian MMPs, but the degrees of divergence were not significant in regard to the relationship of HMMP to specific types of MMPs and therefore an evolutionary tree is not shown.

Expression and biochemical characterization of HMMP protein

We expressed recombinant HMMP in *E. coli*, folded it from inclusion bodies, partially purified it and obtained an active enzyme preparation. Zymography of the folded HMMP fraction showed activity to a be located at approximately 46 kDa and 22 kDa (Fig. 3A, arrows). When this material was then subjected to SDS-PAGE under reducing and non-reducing conditions, at least eight bands were found (Fig. 3B). All eight bands were transferred to PVDF membrane and their N-terminal sequence was determined. The N-terminal sequence of three of these bands

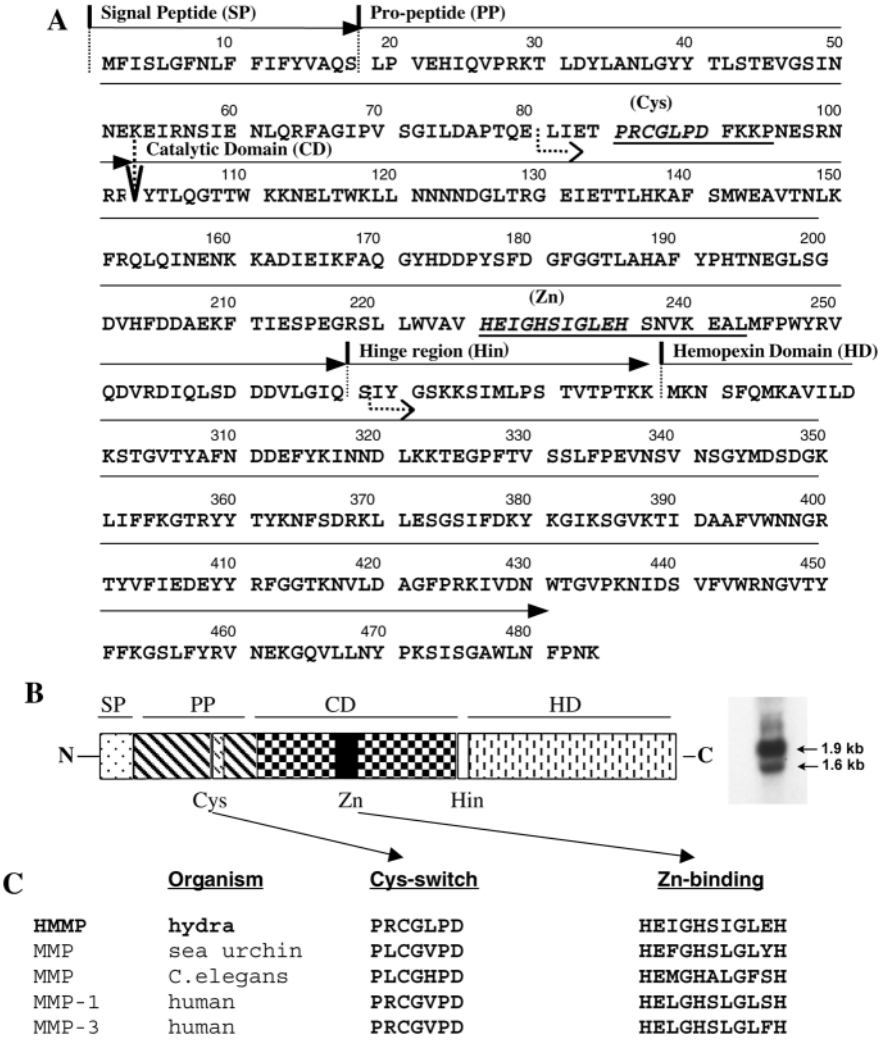

Fig. 1. Amino acid sequence analysis and northern blot analysis of HMMP. (A) cDNA deduced amino acid sequence of HMMP (GenBank accession number AF162688). Domains of HMMP are indicated by the solid arrows above the amino acid sequence. Domains or regions are named at their proposed N terminus. A proposed furin-like intracellular processing site is indicated by the vertical dashed arrow (at residues R^{102}-Y^{103}). The bent dashed arrows at residue Leu^{81} and Ile^{269} indicate the sites where HMMP was cleaved during expression in E. coli (shown in Fig. 3). The Cysteine switch (Cys) region and Zn-binding (Zn) region are indicated in bold italic and are underlined. (B) The left side of B shows a diagram of the domain structure of HMMP protein. SP, signal peptide; PP, propeptide; CD, catalytic domain; HD, hemopexin domain; Cys, cysteine switch region; Zn, Zn- binding region; Hin, Hinge region between CD and HD. The right side of B shows a Northern blot of HMMP. 5. 0 μg of total RNA of *Hydra vulgaris* was separated in 1% agarose/formaldehyde gel and hybridized with α-^{32}P labeled cDNA probe corresponding to a highly conserved region of mammalian HMMP. (C) Comparison of HMMP Cysteine switch and Zn-binding regions to that of vertebrate and invertebrate MMPs.

Table 1. Comparison of the sequence homology of HMMP to that of other MMPs

					Hinge Region	
Enzyme	Organism	Whole length	Catalytic domain	C-terminal (Hemopx) domain	AA length	Number of Pro
MT1 MMP (MMP14)	human	34	43	31		
	dog			35		
	rabbit	35	43	32		
	rat	35	43	31		
	mouse		43	29		
MT2 MMP (MMP15)	human		40	28		
	mouse		41			
MT3 MMP (MMP16)	human	31	39	31		
MT4 MMP (MMP17)	human			34		
MMP-3	human	34	45		25	11
	mouse	35	45		25	5
MMP-10	human	32	47			
	rat	34	45			
MMP-1	human			27	16	4
	pig	32		27	16	5
	bovine			29	16	5
MMP-2	human			28	22	5
	chicken			32		
	rat			30	24	6
MMP-13	human	34	46		16	5
	bovine		47			
	horse		48			
	frog	32			16	5
MMP-12	human	35	44		18	4
	mouse	32	46		18	6
	rabbit	35	46			
	rat		46			
MMP-11	frog			35	32	8
putative MMP	C. elegans	22 -31	33		43/5	1/0
envelisin	sea urchin	31	40		52	4
HMMP	hydra				18	2

Percentage of identity shown for mature enzyme, catalytic domain and hemopexin domain.

corresponded to the deduced sequence within HMMP, while the other bands had no correspondence to the HMMP sequence. The HMMP corresponding sequences were LIETPRCGLP for a band of approximately 46 kDa (Fig. 3B,arrow marked a), and LIETPRCGLP and IYGSKKSIML for bands of about 21.6 kDa and 21 kDa, respectively (Fig. 3B, arrows marked b). The 46 kDa band with the N-terminal sequence LIETPRCGLP and the 21.6 kDa band with the same N-terminal sequence were excised from the non-reducing gels and subjected to gelatin zymography. Both proteins exhibited gelatinoytic activity at expected positions (Fig. 3C, arrows). The 21 kDa band with the N-terminal sequence IYGSSKKSIML had no gelatinolytic activity. Based on these analyses, it was concluded that the 46 kDa band with the N-terminal sequence LIETPRCGLP, represented amino acid residues Leu^{81} through Lys^{484} from the deduced sequence of HMMP. This band therefore represents a partially cleaved form of HMMP containing (1) the cysteine switch region of the pro-domain, (2) the catalytic domain, (3) the hinge region, and (4) the hemopexin domain. The 21.6 kDa band with the N-terminal sequence LIETPRCGLP, represented amino acid residues Leu^{81} through Ser^{268}, lacking the C-terminal hemopexin domain. The 21 kDa band with the N-terminal sequence IYGSKKSIML is a fragment beginning with amino acid residue Ile^{269} and represents an expressed form of HMMP that contains a portion of the hemopexin domain. It is likely that the precursor form of HMMP (original 55.4 kDa contruct) is processed to the 46 kDa, 21.6 kDa and 21 kDa forms during expression in *E. coli* or during the folding of the enzyme. Smaller fragments arising from the C-terminal domain may exist, but were not identified in this analysis.

The optimal activity of recombinant HMMP against the fluorogenic MMP substrate was observed at pH 6.5-7.5 (Fig. 3D). HMMP was inhibited by the MMP inhibitor GM6001 with a calculated IC_{50} of 5.5 nM as compared to an IC_{50} for human MMP of 75 nM (Fig. 3E). As shown in Fig. 4, HMMP was also inhibited by matlistatin and recombinant human TIMP-1.

Degradation of hydra ECM by recombinant HMMP

Recombinant HMMP degraded components of hydra ECM (Fig. 5A). This was most prominent in the high molecular mass range (>200 kDa) by 10-14 hours of incubation. Cleavage of hydra ECM components was blocked when the MMP inhibitor GM6001 (50 μM) was added to the reaction. Because subunits of hydra laminin have a mass >200 kDa, we monitored the effect of HMMP on this ECM component using a β1 laminin mAb. Incubation of hydra ECM with HMMP degraded hydra laminin as monitored by western blot analysis (Fig. 5B).

The ability of HMMP to hydrolyze mammalian ECM components was also tested. Although HMMP was incubated with mammalian type I collagen, type IV collagen, laminin, and fibronectin for up to 24 hours, no degradation of these ECM components was observed (data not shown). Incubation of these components with recombinant human MMP3 caused

a clear cleavage of type IV collagen, laminin and fibronectin under these same conditions (data not shown) as previously reported by Okada et al. (1986).

Expression of HMMP in the adult intact polyp and during foot regeneration

In situ whole-mount analysis indicated that HMMP mRNA was expressed in the endoderm along the entire longitudinal axis of the adult polyp, but at relatively higher levels in the tentacles and in the foot process just superior to the basal disk cells (Fig. 6B). This expression pattern was mirrored in late buds as is also shown in the lower right portion of Fig. 6B. Because recombinant HMMP was shown to degrade hydra laminin, we also determined the in situ expression pattern for the β1 chain of hydra laminin. The expression pattern for hydra laminin mRNA mirrored what was observed for HMMP. As shown in Fig. 6A, laminin mRNA was expressed in the endoderm along the entire longitudinal axis of adult polyps with relatively higher levels in the tentacles and in the foot process just superior to basal disk cells. By adjusting the conditions for probe visualization, we were able to increase our detection levels and determine that the highest levels of laminin and HMMP mRNA were actually inverse of one another in the adult tentacles. As shown in Fig. 6C,D, laminin mRNA expression is highest at the base of the tentacles and progressively decreases distally, whereas, HMMP mRNA is highest at the distal ends of the tentacles and decreases toward the base of the tentacles. It should be noted, however, that this is mainly an issue of degree of staining sensitivity. By comparing Fig. 6B and D, it is clear that HMMP mRNA is expressed at the base of tentacles, although at lower levels than observed at the distal ends.

The expression of HMMP mRNA was monitored during both head and foot regeneration. Because subsequent functional studies indicated that the role of HMMP in head regeneration is a more complicated process than that of foot regeneration, for the purposes of the current study, we have focused only on foot regeneration. As shown in Fig. 7 (upper panel), northern blot analysis during surgically induced foot regeneration indicated that an upregulation of both the 1.6 kb and 1.9 kb transcripts of HMMP occurred by 3 hours of regeneration. This upregulation continued through at least 48 hours of foot regeneration. When monitored by whole-mount in situ analysis, an endoderm-specific expression of HMMP mRNA was detected at the regenerating pole (Fig. 7, lower panel). With surgical removal of the foot process, the normally high expression level of HMMP mRNA is lost between 1 and 3 hours (B). Higher levels of HMMP mRNA expression were observed in the endoderm by 4 and 10 hours of foot regeneration. Alterations in the general morphology of the basal disk cells was also observed during this process. This is particularly clear at 4 and 10 hours when cells changed from an original columnar type morphology (Fig. 7A) to a more low cuboidal type morphology (Fig. 7C,D). By 2 days of foot regeneration, a more typical columnar-type morphology of basal disk cells was observed (Fig. 7E).

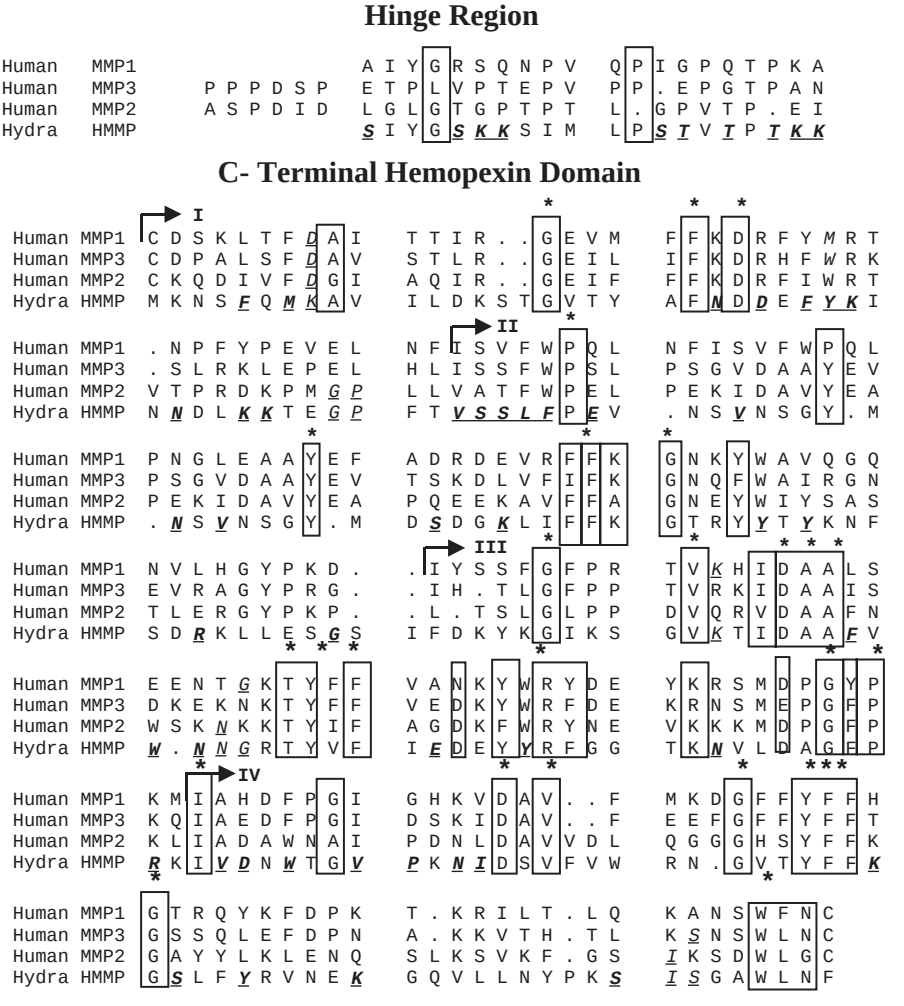

Fig. 2. Comparison of the hinge region and C-terminal hemopexin domain of HMMP to that of vertebrate MMPs. (Upper panel) Comparison of the hinge region of HMMP to that of human MMP-1, MMP-3 and MMP-2. Conserved amino acids are boxed and hydrophilic amino acids are in bold italic and underlined. (Lower panel) Comparison of the hemopexin domain of HMMP to that of human MMP-1, MMP-3 and MMP-2. Absolutely conserved amino acids are boxed and indicated with an asterisk, highly conserved amino acids (2 of 3 forms identical) are boxed, similar substitutions are shown in bold italic and underlined in the HMMP sequence. Three-dimensional studies show that the hemopexin domain of vertebrate MMPs exists as an ellipsoidal disk shape with four bladed B-propeller structures. The bent arrows indicate the beginning of the four (I-IV) proposed propeller blade structures in HMMP.

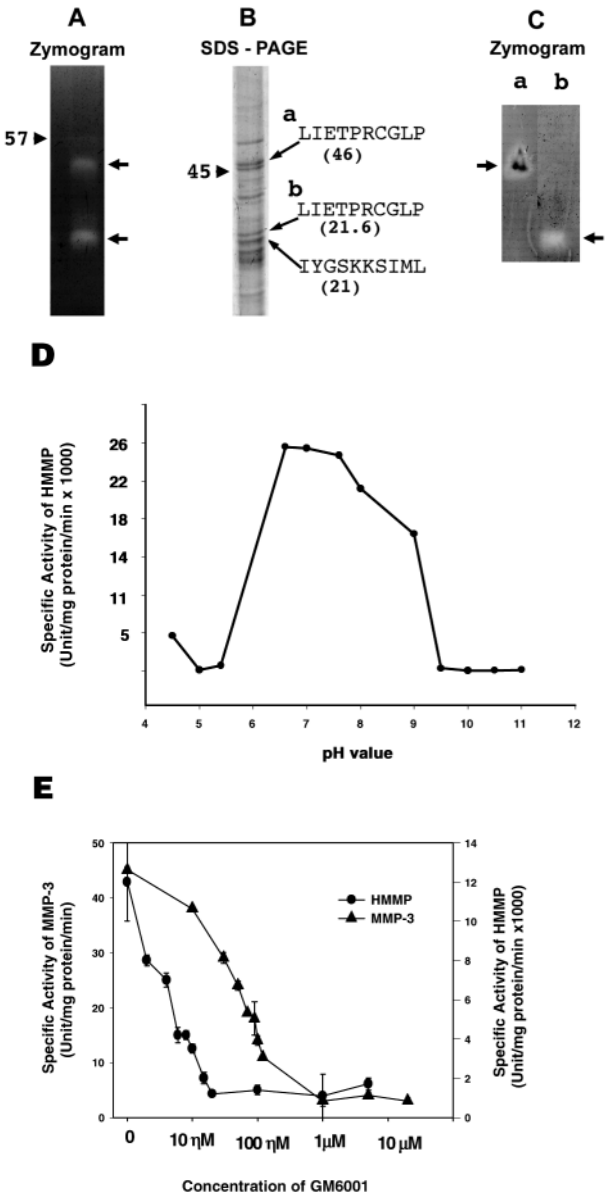

Fig. 3. Biochemical characterization of recombinant HMMP. (A-C) Analysis of enzymatic activity and protein sequence of recombinant HMMP. (A) Gelatin zymogram of recombinant HMMP. 2.5 μg of HMMP was separated on 7.5% acrylamide gel with 0.8mg/ml gelatin; incubated overnight in 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 1% Triton X-100 and stained with Coomassie Blue. (B) SDS-PAGE of recombinant HMMP protein fraction under reducing conditions. HMMP (10 μg) was separated on 7.5% SDS page and transferred on PVDF membrane and stained with Coomassie Blue. Protein bands were cut individually and the sequence of the first 10 N-terminal amino acids was determined. The corresponding amino acid sequences are on the right of the lane. Computer calculated molecular masses (in kDa) of the proteins are in parenthesis. (C) HMMP (10 μg) protein fraction was separated on SDS PAGE under non reducing conditions, protein bands corresponding to the bands a and b (upper 21.6 kDa band) in B were excised and ran separately on 7.5% PAGE with 0.8 mg/ml gelatin and processed as in A. Arrowheads indicate molecular mass markers (in kDa) and the short arrows indicate active forms of HMMP. (D,E) Determination of the optimal pH for HMMP and the IC_{50} for GM6001. (D) pH curve of HMMP activity (pH 4.5-7.5 in Tris-acetate buffer; pH 7-9.5 in Tris HCl buffer; pH 9-11 in CAPS buffer). (E) Inhibition of recombinant HMMP (circles) and recombinant human MMP-3 (trianges) activity by the inhibitor GM6001. For D and E each point represents triplicate determinations and each experiment was repeated three times. Standard error bars are shown.

In vivo functional analysis of HMMP in regenerating and adult intact hydra

The expression pattern of HMMP mRNA in adult intact polyps, in buds and during foot regeneration suggested that the enzyme had a role in morphogenetic and cell differentiation processes. To test this hypothesis, we analyzed the role of HMMP in foot regeneration and in the maintenance of differentiated cells along the longitudinal axis of adult intact polyps. Following the procedures described in the Materials and Methods section, the effects of GM6001 and recombinant human TIMP-1 were analyzed during surgically induced foot regeneration. As shown in Fig. 8 (upper panel), at concentrations known to inhibit recombinant HMMP, both GM6001 and recombinant human TIMP-1 blocked foot regeneration. The fact that TIMP-1 was less effective in blocking foot regeneration than GM6001 may be partially explained by the fact that under the culture conditions utilized for these experiments, recombinant TIMP-1 lost up to 66% of its inhibitory activity as monitored by fluorescent substrate assays with recombinant HMMP. This is in contrast to GM6001 which lost no inhibitory activity under the same culture conditions. Blockage of foot regeneration was accompanied by alterations in the normal columnar morphology (Fig. 8, lower panel) of basal disk cells as compared to groups treated with control peptide or in animals maintained in hydra media alone. Basal disk cells of hydra treated with GM6001 or TIMP-1 failed to produce normal amounts of mucous as reflected by the inability of treated groups to attach to glass probes placed against the base of their foot process. In addition, basal disk cells of GM6001-treated hydra had significant reductions in the expression of peroxidase activity, one of their differentiation markers (data for adult hydra shown in Fig. 10). When the inhibitor were removed, regeneration proceeded indicating that the blockage was reversible. As shown in Fig. 9, blockage of foot regeneration was also observed in antisense experiments performed in parallel with the MMP inhibitor studies. Using a spectrum of antisense thio-oligo nucleotides, we found that foot regeneration was significantly blocked by antisense constructs to the 5′ UTR, initiation region, coding region, but was unaffected by a construct to the 3′ UTR. A mismatch control with a rondomized sequence and a 5′ UTR sense control were also not inhibitory. This effect was reversible, in that hydra recovered from blockage when observed 5-7 days from the initial time of inhibition (48-72 hours) (data not shown).

To determine the potential role of HMMP in the maintenance of differentiated cells along the longitudinal axis of hydra, experiments were performed using adult intact polyps. These studies concentrated on basal disk cells because of the results obtained with foot regeneration and because of the high levels of HMMP mRNA expressed by the endoderm cells at the foot process. These experiments focused on the effect of GM6001 because it allowed us to maintain HMMP in a continuous state of inhibition over a 5-

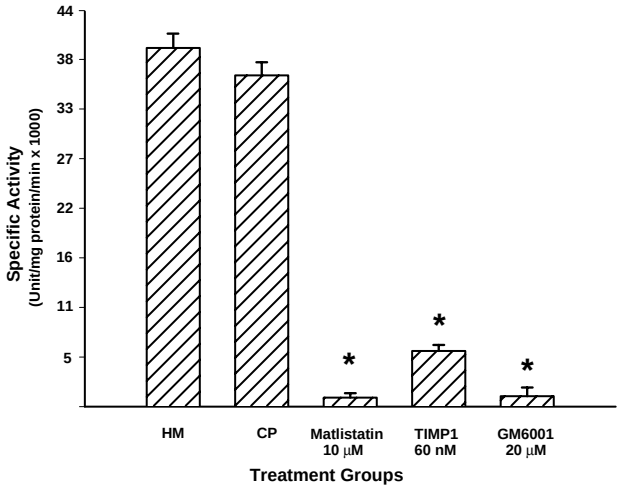

Fig. 4. Effect of various MMP inhibitors on recombinant HMMP. Graphs represent specific activity measurements using the fluorogenic substrate digested with 2.5 μg of the recombinant HMMP fraction. Complete inhibition of HMMP activity was observed at 125 nM TIMP-1 (data not shown). Assays were conducted in triplicate and each experiment was performed three times. Each bar represents the mean from three experiments with standard errors shown. Asterisks indicate groups that were statistically different from the HM and CP controls at $P<0.001$. HM, hydra media, CP GM6001 control peptide, TIMP1, human recombinant TIMP1.

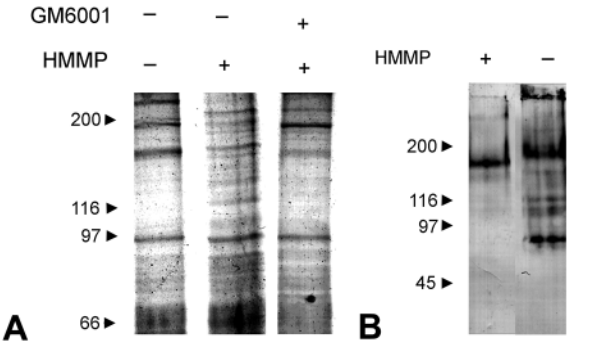

Fig. 5. Effect of recombinant HMMP on hydra extracellular matrix. (A) Hydra ECM was analyzed using 7.5% SDS-PAGE under reducing conditions and stained with Coomassie blue. Aliquots containing 25 μg of hydra ECM were incubated at 22°C overnight under the following conditions: First lane, ECM; Second lane, ECM incubated with 2.5 μg of recombinant HMMP; Third lane, ECM incubated with 2.5 μg of recombinant HMMP and 20 μM of GM6001. (B) Western blot of hydra ECM separated on 7.5% SDS-PAGE under non-reducing conditions. The blot was probed with monoclonal antibody M52 raised against the β1 chain of hydra laminin. First lane, ECM incubated with 2. 5 μg of recombinant HMMP. Following incubation with HMMP, the hydra β1 laminin chain shifted to approximately 160 kDa. Second lane, ECM incubated without HMMP. Arrowheads indicate molecular mass markers in kDa.

8 day period. TIMP-1 was not used for these experiments because, as indicated earlier, we found that recombinant TIMP-1 lost activity over extended periods of time under the culture conditions that we utilized. As shown in Fig. 10, continuous exposure of adult polyps to GM6001 (50 μM) resulted in a de-differentiation of basal disk cells within 80 hours as compared to hydra treated with control peptide. Alternatively, an inhibition of transdifferentiation of body column cells to basal disk cells may also be occurring. The effect was observed in all animals incubated with GM6001 (n=50). This state of de-differentiation or inhibition of transdifferentiation was reflected by (1) the loss of the ability of treated polyps to attach to glass probes placed against the base of their foot process, (2) changes in the morphology of basal disk cells from a columnar type to a low-cuboidal type, and (3) the loss of the differentiation marker, peroxidase in basal disk cells (Fig. 10). This un-differentiated state was observed for as long as the inhibitor was present in the culture wells (Fig. 10C, 5 days of treatment). These effects were reversible, however, since removal of GM6001 from the media resulted in the re-appearance of a normal basal disk cells morphology and peroxidase activity within 2-5 days (Fig. 10F).

DISCUSSION

Structural and biochemical characterization of HMMP

The number of MMP families has grown to over 25 with each type containing members from a broad spectrum of animal species (Massova, 1998; Nagase and Woessner, 1999). In contrast, relatively few MMPs have been identified in invertebrates. These invertebrate MMPs include: envelysin

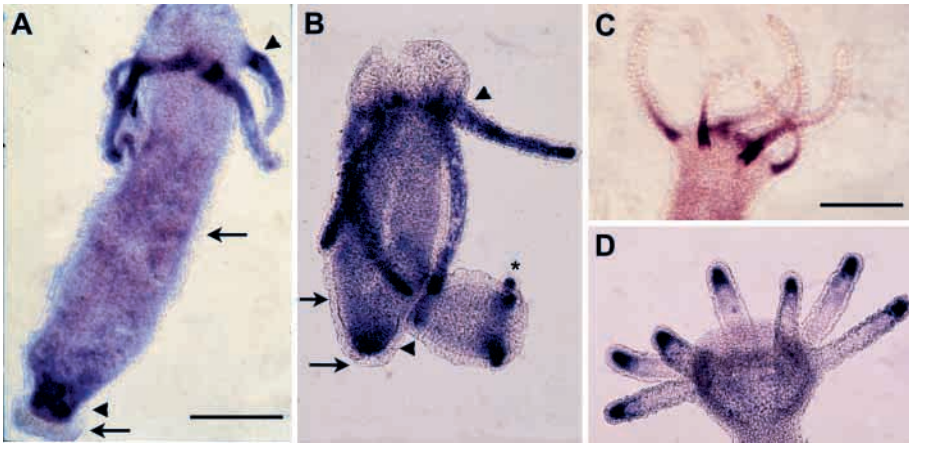

Fig. 6. Localization of HMMP and laminin mRNA as revealed by in situ hybridization in whole-mount preparations of adult intact hydra. (A,C) In situ of hydra laminin β1 chain; (B,D) in situ of HMMP. (C,D) Hydra head pole showing areas of strongest gene expression. Arrowheads, areas of strongest mRNA expression in the cell differentiation zones (see text); arrows point to the outer edge of ectoderm; the asterisk indicates forming tentacles in buds. Bar in A, 500 μm (applies to A and B); Bar in C, 500 μm (applies to C and D).

from sea urchin (Nomura et al., 1991, 1997), and at least three separate MMPs from *Caenorhabditis elegans* (Wada et al., 1998). Two additional putative MMPs have been reported in plants (soybean (*Glycine max*) and *Arabidopsis thaliana*; Massova et al., 1998). The identification of HMMP therefore adds a new member to the MMP family of metalloendopeptidases.

A detailed analysis of the HMMP sequence indicates that this enzyme has many similarities to vertebrate MMPs but also a number of features that make it unique. One region of variation in HMMP is the cysteine-switch region where a leucine residue (Leu^{89}) is substituted for the valine residue typically seen in many vertebrate MMPs. A similar substitution has been reported for MMP-19 in humans (Sedlacek et al., 1995) and MMP-7 in felines (Scalzo et al., 1994). Variations in this region are more common among MMPs as reflected by the divergence of this region in the three MMPs of *C. elegans* (Wada et al., 1998), the MMP of soybean leaf (McGeehan et al., 1992), and the MMP of sea urchin (envelysin). In the case of these invertebrate and plant MMPs, a leucine residue replaces the arginine normally seen as the second amino acid residue of the cystine switch region (Ghiglione et al., 1994). An unusual feature of HMMP is that the hinge region between the catalytic domain and hemopexin domain is short and contains only two proline residues. This region in vertebrate MMPs typically contains four or more proline residues (Bode, 1995); although it should be noted that some vertebrate MMPs completely lack a hinge region (Nagase and Woessner, 1999). In addition, two cysteines commonly found in the hemopexin domain of MMPs are substituted with methionine (Met^{288}) and phenylalanine (Phe^{481}) in HMMP. These cysteines are conserved in the hemopexin domain of all vertebrate and invertebrate MMPs that we analyzed (see Table 1). Despite the absence of all cysteines from the hemopexin domain, the deduced sequence of this domain in HMMP is compatible with the existence of an ellipsoidal disk shape consisting of a bladed B-propeller structure as has been reported for the hemopexin domain of vertebrate MMPs (Gohlke et al., 1996; Gomis-Ruth et al., 1996). At this time, the functional significance of these structural differences in HMMP remains to be determined.

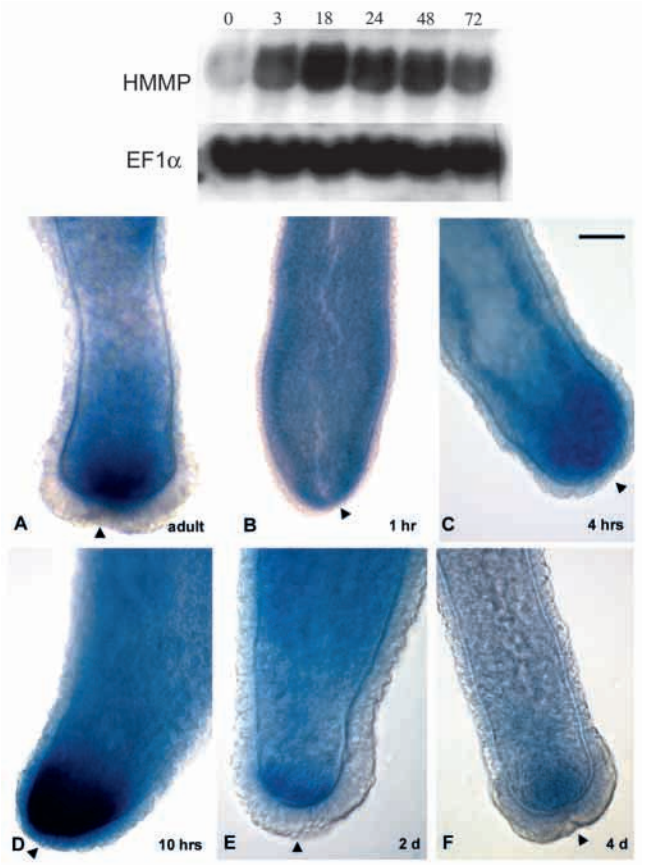

Fig. 7. Time course of HMMP mRNA expression during foot regeneration. (Upper panel) Northern blot analysis. 50 segments of foot regeneration were taken for RNA preparation and northern blot analysis. Time points included: 0 hours (immediately following excision of the foot), 3, 18, 24, 48 and 72 hours after excision of the foot. The blot was hybridized with α-^{32}P-labeled HMMP cDNA probe (upper row), washed and hybridized again with ^{32}P-labeled EF1 cDNA probe as a loading control (lower row). (Lower panel) Whole-mount in situ hybridization. Hydra feet were surgically removed and specimens were allowed to regenerate. Animals were fixed 1 hour (B), 4 hours (C), 10 hours (D) 2 days (E), 4 days (F) and further processed for in situ hybridization. The foot process of an intact polyp is shown in A. Arrowheads point to the apical border of basal disc cells. Bar in C, 100 μm (applies to A-F).

Recombinant HMMP has maximal activity at about pH 6.5-7.5; a typical optimal pH for known MMPs (Nagase, 1995). The fact that the activity of recombinant HMMP was blocked by a specific MMP inhibitor such as recombinant human TIMP-1 (Itoh et al., 1998) and by synthetic MMP inhbitors such as GM6001 (Grobelny et al., 1992) and Matlistatin provided strong evidence that this enzyme had characteristics reflective of bonafide MMPs.

Our studies demonstrated that HMMP is able to cleave a number of hydra ECM components such as hydra laminin. While HMMP was unable to cleave mammalian laminin, fibronectin, type IV collagen and type I collagen, it was able to cleave heat-denatured type I collagen (gelatin). The structural features of HMMP that preclude the digestion of these mammalian ECM components is not obvious, but may be related to the structure of HMMPís C-terminal hemopexin domain. Previous studies have shown that the hemopexin domain of MMPs can be important for substrate interactions. For example, MMP-1, MMP-8 and MMP-13 lacking their hemopexin domain lose the ability to cleave native interstitial collagens (Clark and Cawston, 1989; Knauper et al., 1993, 1996). On the contrary, the hemopexin domain is not required for the ability of MMP-2 and MMP-9 to degrade native type IV and V collagen or fibronectin (Murphy et al., 1992a,c; 1994). Future studies involving domain swaps between HMMP and mammalian MMPs will help to clarify this question of substrate specificity.

Another aspect of HMMP substrate specificity relates to the question of the role of the activation process in creating an optimally active mature MMP. Mechanistic studies have shown that the SH group of the cysteine in the cysteine-switch region interacts with Zn^{2+} of the active site to prevent association of the Zn^{2+} ion with a water molecule (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990). During the stepwise activation of the enzyme, the propeptide is finally cleaved at

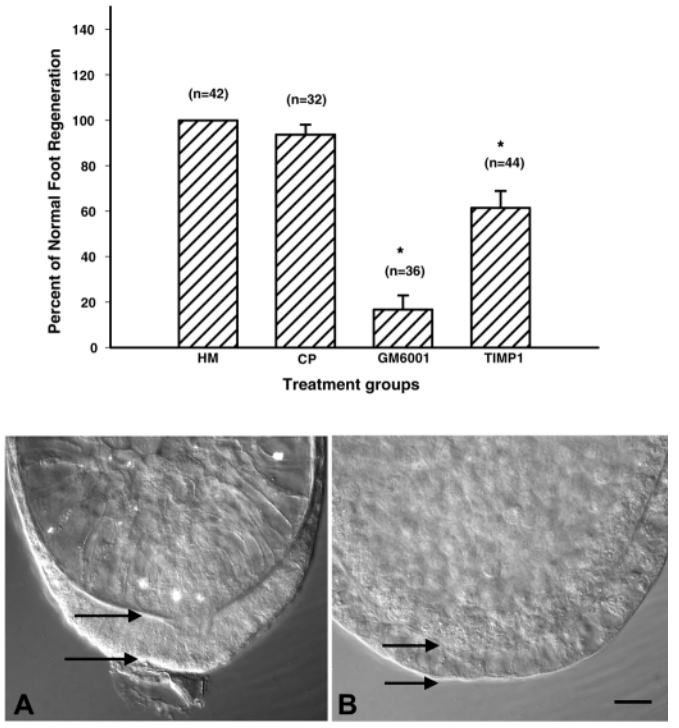

Fig. 8. Effect of MMP inhibitors on hydra foot regeneration in vivo. The foot processes of hydra were surgically removed and the animals were allowed to regenerate in media containing: (1) fresh hydra media (HM); (2) 50 μM control peptide (CP), (3) 50 μM GM6001, or (4) 2.5 μM human TIMP-1. (Upper panel) The fraction of animals that regenerated normal feet within 3 days is shown (± standard errors). Number of animals in each group is given in parenthesis and asterisks indicate experimental groups that are significantly different from control groups at $P<0.05$. (Lower panel) Morphology of basal disc cells as revealed by Nomarski optics. A control hydra is shown in A and a hydra in which foot regeneration was blocked with GM6001 is shown in B. Arrows point to the apical and basal borders of ectodermal cells. Bar in B, 100 μm (applies to A and B).

specific amino acids downstream of the cysteine-switch region (Murphy et al., 1992b; Cao et al., 1998). Microsequence analysis of the N-terminal of recombinant HMMP showed that active recombinant HMMP has an N-terminal end upstream from the cysteine-switch region. This partially processed protein should be considered as an intermediate in the stepwise activation of MMPs (Nagase, 1996). In our experiments, recombinant HMMP was able to cleave not only gelatin, transferrin and synthetic fluorogenic substrate, but also hydra extracellular matrix. This suggests that cleavage at Leu^{81}, four amino acids upstream of the cysteine-switch, can result in an active HMMP enzyme; although we are not certain whether our recombinant HMMP is functioning at its 'maximal' activity level. In this regard, human MMP-1 at intermediate stages of activation, possesses only 15-40% of its maximal activity (Suzuki et al., 1990). Additional studies also suggest that the presence of Phe^{81} at the N-terminal of activated human MMP-1 and MMP-3 is critical for their maximal activity (Crabbe et al., 1992; Abramson et al., 1995; Imai et al., 1995; Benbow et al., 1996) and for substrate specificity of MMP-3 (Benbow et al., 1996). HMMP has a Tyr (Tyr^{103}) substituted for the Phe found at the N terminus of MMP-1 and MMP-3. It is important to note that Phe (or Tyr) is highly conserved as a potential N terminus of active MMPs. Alignment of the catalytic domain of HMMP with other members of the MMP family indicates that Tyr^{103} is an expected N terminus of active HMMP because it is preceded by the potential pro-protein processing proteinase cleavage site, RNRR (amino acid residues 99-102). This suggests that HMMP may be activated intracellularly by a furin-like enzyme as demonstrated for MMP-11 and MT1–MMP (Pei and Weiss, 1995, 1996). It should also be noted, that an N-terminal Phe is present in sea urchin envelysin but is missing in all *C. elegans* and soybean MMPs.

Functional studies indicate that HMMP has a role in foot regeneration and in the maintenance of basal disk cells of adult intact polyps

Previous studies have established that cell-ECM interactions have an important role in the developmental biology of hydra. Initial biochemical and cloning studies determined that hydra ECM has a similar molecular composition to that of other invertebrate and vertebrate organisms (Sarras et al., 1991a,b, 1993, 1994). Functional studies have shown that (1) regenerative processes in hydra are contingent upon and preceded by the de novo biogenesis of ECM (Sarras et al., 1991b, 1993, 1994; Zhang et al., 1994), (2) cell transdifferentiation along the longitudinal axis of hydra can be coupled to cell-ECM interactions (Yan et al., 1995; Sarras, 1996), (3) cell migration events in hydra can involve interactions with ECM components (Zhang and Sarras, 1994), and (4) any perturbation of ECM assembly or cell-ECM interactions results in abnormal morphogenesis of hydra (Sarras et al., 1991b, 1993; Zhang et al., 1994) In addition, pulse-chase studies have also indicated that hydra ECM is in a constant state of turnover (Sarras et al., 1991b); thus indicating that matrix-degrading enzyme systems must be in place in hydra to execute these proteolytic processes. These observations coupled with numerous other studies that have tied MMP action to developmental processes (see review by Werb et al., 1999) lead to the logical conclusion that an important aspect of cell-ECM interactions in hydra involves MMPs. The results of the current study provide substantial support to this proposal. The cloning and biochemical data of this study clearly establish that hydra has at least one MMP that has the functional characteristics of a matrix-degrading enzyme based on in vitro ECM substrate analysis studies and MMP inhibitor studies. Our in vivo studies demonstrated that these same MMP inhibitors reversibly blocked foot regeneration and caused de-differentiation or inhibition of transdifferentiation of basal disk cells in adult polyps just as has been observed for differentiating parietal endoderm cells in the mouse (Behrendtsen and Werb, 1997). The blockage of foot regeneration by antisense thio-oligo nucleotides reinforced the inhibitor data by showing that the same result could be obtained using a separate but convergent approach. The expression patterns of HMMP mRNA in regenerating hydra were consistent with the functional blocking studies and provided additional evidence that HMMP was intimately tied to morphogenesis. The higher levels of expression of HMMP

and laminin mRNA in regions of cell transdifferentiation (foot process and tentacle region) together with the fact that the MMP inhibitor GM6001 caused de-differentiation or inhibition of transdifferentiation of basal disk cells, strongly supports the functional relationship of HMMP and ECM to those mechanisms controlling the regional distribution of differentiated cells along the longitudinal axis of adult hydra.

The precise mechanism(s) by which HMMP effects morphogenesis and cell transdifferentiation are not clear from these studies; however, based on an extensive body of literature it is likely that MMPs in general and HMMP in regard to hydra, interact with the ECM at a number of levels to facilitate these developmental processes (Werb and Chin, 1998; Werb, 1999; Werb et al., 1999; Schwarzbauer, 1999). These modes of action may be divided into three major areas of cell-ECM interaction, which include: (1) the role of ECM as an extracellular structural entity whose assembly and presence affects the three-dimensional shape of tissues, (2) the role of ECM as a polymerized network of macromolecules that have endogenous signaling sequences such as RGD or YIGSR that can interact with cell surface matrix receptors, and (3) the role of ECM as a scaffolding for the attachment of signaling molecules such as growth factors, small peptides, or other signaling compounds.

In regard to the ECM as a structural entity whose assembly and presence contributes to the overall shape of tissues, it is important to note that the entire body wall of hydra is structurally reduced to a simple epithelial bilayer with an intervening matrix. Previous studies have shown that the assembly of ECM is intimately tied to morphogenetic processes in hydra (Sarras et al., 1991b, 1993, 1994; Zhang et al., 1994). Through the use of convergent approaches utilizing a broad spectrum of reagents that affect ECM components (e.g. drugs, blocking antibodies, peptides and protein domains), it is clear that any perturbation of ECM assembly results in a blockage of morphogenesis in hydra (Sarras et al., 1991b, 1993, 1994; Zhang et al., 1994). The co-expression of high levels of ECM components such as the β1 chain of laminin and

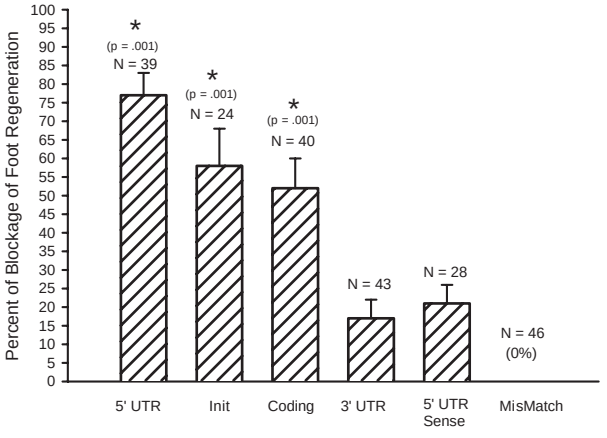

Fig. 9. Effect of antisense thio-oligo nucleotides to HMMP on hydra foot regeneration. The percentage of blockage of foot regeneration is plotted for each antisense or control thio-oligonucleotide tested. *n*, total number of animals monitored. Asterisks indicate groups that were significantly different from controls at $P \leq 0.001$. Experiments were repeated at least three times and standard errors are indicated.

HMMP at specific regions along the longitudinal axis of hydra suggests that the processes of matrix biogenesis and degradation may be coupled in this simple metazoan. Numerous studies from a number of vertebrate systems indicate that MMPs are critical to tissue remodeling processes that are known to involve the reformation of ECM (Werb and Chin, 1998; Werb et al., 1999). This is the case for such normal processes as angiogenesis (Preissner et al., 1997; Sang, 1998) and for such abnormal processes as glomerulosclerosis (Schnaper, 1995) and restenosis of the human coronary artery (Tyagi et al., 1995). In the case of hydra, ECM biogenesis and HMMP expression coincide at regions where the structure of the epithelial bilayer is distinct from other regions of the organism. For example, the foot process represents a region where the epithelial tube narrows relative to the gastric tube proper. Significant changes in cell shape are observed at the distal end of the foot process where the basal disk cells are located and where high levels of laminin and HMMP mRNA are expressed. These structural alterations occur at regions were ECM biogenesis (Sarras et al., 1991b, 1993) and presumably degradation, are occurring. While the precise relationship of HMMP activity to ECM biogenesis is not known at this time, it is notable that inhibition of HMMP activity during foot regeneration did affect the general morphology of the foot process. This raises the interesting question as to what degree HMMP is directly involved with the assembly of matrix components during the biogenesis of hydra ECM.

From another perspective, ECM must be considered as a polymerized network of macromolecules containing an almost infinite array of endogenous signaling sequences. When properly presented, these sequences can interact with cell surface receptors and stimulate signal transduction pathways. The role of MMPs in exposing cryptic signaling sites in the ECM has been reported in a number of vertebrate systems. For example, MMP-2 has been shown to cleave laminin-5 to expose cryptic sequences in this matrix component that then induce migration of mammary epithelial cells (Giannelli et al., 1997). Likewise, exposure of cryptic type I collagen sites by the action of MMP-1 is involved in keratinocyte migration during wound healing (Pilcher et al., 1997). Holliday et al. (1997) has reported that MMP-13 degrades interstitial collagen to produce collagen fragments which then function as 'coupling factors' that activate osteoclasts during bone resorption. In additional, Zhao et al. (1999) report that the stimulation of bone resorption by parathyroid hormone (PTH) is blocked in transgenic mice containing mutations in type I collagen (α1 chain) that prevent degradation of this interstitial matrix component by collagenase. Their studies indicate that the cleavage of type I collagen is necessary for PTH-induced bone resorption. Because the ECM of hydra has a similar composition to that of vertebrate ECM, this opens the potential for similar mechanisms of action by HMMP, which is expressed at high levels at regions along the longitudinal axis of hydra where cell transdifferentiation is occurring.

ECM can also be considered as a scaffolding for the attachment of signaling molecules such as growth factors, small peptides or other non-protein signaling compounds. In this sense, the ECM can be considered a reservoir of soluble

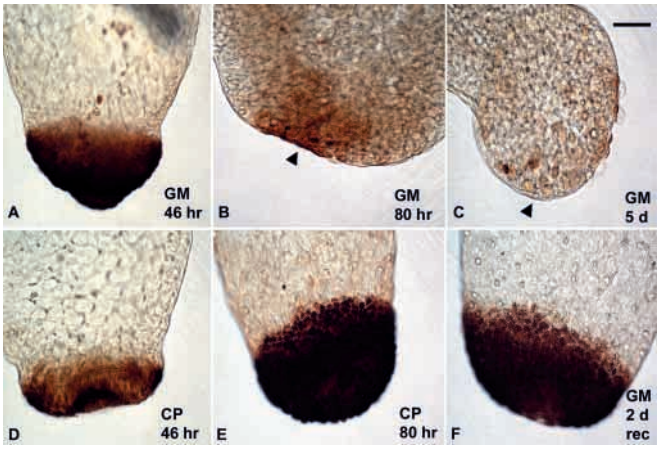

Fig. 10. Effect of MMP inhibitor GM6001 on the maintenance of differentiated basal disk cells as monitored by endogenous peroxidase activity. (A-C) Hydra were incubated in 50 μM GM6001 for 46 hours, 80 hours or 5 days, respectively. (F) Hydra incubated with 50 μM GM6001 for 5 days and then maintained in fresh HM for 2 days. (D,E) Control animals were incubated in 50 μM control peptide for 46 hours or 80 hours, respectively. Bar in C, 100 μm (applies to A-F).

signaling molecules. Proteinases such as MMPs are thought to have an important role in releasing these signaling molecules from ECM components so that cell surface receptors may subsequently be stimulated (see review by Rifkin et al., 1999). In this regard, it has been recently shown that stromelysin and rat collagenase (MMP-3 and MMP-9) can release basic FGF bound to perlecan (Whitelock et al., 1996). Watanabe et al. (1998) have shown that the transmembrane precursor form of TNFα is released by MMPs and that this release is blocked by GM6001. Similarly, Suzuki et al. (1997) have demonstrated that the membrane-anchored precursor of EGF is released by MMP-3 but not by MMP-2 or MMP-9. In this regard, growth factors and peptides have been shown to be involved with pattern formation and cell differentiation pathways in hydra. Thus it is possible that HMMP may act to release potential signaling molecules from the ECM of hydra. The relationship of HMMP to the function of potential gradients of 'morphogens' (Bode and Bode, 1984; Muller, 1996) that provide positional cues along the longitudinal axis of hydra is unclear. These gradients could actually be associated with the ECM which, as stated, could act as a scaffolding for their attachment. Consistent with this proposal is the fact that (1) mammalian growth factors are biologically active in hydra (Yan et al., 1995; Sarras et al., 1997), (2) receptors to growth factors have been cloned in hydra (Steele et al., 1996), (3) a variety of peptides with growth factor-like activity have been identified in hydra (Schaller, 1973; Hobmayer et al., 1997; Schaller et al., 1996; Hoffmeister, 1996; Grens et al., 1999), and (4) astacin-class metalloproteinases have been identified in hydra whose function appears to be related to the processing of ECM-associated proteins that function in morphogenesis and cell-transdifferentiation (Yan et al., 1995, 2000b). When one considers these data with the previously mentioned fact that MMPs have been tied to growth factor release from the ECM, it becomes reasonable to propose that one aspect of HMMP function may involve the activation of latent ECM-associated signaling proteins.

In summary, these findings indicate the MMPs arose early in metazoan evolution. Our studies with HMMP also indicate that structural motifs and functional characteristics of MMPs have also been well conserved during evolution. Based on our expression studies and functional analysis studies we hypothesize that HMMP is a multifunctional enzyme that acts on the ECM at different levels to facilitate morphogenesis and cell transdifferentiation processes in hydra.

It should be noted that the original cloning, sequencing and northern blot analysis of HMMP was conducted by Jinsong Zhang and all other aspects of the study were performed by Alexey Leontovich with in-put from the other co-authors. The authors wish to thank Sharon Dexter for assistance with the antisense studies, Olga Leontovich for assistance with establishing the original conditions for in situ analysis of HMMP mRNA, Drs Linda Frisse and Kelly Thomas of the University of Missouri, Kansas City for assistance with evolutionary tree analysis, and Eileen Roach for assistance with preparation of the figures. This work was supported by NIH grants DK47840 awarded to MPS and AR39189 to H. N.

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