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Identification and characterization of hydra metalloproteinase 2 (HMP2): a meprin-like astacin metalloproteinase that functions in foot morphogenesis

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SUMMARY

Several members of the newly emerging astacin metalloproteinase family have been shown to function in a variety of biological events, including cell differentiation and morphogenesis during both embryonic development and adult tissue differentiation. We have characterized a new astacin proteinase, hydra metalloproteinase 2 (HMP2) from the Cnidarian, *Hydra vulgaris*. HMP2 is translated from a single mRNA of 1.7 kb that contains a 1488 bp open reading frame encoding a putative protein product of 496 amino acids. The overall structure of HMP2 most closely resembles that of meprins, a subgroup of astacin metalloproteinases. The presence of a transient signal peptide and a putative prosequence indicates that HMP2 is a secreted protein that requires post-translational processing. The mature HMP2 starts with an astacin proteinase domain that contains a zinc binding motif characteristic of the astacin family. Its COOH terminus is composed of two potential protein-protein interaction domains: an "MAM" domain (named after meprins, A-5 protein and receptor protein tyrosine phosphatase μ) that is only present in meprin-like astacin proteinases; and a unique C-terminal domain (TH domain) that is also present in another hydra metalloproteinase, HMP1, in *Podocoryne* metalloproteinase 1 (PMP1) of jellyfish and in toxins of sea anemone. The spatial expression pattern of HMP2 was determined by both mRNA whole-mount in situ hybridization and immunofluorescence studies. Both morphological techniques indicated that HMP2 is expressed only by the cells in the endodermal layer of the body column of hydra. While the highest level of HMP2 mRNA expression was observed at the junction between the body column and the foot process, immunofluorescence studies indicated that HMP2 protein was present as far apically as the base of the tentacles. In situ analysis also indicated expression of HMP2 during regeneration of the foot process. To test whether the higher levels of HMP2 mRNA expression at the basal pole related to processes underlying foot morphogenesis, antisense studies were conducted. Using a specialized technique named localized electroporation (LEP), antisense constructs to HMP2 were locally introduced into the endodermal layer of cells at the basal pole of polyps and foot regeneration was initiated and monitored. Treatment with antisense to HMP2 inhibited foot regeneration as compared to mismatch and sense controls. These functional studies in combination with the fact that HMP2 protein was expressed not only at the junction between the body column and the foot process, but also as far apically as the base of the tentacles, suggest that this meprin-class metalloproteinase may be multifunctional in hydra.

Key words: Hydra, Epithelial morphogenesis, Meprin, Astacin, Metalloproteinase

INTRODUCTION

Initial interest in the astacin metalloproteinase family resulted from the ability of two representative members, human Bone Morphogenetic Protein 1 (huBMP1) and *Drosophila* TOLLOID, respectively, to induce ectopic bone formation in adult vertebrates (Wozney et al., 1988), and to regulate axis formation in *Drosophila* embryonic development (Shimell et al., 1991). Recently, more than 20 proteinases sharing the astacin signature, HEXXHXXGFXHEXXRXDR, have been identified from a number of different species with a wide variety of functions during developmental processes (reviewed by Bond and Beynon, 1995; Sarras, 1996). Most of them, namely, sea urchin BP10 (Lepage et al., 1992), SpAN (Reynolds et al., 1992) and suBMP (Hwang et al., 1994), *Drosophila* tolloid-related (Nguyen et al., 1994), mouse BMP1 (Fukagawa et al., 1994), zebrafish tolloid (Blader et al., 1997), *Xenopus* Xolloid (Piccolo et al., 1997) and human mammalian tolloid (mTld) and tolloid-like (Takahara et al., 1994, 1996), resemble the overall structure of huBMP1 and TOLLOID. This common domain structure includes an NH_2-terminal hydrophobic signal peptide, followed by a prosequence, a consensus astacin proteinase domain at the NH_2 terminus of the mature protein, and a variable number of COOH-terminal CUB (complement-Uegf-BMP1) (Bork and Beckmann, 1993) and EGF-like (epidermal growth factor-like) repeats (Davis,

1990). Functional analysis as well as spatial and temporal expression indicated that these proteinases are developmentally important at different embryonic stages. Genetic and biochemical studies have linked astacins to the signaling pathway of members of the transforming growth factor β (TGFβ) superfamily of growth factors (Wozney et al., 1988; Ozkaynak et al., 1992; Finelli et al., 1994; Blader et al., 1997). Most recently, the astacin metalloproteinase, huBMP1 has been shown to be identical to type I procollagen C-proteinase (Kessler et al., 1996), which is known to be involved in processing type I collagen. This finding suggests that huBMP1 is capable of modifying extracellular matrix (ECM) biosynthesis and thus can influence cell events by its effects on cell-matrix interactions. Taken together, these astacin metalloproteinases are multifunctional proteinases that play a central role in regulating interactions among cells, growth factors and the ECM.

Meprins are a subgroup of the astacin metalloproteinases that are distinguished from other members of the family by the presence of a unique COOH-terminal MAM domain (domain name derived from the proteins: meprin, Xenopus A5 and protein tyrosine phosphatase “Mu”) (Beckmann and Bork, 1993), and the absence of the CUB domain (Jiang and Bond, 1992; Bork and Beckmann, 1993; Bond and Beynon, 1995). These cell surface proteinases were isolated from kidney brush border membranes and from the small intestine of rodents (Butler et al., 1987; Jiang et al., 1992; Johnson and Hersh, 1992; Corbeil et al., 1992; Gorbea et al., 1993), as well as from human tissues (Dumermuth et al., 1993). Although it has been shown that meprins have a wide spectrum of substrates, including bradykinin, insulin B chain, glucagon, TGFα, collagen and azocasein (Butler and Bond, 1987; Choudry and Kenny, 1991; Wolz et al., 1991; Wolz and Bond, 1995), the potential biological functions of these enzymes in vivo are still unclear.

Our laboratory has utilized a unique epithelial animal model, the Cnidarian, *Hydra vulgaris*, to approach this problem. Hydra is one of the earliest divergent forms of existing multicellular organisms and has a simple body structure consisting of a head, a body column and a foot process. Its body wall is composed of an epithelial bilayer (outer ectoderm layer and inner endoderm layer) with an intervening ECM that has molecular components similar to those seen in higher invertebrates and vertebrate species (Sarras et al., 1991, 1994). In addition, hydra has unique cell dynamics in which epithelial cells in the body column divide continuously, thus causing subsequent differentiated daughter cells to be displaced into the head and the foot regions. Once these cells move across the junctional areas between the body column and the head, or between the body column and the foot process, they transdifferentiate into cell types that are specific to the head or the foot regions, respectively (Bode et al., 1986). This process has been shown to be controlled by positional signals along the longitudinal axis of hydra (Bode and Bode, 1984; Javios, 1992). Previous studies have shown that, as a consequence of this extensive cell turnover and continuous cell transdifferentiation, hydra is highly regenerative (Bode and Bode, 1984; Javios, 1992; Sarras et al., 1993). The simple structure and unique cellular dynamics of hydra make the organism an ideal model for studying the role of cell-cell and cell-matrix interactions during developmental processes.

In the current study, we report the molecular characterization of a meprin-like astacin metalloproteinase, HMP2, from *Hydra vulgaris*. Because higher levels of mRNA expression were observed at the basal pole of adult hydra, functional studies were conducted to determine if HMP2 was involved in processes related to foot morphogenesis. In these functional studies, foot regeneration was specifically blocked by local administration of antisense thio-oligo constructs into endodermal cells at the basal pole of the organism. The results demonstrated that one function of HMP2 involves cellular processes related to foot morphogenesis in hydra.

MATERIALS AND METHODS

Molecular cloning and sequence analysis

A *Hydra vulgaris* λZAPII cDNA library was constructed using a mixture of oligo(dT) and random primers (Sarras et al., 1994) and the library was screened to isolate cDNA clones encoding novel hydra metalloproteinases. 1×10^6 clones were screened by α^{32}P randomly labeled cDNA fragments corresponding to the astacin domain of hydra metalloproteinase 1, HMP1 (Yan et al., 1995). Sequence analysis indicated that one of the positive clones, HMP53, contained an 802 bp region that gave a conceptional translation including a distinct astacin proteinase domain. The corresponding cDNA was named HMP2. To obtain the full-length cDNA of HMP2, a PCR primer, HMP2JUNC2, was designed based on the sequence obtained from the 3′-region of HMP53, (5′-CAGAGCATGATC-AGTATCAA-3′). This primer was then used in pair with a T7 primer to amplify the rest of HMP2 sequence from an oligo(dT) primered UNI-ZAP cDNA library of *Hydra vulgaris*. The PCR product was subsequently cloned into PCR II TA-cloning vector (Invitrogen, CA) for DNA sequencing.

cDNA sequencing was performed using both manual sequencing (USB, Ohio) and automated methods (Biochemical Supporting Facility, University of Kansas Medical Center). DNA sequence analysis, as well as protein analysis including hydrophilicity plot analysis was conducted using the MacVector 5.0 DNA sequence analysis software package (Kodak, NY). Multiple sequence alignment was performed at a GCG workstation (University of Wisconsin). Database searches were assessed at the NCBI using the BLAST network service.

Northern and Southern analysis

The proteinase domain of HMP2 spanning from 190 bp to 786 bp was amplified by PCR. The PCR product was subcloned into the TA-cloning vector (Invitrogen, CA) and was random primer-labeled with α^{32}P-dCTP using a NE Blot Kit (New England BioLabs, MA). Total RNA was isolated from 2 day starved hydra as previously described (Sarras et al., 1994). After separation by 1.0% agarose electrophoresis containing formaldehyde, RNA was transferred to nylon membranes (S&S, NH). A northern blot of 5 μg *Hydra vulgaris* total RNA was hybridized overnight at 65°C with the radiolabeled probes at a concentration of 2×10^7 cts/minute/10 ml. The blot was washed to a final stringency of 0.1× SSC and 0.1%SDS at 65°C.

For Southern analysis, genomic DNA was isolated from *Hydra vulgaris* according to Sambrook et al. (1989), and digested with *Eco*RI, *Hin*dIII or *Bam*HI. 10 μg of digested genomic DNA was separated by 1% agarose gel electrophoresis and transferred onto S&S Nytran membranes. Hybridization was carried out under the same conditions as described for northern analysis.

In situ hybridization

As described earlier, the proteinase domain of HMP2 was PCR amplified and subcloned into PCR II TA-cloning vector. T7 or SP6

RNA polymerases were used to make either sense or antisense digoxigenin(DIG)-labeled ribonucleotide probe (Ambion, TX). In addition, using these same procedures, a second probe was developed that included the proteinase and MAM domains.

Whole-mount in situ hybridization of hydra was performed as previously described (Grens et al., 1995). Analysis was performed on intact adult polyps and on hydra in which foot regeneration was initiated. For analysis of regeneration, the foot process was excised at the apical region of the peduncle. For the purposes of this study, the foot process is defined as the basal disk cells and the peduncle that represents the narrowed region of the body between the basal disk cells and the gastric tube proper. Regenerating hydra were collected at various time points following excision of the foot process (indicated in the results section), fixed and processed for whole-mount in situ analysis. Briefly, 2 day starved hydra were fixed overnight in 4% paraformaldyhyde followed by processing in 10 μg/ml proteinase K to increase tissue permeability and heat treated at 80°C for 30 minutes to eliminate endogenous alkaline phosphatase activity. Hybridization was carried out in 100 μl hybridization buffer (50% formamide, 5× SSC, 200 μg/ml yeast transfer RNA, 0.1% Tween 20, 1% CHAPS, 1× Denhardt's solution and 100 μg/ml heparin) containing 40 ng DIG-labeled RNA probes, at 60°C overnight. After extensive washing, anti-DIG antibodies conjugated with alkaline phosphatase were used to localize mRNA. Color development was carried out using BM-purple under conditions recommended by the manufacturer (Boehringer Mannheim, IN).

Preparation and characterization of anti-HMP2 antibodies

(a) Design of a multiple antigenic peptide (MAP) and preparation of anti-HMP2 antibodies

cDNA-deduced amino acid sequence of HMP2 was analyzed using Protein Analysis Toolbox to select a region with both a high surface probability and a high antigenic index (MacVector 5.0). A multiple antigenic peptide (MAP) was generated based on a 11 residue region in the MAM domain of HMP2, with sequence SKSEPNNDWKN (Biomedical supporting facility, KUMC). Antibodies against this MAP were generated in rabbits according to standard procedures (Sarras et al., 1994). Rabbit antibodies generated against mouse meprin A was kindly provided by Dr Judy Bond (Pennsylvania State University, Hershey, PA).

(b) Western blot of HMP2 antibodies

Proteins from sonicated *Hydra vulgaris* were resolved on SDS-PAGE, 68 μg/lane and transferred to Nitrocellulose membranes (Bio-Rad, CA), as previously described (Sarras et al., 1994). After preincubation in TTBS buffer (0.2% Tween-20, 10% Fetal Bovine Serum, 1% BSA in PBS) for 30 minutes, the membranes were incubated for an another 30 minutes at room temperature in a 1:500 dilution of anti-HMP2MAP antiserum. After three washes in PBS, membranes were incubated in a 1:5000 dilution of a secondary antibody conjugated with alkaline phosphatase (Promega, WI). Protein bands were visualized with BM-purple (Boehringer Mannheim, IN). Preimmune serum was used as a negative control.

(c) Immunocytochemical localization of HMP2

Immunofluorescent staining was carried out as previously described (Yan et al., 1995). Briefly, 2 day starved hydra were relaxed in 2% urethane for 2 minutes at room temperature and fixed in Lavdowsky's fixative at 4°C overnight. After blocking nonspecific epitopes with 1% BSA, 10% FBS in PBS, hydra were incubated in a 1:200 dilution of primary antibodies for 1 hour at RT. Protein localization was detected by secondary antibodies conjugated to FITC (ZYMED, CA). The same dilution of the preimmune serum was used as a negative control.

Functional analysis of HMP2 using localized electroporation (LEP) and thio-oligo antisense constructs

Because transfection approaches have not been successfully applied to Cnidarians, we developed a procedure to test specifically the effect of antisense constructs on head or foot regeneration in hydra. This approach was developed based on discussions with Dr Hans Bode (Developmental Biology 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 LEP procedure initially outlined by H. Bode (personal communication), we tested the hypothesis that HMP2 was involved in processes related to foot morphogenesis. Based on the work of Dr Richard W. Wagner (Wagner, 1994, 1995; Flanagan et al., 1996) a series of 20-mer oligonucleotides with phosphorothioate linkages were designed. Six oligonucleotides were synthesized to include four antisense sequences to portions of the 5′ UTR, initiation site, coding sequence and 3′ UTR, a sense strand of the 5′ UTR, and a mismatch construct. The oligonucleotides were introduced into hydra cells using electroporation (BioRad Gene Pulser) with a micropipette drawn on a pipette puller. The ends of the micropipettes brought in contact with the hydra were polished with a microforge. Because HMP2 is expressed in the endoderm layer of cells, it was necessary to bisect the basal pole of the hydra to expose the inner layer of cells lining the gastric cavity. Because foot regeneration was being investigated, the foot process was surgically removed just prior to electroporation. To maximally retain the oligonucleotides within the area where the electroporation was performed and also to reduce movement of the animal during LEP, hydra were chilled in a 10% heptanol solution (heptanol, in hydra media) for a maximum of 1 hour. A 100 μM stock solution of 20-mer thiolated oligonucleotide (Genset Corp, www.genset.fr/cgi/gorders.pl) was mixed with FITC-dextran 10,000 M_r (Molecular Probes, Eugene, OR) in a ratio of 3:1 (typically 6 μl of DNA + 2 μl of FITC-dextran). After fitting the micropipette over the microelectrode (World Precision Instruments, Inc), the oligonucleotide/FITC-dextran mixture was loaded into the micropipette. The BioRad gene pulser was set at 100 ohms, 25 μFD, 50 V and the average time for the pulse was 3.6 msec. Hydra were placed on a Nytex net attached to a plastic Petri dish with soft wax. The dish was placed on an incline to facilitate positioning of the micropipette. The micropipette was placed in contact with the endoderm at the cut basal pole of the hydra. After charging the gene pulser to 50 volts, the pulse was initiated for a 3.6 msec period. An additional pulse could be applied if the width of the endoderm area at the cut pole was greater than the micropipette diameter. The hydra were then placed in hydra media and, at 24 hours, all animals were screened on a Leitz fluorescent microscope to ensure that each specimen had retained the DNA/FITC-dextran mixture at its regenerating basal pole. As a general note, we found that experiments involving head regeneration (not part of the present study) varied from this procedure. In this case, decapitation had to be performed at 2-4 hours when the fluorescent screening was initially conducted. This modification was necessary because, if decapitation was performed prior to electroporation, an extensive loss of electroporated cells would occur at the cut edge of the apical pole. To ensure that the DNA/FITC mixture was retained in cells at the apical pole after decapitation, electroporation was performed with multiple pulses on the endoderm or ectoderm (depending on the gene being studied), just inferior to the mouth region to make sure that a more extensive area of the apical pole received the construct. In this way, cells with the DNA/FITC mixture constituted the apical pole following decapitation. Electroporated hydra were observed every 24 hours and the degree of regeneration was compared to mock electroporated controls. In the current study, animals were cut in the lower body column just apical to the peduncle and then utilized for LEP. 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 mucous by placing a glass probe against the basal pole of the regenerating hydra and monitoring cells adhesion 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. In controls, foot regeneration is normally completed within 72 hours and, therefore, experimental groups in which blockage was observed were monitored for an additional 5 days to determine if recovery from blockage occurred. Controls animals were treated with mismatched thio-oligo 20-mer constructs (randomized sequence) or sense constructs if a particular antisense construct was found to block morphogenesis. Control and experimental groups were statistically compared using an ANOVA test. Lastly, the effect of antisense thio-oligonucleotides on the translation of a putative HMP2 molecule in the basal pole was monitored using [^{35}S]methionine incorporation studies coupled with immunoprecipitation and SDS-PAGE as previously described by Sarras et al. (1993).

RESULTS

Cloning and sequence analysis of hydra metalloproteinase 2 (HMP2)

By screening a λZAP II cDNA library of *Hydra vulgaris* (Sarras et al., 1994) using the proteinase domain of hydra metalloproteinase 1 (HMP1) (Yan et al., 1995), a cDNA fragment, HMP53, of 802 bp was isolated. Sequence analysis revealed a putative protein product containing a novel astacin metalloproteinase domain that was distinct from HMP1. The conceptional protein encoded by this clone was designated hydra metalloproteinase 2 (HMP2), in order to distinguish it from the previously identified HMP1. Clone HMP53 contains a signal peptide, a prosequence, and an astacin proteinase domain, which were confirmed by later cloning and sequencing studies. The COOH-terminal region of this protein was PCR cloned from a UNI-ZAP cDNA library of *Hydra vulgaris* as described in the Materials and Methods. Nucleotide sequence of the entire 1723 bp HMP2 cDNA (GenBank Accession No. AF140020)

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                                                                ggtgctta tattttagaa
   1  ATGACGAAAC TGGTTTTGAT TTTGCTTTCT GTTGCTCTGT GCCACTCTTT CCCTGAGGAA
        M  T  K  L  V  L  I  L  L  S  V  A  L  C  H  S  F  P  E  E   20
  61  GAAAATAATG ATCCTCAAGG TATTTTGTTT GGTGGTGACA TTTTACTTAC ACCAGAACAA
        E  N  N  D  P  Q  G  I  L  F  G  G  D  I  L  L  T  P  E  Q   40
 121  AAATCCATAA TTGAAGTTGG TGGAGATATT AGTCAAGCTG GACTACTCAA GACATTAAGA
        K  S  I  I  E  V  G  G  D  I  S  Q  A  G  L  L  K  T  L  R   60
 181  CAAAAACGTG CTGCTTTGAG TAATTCGAGT ATATTGTGGT TACCCAACAA CAAGGTGGTT
        Q  K  R  A  A  L  S  N  S  S  I  L  W  L  P  N  N  K  V  V   80
 241  CCATGGAGTA TTACAAAACA GTTAGAAAAT ACTGCTGAAG CTACATTCGG ATTAATGGCA
        P  W  S  I  T  K  Q  L  E  N  T  A  E  A  T  F  G  L  M  A  100
 301  GCTTTTAGAG AATGGGAAGA AAGATCTTGT CTCACATTCA AAAGAAGAAC AGATGAGAAG
        A  F  R  E  W  E  E  R  S  C  L  T  F  K  R  R  T  D  E  K  120
 361  GATTACATAG AGTTTTTTCA AGGTAGTGGT TGTTGGAGTT ATCTGGGAAG AGTAGGTGGG
        D  Y  I  E  F  F  Q  G  S  G  C  W  S  Y  L  G  R  V  G  G  140
 421  CTTCAAAACA TTTCTTTAGA TGATGGCTGT TGGGGAAAGG GGACCATTGT TCACGAAATA
        L  Q  N  I  S  L  D  D  G  C  W  G  K  G  T  I  V  H  E  I  160
 481  GGTCATGCTA TGGGATTTGG ACATGAGCAA AATCGTCCAG ATAGAGATCA GTATATTACT
        G  H  A  M  G  F  G  H  E  Q  N  R  P  D  R  D  Q  Y  I  T  180
 541  ATTCGATGGG AAAACATTCC TGAATCTAAG AAGCACAACT TCAGATTGTA TTCCAACTCC
        I  R  W  E  N  I  P  E  S  K  K  H  N  F  R  L  Y  S  N  S  200
 601  TTAGTTGACA GCCTCAACTC ACCATATGAT TATAGAAGTT ATATGCAATA TTCTAAAACT
        L  V  D  S  L  N  S  P  Y  D  Y  R  S  Y  M  Q  Y  S  K  T  220
 661  GCATTTGGAA TTAATGACTC AGTAACATTA GATCCTAAAC TTCCTGGTAT TTTCCAGCTT
        A  F  G  I  N  D  S  V  T  L  D  P  K  L  P  G  I  F  Q  L  240
 721  GGTCAAAGAG TTGGATTTAC AGAGCATGAT CAGTATCAAG CCATGCAGCT TTATCGATGT
        G  Q  R  V  G  F  T  E  H  D  Q  Y  Q  A  M  Q  L  Y  R  C  260
 781  CAAGGTAAAA CGACTCGCCC AACAACTTTT TTTCCAAGAT ACGATCTGAA AGGTGATTAT
        Q  G  K  T  T  R  P  T  T  F  F  P  R  Y  D  L  K  G  D  Y  280
 841  ACTTGTGATT TTGAAACTGA TTTGTGTGGG TTTACTCATG ATAAAACTGC AACTTTTGAA
        T  C  D  F  E  T  D  L  C  G  F  T  H  D  K  T  A  T  F  E  300
 901  TGGGCTCAAA TTTATGGTGA AACACCTTCT AGAGGAACTG GACCCGATTC TGATCATACT
        W  A  Q  I  Y  G  E  T  P  S  R  G  T  G  P  D  S  D  H  T  320
 961  ACTGGAAGAC TAGGAACCTA TGTATATATT GAAGCATCCT ATCCACAAAA GAAAAATGAT
        T  G  R  L  G  T  Y  V  Y  I  E  A  S  Y  P  Q  K  K  N  D  340
1021  AAAGCTCGTC TTAAAACAAA ATGGTTTGAA AAACCATTAT CTGCACAATG TTTCTCTGTT
        K  A  R  L  K  T  K  W  F  E  K  P  L  S  A  Q  C  F  S  V  360
1081  TTTTACAACA TGTTTTCTCG AAATGCTTCA ATGGTTGGTG AATTTAATAT TTATATTGAT
        F  Y  N  M  F  S  R  N  A  S  M  V  G  E  F  N  I  Y  I  D  380
1141  GACACAGTAA CAATAAAAAA TATCTTTTCT CAATCAAAGA GTGAGCCAAA TAATGATTGG
        D  T  V  T  I  K  N  I  F  S  Q  S  K  S  E  P  N  N  D  W  400
1201  AAAAACTTAT TAATTAATAT TAAACCCGAA GGTCCTTATC AGGTTATATT TGAAGGTATT
        K  N  L  L  I  N  I  K  P  E  G  P  Y  Q  V  I  F  E  G  I  420
1261  ATTGGTAATG GATGGCAAGG TGATATTGCT ATTGACGATA TAAGTATAAC AGCTGGATAT
        I  G  N  G  W  Q  G  D  I  A  I  D  D  I  S  I  T  A  G  Y  440
1321  TGTCCAACAA ATATCGAAAT TTCTGACAAT TTCTCAAATG ACTCAAGTTC GTGTGAAGAT
        C  P  T  N  I  E  I  S  D  N  F  S  N  D  S  S  S [C  E  D  460
1381  ATAAATGATA GAGAAGCTGA ATATTGTCGT CAATGGGAAC AAGCGGGGTA TTGTGTAAGC
        I  N  D  R  E  A  E  Y  C  R  Q  W  E  Q  A  G  Y  C  V  S  480
1441  GAAGAAAAAA CTATGAAGTT GTATTGTAGA AAGACATGTA ATTTTTGCTA Aatgttaaat
        E  E  K  T  M  K  L  Y  C  R  K  T  C  N  F  C] *           496
1501  gttaaatgac ttttttttcat atttattttt aaggttatcc aaaattttgc agttgtttat
1561  ttcaataata aactgactat agtaagttat gttactgcag taaccaatta acagtaagtt
1621  atgttactat cggttatcgt tgtataacca tagaattata attttatgat caaacagtat
1681  ttttaaaaaa aaaaaaaaaa aaaaa
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Fig. 1. cDNA and deduced amino acid sequence of hydra metalloproteinase 2 (HMP2). The 1723 bp HMP2 cDNA has an open reading frame of 1488 bp, from the conceptional translational start codon ATG (Met) to the stop codon TAA (*) (double underlined and in bold), encoding a protein product of 496 amino acids. The first 63 amino acids represent the signal sequence and the proregion which are presumptively processed by unknown proteinase activities. The potential proteolytic cleavage site for releasing the mature protein is identified by an arrow between Arg^{63} and Ala^{64}. The mature HMP2 is composed of an astacin proteinase domain, and two COOH-terminal protein-protein interacting domains: an MAM domain (Lys^{263} to Ser^{457}) and a Cys-rich terminal domain (TH domain) (Cys^{458} to Cys^{496}). The sequence Ala^{64} to Gly^{262} representing the astacin domain is in bold and italic type. The Cys-rich region is marked by brackets. Potential N-glycosylation sites are underlined.

was obtained by both manual and automated DNA sequencing (Fig. 1). It contains an 18 bp 5′-untranslated region (UTR) and a 217 bp 3′-UTR, which ends with a poly(A) tail. A 1488 bp open reading frame encodes a 496 amino acids protein product, including a translational starting methionine and a 3′-stop codon. Hydrophilicity plot analysis of this sequence revealed a 21-residue hydrophobic region located at the NH_2-terminus, which most likely represents a cleavable transient signal peptide (von Heijne, 1983) as found in most members of the astacin metalloproteinase family (Jiang and Bond, 1992) (Fig. 3B). The signal peptide was followed by a putative prosequence which is presumptively removed by hydrolysis of the R-63-A-64 bond at a consensus RXXR proteolytic processing site (Steiner et al., 1992). The resulting mature HMP2 proteinase has 433 amino acid residues with a calculated molecular mass of 54.5×10^3, which closely resembles a group of three gelatinase activities with intermediate molecular sizes from 51×10^3 to 58×10^3 M_r that were previously identified from *Hydra vulgaris* (Yan et al., 1995). There are six potential N-glycosylation sites distributed in the whole sequence of mature HMP2. Two of the three sites located within the astacin proteinase domain are well conserved in the corresponding regions of mouse meprin α subunit. It has been reported that different glycosylation modifications of meprin α subunit could account for its multiple forms with different molecular sizes (Jiang et al., 1992). These studies indicate that, while no glycosylation of the proteinase domain has been detected, evidence for glycosylation of the MAM domain does exist. Therefore, a similar situation may explain the three intermediate gelatinase activities observed in our previous studies (Yan et al., 1995).

A Blast Genbank database search revealed that HMP2 is most homologous to the meprin α and β subunits of different species, including mouse, rat and human (Jiang et al., 1992; Gorbea et al., 1993; Johnson and Hersh, 1992; Corbeil et al., 1992). Multiple alignment analysis of HMP2 with mouse meprin α and β chain demonstrated a well-conserved primary structure as shown in Fig. 2A. The overall identity of HMP2 at the protein level is about 30% as compared with

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            1          Signal Peptide       ▼         Pro-region        65
MepAα.mou   M.ARrlgRss sfaiMlWIqp AcLLSliFsa hiaAvsiKh. ...LLnGsdh Dt.DvGEQKD IFEIN
MepAβ.mou   MdARhqpW.. ...fLVF... AtFL...Las gLPApE.K.. ...Fvk.... DI.DgGidqD IFDIN
HMP-2.hyd   .......... .mtkLVLI.. ..LLSvaLch sFPeeEnndp qgiLFgG... DIlltpEQKs IiEV.
Consensus   M-AR------ ----LV-I-- A-LLS--L-- PA-E-K---- ---L--G--- DI-D-GEQKD IFEIN
            66    Pro-region   ▼                        Astacin Domain      130
MepAα.mou   laaGLnLFQG D.I.LLprtR NAMrdpSs.R Wkl..pIPYI LaDnLELNAK G..aILhAFE mFRLK
MepAβ.mou   qglGLDLFeG D.IKLeangk Nsiigdhk.R WPh..tIPYV LeDsLEMNAK G..vILnAFE rYRLK
HMP-2.hyd   ...GgDisQa gllKtLrqkR aALsnsSilW lPnnkvVPWs itkqLEntAe atfglMaAFr eWeer
HMP-1.hyd                         ...kgvtldr iwpgaqipyv fdnnfdnrrr ql..vnqAit synqh
Consensus   ---GLDLFQG D-IKLL---R NA----S--R WP----IPY- L-D-LE-NAK G---IL-AFE --RLK
            131                    Astacin Domain                           195
MepAα.mou   SCVDFKPYeG EssYI.IFQK lSGCWSmIGd qqvG.QNISI GEGCDF.KAT IeHEILHALG FFHEQ
MepAβ.mou   tCIDFKPWsG EanYIsVF.K GSGCWSsVGn IhaGkQelSI GtnCDR.iAT VqHEfLHALG FWHEQ
HMP-2.hyd   SCltFKrRtd EkdYIefFQ. GSGCWSylGr V.gGlQNISl dDGC.WgKgT IvHEIgHAMG FgHEQ
HMP-1.hyd   tCvrFvprtnqrdfvhmvneq ..GCWSiiGr qklklgdw      Cldiga  I.HEMMHAAG FyHEQ
Consensus   SC-DFKP--G E--YI--FQK GSGCWS--G- ---G-QNISI G-GCD--KAT I-HEILHALG F-HEQ
                                                                     (Astacin
            196                    Astacin Domain                           260
MepAα.mou   SRtDRDDYVn IWWDqIitdy EHNFNtYdDN tItD.LNtPY DYeSlMHYgp fsF.NkNESI pTItT
MepAβ.mou   SRaDRDDYVi IvWDrIqpgK EHNFNiYnD. SVsDSLNvPY DYtSvMHYSK TAFqNgtES. .TIvT
HMP-2.hyd   nRpDRDqYIt IRWEnIpesK kHNFrlYs.N SlvDSLNsPY DYrSyMqYSK TAF.giNDSV .Tldp
HMP-1.hyd   SrnDRDDhvv ifWnnIqrge ynfqk.Yr.. teyyqqty.. DhnSIMHYrn deFsrngkn...TIqa
Consensus   SR-DRDDYV- I-WD-I---K EHNFN-Y-DN S--DSLN-PY DY-S-MHYSK TAF-N-NES- -TI-T
            Signature)                                (Met-Turn)
            261   Astacin Domain            ▼          MAM Domain           325
MepAα.mou   KIPe.FntII GQlpDFSaiD LirLNrMYNC ....TathTL L.......D. hCDFEkTNVC GMIQg
MepAβ.mou   rIPr.FedVI GQRmDFSDyD LlkLNQLYNC ....TsslsF M.......D. sCDFEleNIC GMIQs
HMP-2.hyd   KlPgiFq..l GQRvgFtEhD qyqamQLYrC qgktTrptTF FprydlkgDy tCDFE.TdlC Gfthd
HMP-1.hyd   Knnpgldlgn nq...FSksD iaaiNQmYkC sgvt
Consensus   KIP--F---I GQR-DFS--D L--LNQLYNC ----T---TF --------D- -CDFE-TN-C GMIQ-
            326                    MAM Domain                               390
MepAα.mou   trDdADWAhg dS...S...q PEqvDHTlve QCKgaGYFMF FNTSlga.rG EaALLESRiL YPKR.
MepAβ.mou   sgDsADWqrV .SqvlS...G PE.SDHskmG QCKdsGFFMh FNTSiln.eG atAMLESRlL YPKR.
HMP-2.hyd   ktatfEWAqI ygetpSrgtG PD.SDHT.tG r...lGtYvY ieaSypqkkn DkArLktk.w FeKpl
Consensus   --D-ADWA-- -S---S---G PE-SDHT--G QCK--G-FM- FNTS-----G --A-LESR-L YPKR-
            391                    MAM Domain                               455
MepAα.mou   kqQCLq.FF. .YkmtGSpaD rFeVWVrRDD naGkvrQlak IqTFQgdSdh ....nWKiah VTLne
MepAβ.mou   gfQCLe.FY. LYn.sGSgnD qLNIYt.REy TtG...Qqgg VlTLQrQiKe vPigsWqlhy VTLqv
HMP-2.hyd   saQCFsvFYn MFsrnaSmvg eFNIYI..DD Tvt....... IknifsQSKs ePnndWKnll Inikp
Consensus   --QCL--FY- -Y---GS--D -FNIY--RDD T-G---Q--- I-T-Q-QSK- -P---WK--- VTL--
            456                    MAM Domain            505
MepAα.mou   EKKFRyVFlG tkGdPGnSSG GIylDDItLT ETpCPagVWt IrNISQILeN
MepAβ.mou   tKKFRVVFEG lrG.PGtSSG GlsIDDInLs ETrCPhhIWh IqNftQILgg
HMP-2.hyd   EgpYqVIFEG iiGn.G.wqG dIaIDDIsiT agyCPtnI.. ..eISdnFsN
Consensus   EKKFRVVFEG --G-PG-SSG GI-IDDI-LT ET-CP--IW- I-NISQIL-N                 A
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Comparison of Cys-rich domain of HMP-1 and HMP-2

```
HMP-1  CED....shsnCaaWakAneCnknpnwMrpnCkKsCgtC
       :::     . . : .:..:. : .. . :.  :.:.:. :
HMP-2  CEDindreaeyCrqWeqAgyCvseektMklyCrKtCnfC              B
```

Fig. 2. Multiple alignment analysis of the cDNA-deduced amino acid sequences from hydra HMP2, hydra HMP1, mouse meprin Aα and Aβ. (A) Sequences of the signal peptide, pro-region, the astacin domain and the MAM domain are aligned using GCG 'pileup' multiple sequence analysis software. A consensus sequence is also shown. The conserved amino acid residues of HMP2 are shown in capital case and bold type. The astacin signature sequence, HEXXHXXGFXHEQXRXDR, is double underlined. The characteristic Met-turn, SXMXY, is underlined. HMP2-MAP sequence SKSEPNNDWKN (position 392 to 402) is shown in shadow. Abbreviations: HMP2, hydra metalloproteinase 2 (AF140020); MepA.mou, mouse meprin A chain subunit (gi 2827776); MepA.mou, mouse meprin A chain subunit (gi 2499913); HMP1, Hydra Metalloproteinase 1 (catalytic domain; gi 726483). (B) Comparison of the C-terminal domain (TH domain) of HMP1 and HMP2. Strictly conserved cysteine residues are shown in bold. HMP1, hydra metalloproteinase 1 (gi 726483).

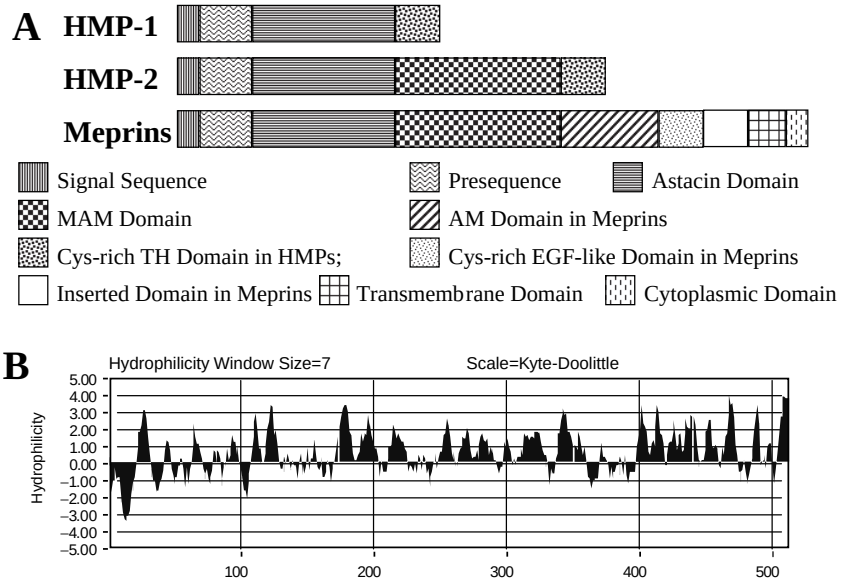

Fig. 3. Domain structure and hydrophilicity plot analysis of hydra metalloproteinase 2 (HMP2). (A) Domain structure of hydra metalloproteinase 2 (HMP2) is shown together with that of HMP1 (catalytic domain and C-terminal domain shown) and meprin. There is a putative signal sequence at the NH_2 terminus of all of these astacin metalloproteinases, which is followed by a relatively conserved pro-sequence of about 35 amino acids. The homologous proteinase domains are aligned. In HMP2 and meprins, a MAM domain, which was proposed to function in protein-protein interaction, was found between the proteinase domain and the C-terminal cys-rich domain (TH domain). However, the MAM domain of HMP2 does not exist in HMP1. No transmembrane domain or cytoplasmic domain were found in HMP1 or HMP2, suggesting that these two proteinases are secreted into extracellular milieu post-translationally. The domain structure of meprin is summarized from sequence data of mouse and rat meprin subunit α and β. (B) Hydrophilicity plot analysis of HMP2. Hydrophilicity of HMP2 was analyzed using Protein Analysis Toolbox provided by Macvector 5.0 (Kodak, NY). The plot was generated according to Kyte-Doolittle method with a window size of 7 amino acid residues. Overall, HMP2 is a very hydrophilic protein except for the first 20 residues at the NH_2-terminus. This hydrophobic region most likely represents a signal sequence, indicating that HMP2 is a secreted protein.

mouse meprin α and β subunits. As in other astacin members, there is little conservation in the NH_2 termini, except they all share a hydrophobic region, the putative signal peptide. The prosequences of these proteinases are relatively more homologous. The NH_2 terminus of the mature HMP2 begins with an astacin proteinase domain that is composed of 199 residues, starting at A^{64} and ending at G^{262}. It contains a conserved zinc-binding motif HEXXHXXGFXHEXXRXDR, (HEIGHAMGFGHEQNRPDR in HMP2), which is characteristic for all metalloproteinases of the astacin family (Sarras, 1996). The astacin domain is followed by a segment of 200 residues that is homologous to a newly identified MAM domain (Beckmann and Bork, 1993). This domain is also present in a group of proteins with diverse functions, namely, meprin α (Jiang et al., 1992) and meprin β (Gorbea et al., 1993), the A5 protein of *Xenopus laevis* (Takagi et al., 1991) and the extracellular domain of receptor protein tyrosine phosphatase μ (Gebbink et al., 1991; Jiang et al., 1993). The COOH terminus of mature HMP2 is composed of a Cys-rich region (Cys^{458} to Cys^{496}) that closely resembles the similar region found in HMP1. All six Cys residues in this 39-amino acid stretch were conserved and the overall homology is 30.8% (Fig. 2B). This domain has been reported in sea anemone toxins specific for potassium plasma membrane channels and in a jellyfish astacin-class metalloproteinase, PMP1 (Pan et al., 1998). The exact function of this region in either HMP1 or HMP2 is currently unclear. Like other meprins, HMP2 lacks the COOH-terminal CUB domain (Bork and Beckmann, 1993), which is commonly found in other astacin metalloproteinases. A comparison of the overall domain structure of HMP2, HMP1 and meprins is shown in Fig. 3A.

HMP2 is encoded by a single message derived from a single gene

The previously identified HMP1 was encoded by two differentially polyadenylated mRNA species derived from a single gene (Yan et al., 1999). A northern analysis was carried out to investigate whether HMP2 is also encoded by multiple messages. The proteinase domain of HMP2 was PCR amplified, subcloned and randomly labeled by [α^{32}P]dCTP. Northern blot analysis of *Hydra vulgaris* total RNA indicated that there exists as only a single 1.7 kb message for HMP2, which correlates well with the cloned cDNA of 1723 bp (Fig. 4A). A faint band of 620 bp was also observed (Fig. 4A), but its presence was inconsistent. Southern blot analysis was also performed to study the complexity of the HMP2 gene at the genomic level. Genomic DNA was isolated from *Hydra vulgaris* and probed with the HMP2 proteinase domain after digestion with different restriction enzymes. As shown in Fig. 4B, each digested DNA gives only one significant band, suggesting that HMP2 is likely a single copy gene.

Expression of HMP2 mRNA is highest at the basal pole of the body column

As previously demonstrated, another hydra astacin metalloproteinase, HMP1, is specifically expressed in the upper body column, in a gradient-like manner from the head to the foot process (Yan et al., 1999), correlating with its functions during cell differentiation and hydra head morphogenesis (Yan et al., 1995). We were particularly interested in the distribution of HMP2 mRNA along the longitudinal axis and its relationship with positional cell differentiation and morphogenesis. The spatial localization of HMP2 mRNA was studied by whole-mount in situ hybridization. As shown in Fig. 5B, using DIG-labeled antisense RNA probe corresponding to the astacin proteinase domain of HMP2, it was revealed that the highest levels of HMP2 mRNA are located toward the basal pole of the body column. The signal was absent in animals hybridized with

sense RNA probes as shown in Fig. 5A. This localization pattern was confirmed using a second probe that included the proteinase and MAM domain of HMP2 as shown in Fig. 5E-I. This probe provided a stronger signal as compared to the proteinase domain probe and showed that HMP2 expression, while being highest at the basal pole, also extended along the body column toward the apical pole of the animal (Fig. 5E). Therefore, the spatial localization of HMP2 mRNA forms a reciprocal image to that of HMP1, which is highest at the apical pole of the body column.

At higher magnification, it was also noticed that HMP2 mRNA in adult hydra is at relatively higher levels in cells of the endodermal layer in the lower body column (Fig. 5C,E,F). The highest level of HMP2 mRNA was detected in the junctional area between the body column and the foot process, a region that is just apical to the position where active cell differentiation/transdifferentiation occurs (best observed in Fig. 5C). Once cells in the body column are displaced across this region into the foot process, HMP2 expression disappears.

The distribution of HMP2 mRNA along the body column was also confirmed by northern analysis of hydra total RNA isolated from the upper half and the lower half of the animal. As shown in Fig. 5D, northern analysis results clearly demonstrated that HMP2 mRNA is at higher levels in the lower body of the organism.

Analysis of HMP2 expression during foot regeneration indicated expression of HMP2 mRNA within 1 hour of surgical removal of the foot process (Fig. 5G). Expression of HMP2 mRNA continued as foot regeneration proceeded (monitored over a period of 1 hour to 3 days; Fig. 5H,I).

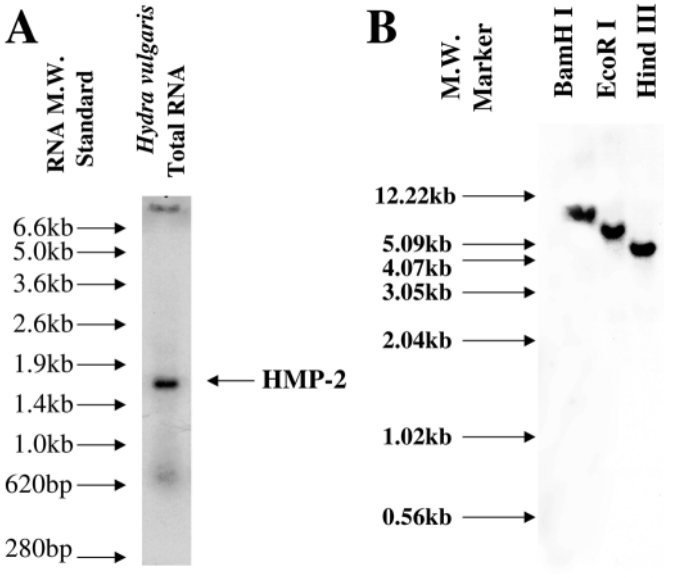

Fig. 4. Northern blot and Southern blot analysis of HMP2. (A) Northern blot analysis of HMP2 mRNA. *Hydra vulgaris* total RNA (5 μg/lane) was separated by 1.0% agarose gel electrophoresis and transferred onto nylon membrane. The blot was hybridized with ^{32}P-labeled cDNA probes corresponding to the sequence of HMP2 astacin proteinase domain. A major transcript of 1.7 kb was detected and is marked by an arrow. (B) Southern blot analysis of HMP2 gene. 10 μg *Hydra vulgaris* genomic DNA was digested by *Bam*HI, *Hin*dIII or *Eco*RI. After the digestion, fragments were separated by electrophoresis and transferred to nylon membrane, the blot was hybridized with the same probe used in Northern blot analysis. 1.0 kb DNA size ladder is shown on the left.

Characterization of antibodies to HMP2

Antiserum was prepared using a Multiple Antigen Peptide (HMP2-MAP) which was designed based on a stretch of 11 residues corresponding to the MAM domain of HMP2 (Fig. 6A). As shown in Fig. 6B, western blot analysis of hydra homogenates using anti-HMP2-MAP demonstrates that the polyclonal antibodies recognized two major proteins with approximate molecular sizes of 56.2×10^3 and 79.4×10^3. A weak protein band of 112.2×10^3 M_r was also detected. The size of the smallest protein species closely resembles the calculated molecular mass of HMP2, which is 54.5×10^3. Polyclonal antibodies raised against mouse meprin A (a generous gift from Dr Judy Bond) detected three proteins with same molecular sizes observed with the anti-HMP2 antibody (Fig. 6B, right lane). In addition to these three bands, a weak band of approximately 180×10^3 M_r was also observed with the anti-mouse meprin A antibody (Fig. 6B, right lane).

The anti-HMP2 MAP antibodies were also used to examine the distribution of HMP2 protein in adult hydra. Immunofluorescent studies indicated that HMP2 was localized to endodermal cells of the body column (Fig. 7B, insert) as previously demonstrated by the in situ hybridization studies. As shown in Fig. 7B, the immunofluorescent signal extended as far basally as the junction between the body column and the foot process (the region of highest mRNA expression) but also extended apically to the base of the tentacles. No signal was observed in the tentacles. This immunofluorescent pattern matched with the in situ analysis that indicated that while at lower levels compared to the basal pole, HMP2 mRNA expression does extend to the apical pole of the body column. Preimmune controls are shown in Fig. 7A.

Antisense analysis of HMP2 function in foot regeneration

To determine if HMP2 had a function in foot regeneration, antisense studies were performed. The unique morphology and regenerative capacity of hydra allowed us to develop and apply the LEP technique for functional analysis of antisense oligonucleotides. This technique is novel in that it allows the investigator to analyze regionally the function of any given gene product along the longitudinal axis of hydra. As shown in Figs 8 and 9, when HMP2 antisense thio-oligonucleotides (20-mers) were electroporated into the endodermal layer of cells at the basal pole of hydra, blockage of foot regeneration was observed as compared to control groups. Statistically significant blockage of foot regeneration was observed with antisense constructs designed to the 5′ UTR (87% inhibition), initiation site (69% inhibition) and coding region (82% inhibition) of HMP2. As described in the Materials and Methods section, these percentages were based on analysis of (a) the ability of basal disk cells to produce mucous, (b) use of Nomarski optics to monitor the cellular morphology of basal disk cells that normally are of a columnar classification, (c) the expression of the basal disk cell

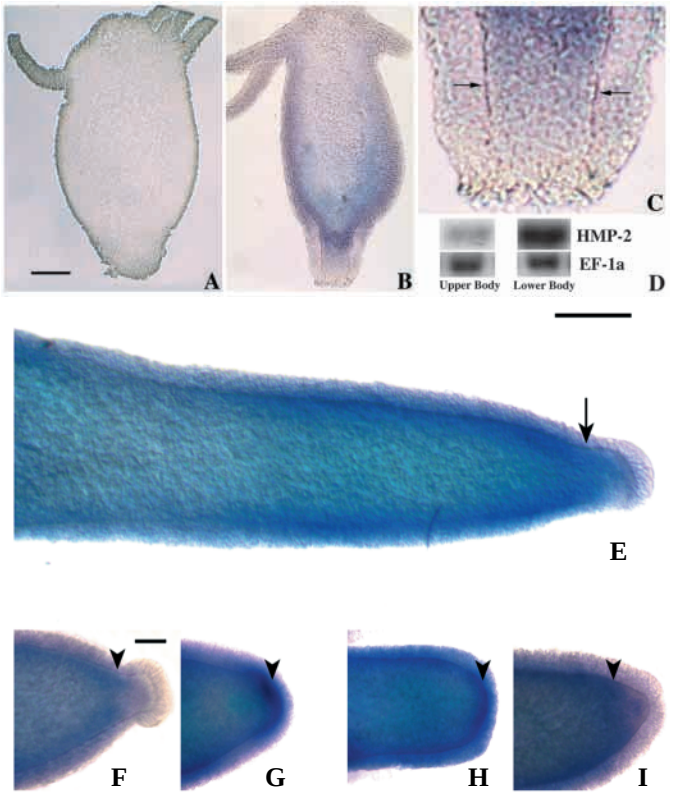

Fig. 5. Whole-mount in situ hybridization of HMP2 mRNA. *Hydra vulgaris* was fixed and treated as described in Material and Methods. Whole-mount in situ hybridization was carried out in 100 µl hybridization buffer containing 40 ng DIG-labeled RNA probe representing the proteinase domain of HMP2. mRNA localization was detected by an alkaline phosphatase reaction using BM-purple as substrate. (A) Sense probe control; (B) antisense probe: HMP2 mRNA is expressed by the endoderm cells that localize to the lower part of the body column of hydra in a gradient from the foot process to the head. (C) High magnification shows a restricted expression of HMP2 mRNA to cells of the endodermal layer in the lower body column. The highest level of HMP2 mRNA expression correlates to a cell differentiation/transdifferentiation zone at the junction between the body column and the foot process of hydra (peduncle and basal disk). Arrows point to the ECM that separates the outer ectodermal layer and the inner endodermal layer. (D) Northern analysis of HMP2 mRNA distribution in the upper body vs. lower body. The same blot was hybridized with a hydra EF-1 cDNA for internal control. (E-I) In situ analysis using a second ribo-probe developed from the proteinase and MAM domain of HMP2. The stronger signal obtained with this probe again shows high levels of HMP2 mRNA in the endoderm at the basal pole (E,F), but also shows the signal extending apically toward the head pole (E). Following excision of the foot process, HMP2 expression is observed by 1 hour of foot regeneration (G). This expression continues through 4 hours (H) to 24 hours (I). (Scale bar in A, 200 µm, applies to A-C; scale bar in E, 200 µm; scale bar in F, 100 µm, applies to F-I).

marker, peroxidase, and (d) the general morphology of the entire foot process. No inhibition was observed with an antisense oligonucleotide designed to the 3′ UTR region as compared to mismatch and sense oligonucleotides that gave a background blockage of 20%. As shown in Fig. 8, blockage of foot regeneration correlated with a disruption of the normal morphology of the basal disk cells and a loss of the basal disk cell marker, peroxidase, as compared to control specimens. Antisense-inhibited groups were able to completely recover from blockage if monitored for 5-8 days post electroporation (data not shown). [^{35}S]methionine incorporation studies coupled with SDS-PAGE indicated that translation of putative HMP2 (mass of approx. 56 kDa) was blocked under the conditions of these antisense experiments (inset in Fig. 9).

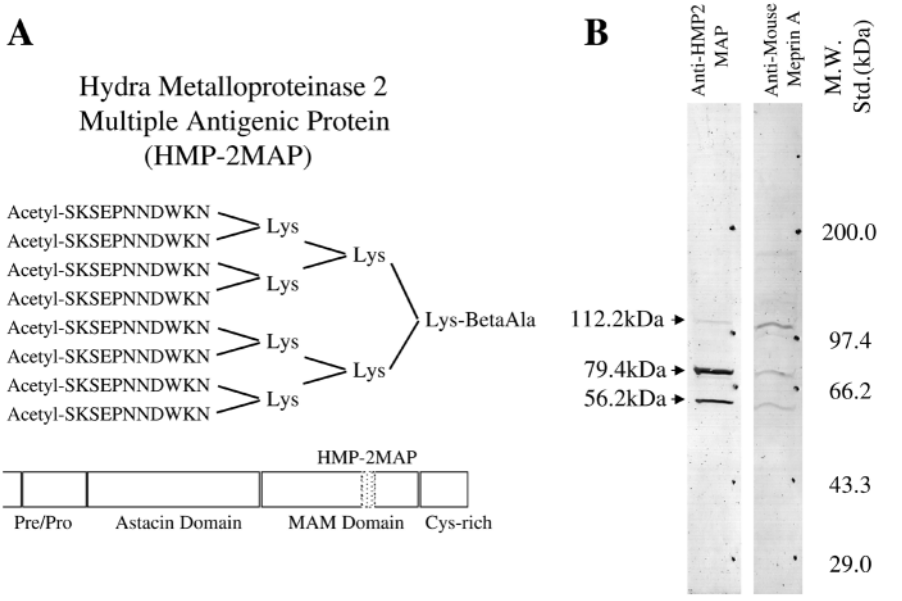

Fig. 6. Western blot analysis of HMP2 in *Hydra vulgaris*. (A) Sequence and structure of HMP2 MAP. A multiple antigenic peptide (MAP) of 11 residues was designed based on the cDNA deduced amino acid sequence of HMP2 MAM domain. (B) Western blot analysis of *Hydra vulgaris* protein using anti-HMP2-MAP antibodies. 2 day starved *Hydra vulgaris* were sonicated and suspensions were resolved by 7.5% SDS-PAGE under reducing condition. Nitrocellulose membranes with separated proteins were immunoblotted with 1:500 anti-HMP2 MAP-2 antiserum. Three protein products with molecular masses of 56.2×10^3, 79.410^3, and 112.2×10^3 were detected. Three protein products with the same molecular masses were also detected using a dilution of 1:2000 of polyclonal antibodies against mouse meprin A. A weak signal from a fourth protein of approximately 180×10^3 M_r was also observed with the anti-mouse meprin A antibody. The positions of protein molecular size markers are on the left (myosin, 200 kDa; β-galactosidase 116 kDa; phosphorylase B, 97.4 kDa; bovine serum albumine, 66.2 kDa; ovalbumin, 43.3 kDa; carbonic anhydrase 29 kDa, BRL).

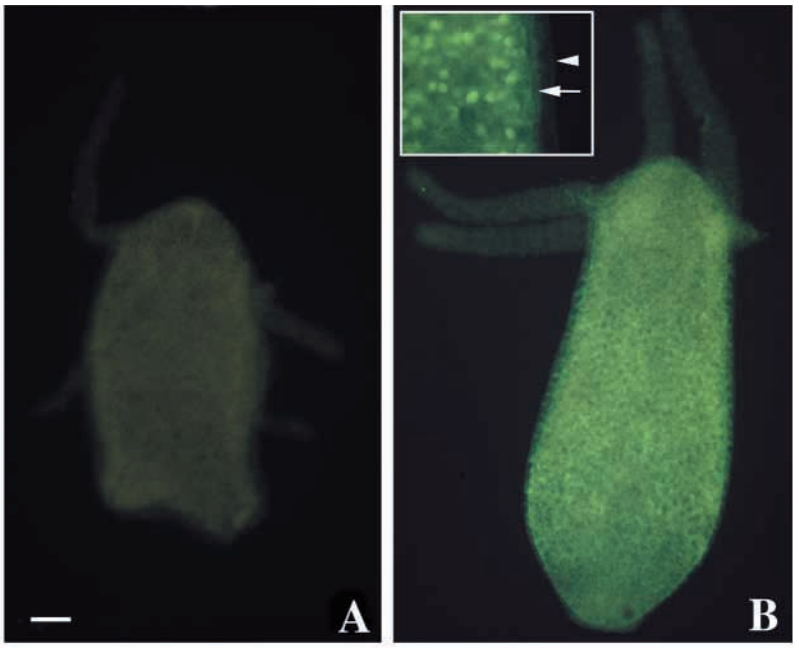

Fig. 7. Immunocytochemical localization of HMP2 protein. Immunofluorescent staining of HMP2 distribution along the longitudinal axis. 2 day starved hydra was processed as described in Materials and Methods. (A) Preimmune control; (B)anti-HMP2MAP antiserum was used in a dilution of 1:100. HMP2 is localized to cells of the body column. In the lower body column, the signal is associated with cells at the region just apical to the foot process. The signal extends apically to the base of the tentacles and into the hypostome (mouth). The signal is localized to cells of the endodermal layer (B, insert). For positional reference, the arrowhead points to the outer edge of the ectodermal layer while the arrow points to the extracellular matrix. The signal is absent in the tentacles (the apical most extent of the organism). Scale bar in A, 100 μm, applies to A and B.

DISCUSSION

HMP2 encodes a meprin-like astacin metalloproteinase

Based on the cDNA-predicted amino acid sequence, HMP2 is composed of a domain structure that is similar to meprin-like astacin metalloproteinases as shown in Figs 2, 3A. Its primary structure includes a signal sequence, a proregion, an astacin proteinase domain, a characteristic MAM domain and a C-terminal domain also observed in HMP1 and other Cnidarians that has been termed a Tox1 (TH) domain (Pan et al., 1998). Although the overall identity of HMP2 is only 30% to that of other meprin molecules, several lines of evidence support the classification of HMP2 as a meprin-like astacin metalloproteinase.

First, the 199 amino acid astacin proteinase domain of HMP2 is about 45% identical to that of other members of the astacin family. Two characteristics of astacin metalloproteinases, the astacin signature, HEXXHXXGFXHEXXRXDR (His^{158}-Arg^{175} in HMP2), and the Met-turn, SXMXY (Ser^{213}-Tyr^{217}) are well conserved in HMP2 (Fig. 2). These two sequences are responsible for (1) Zn^{2+} binding and (2) maintaining the conformation of the catalytic domain and, therefore, are both critical for enzymatic activity. The isoelectric point calculated from the deduced amino acid sequence of the mature HMP2 is 5.12 (MacVector

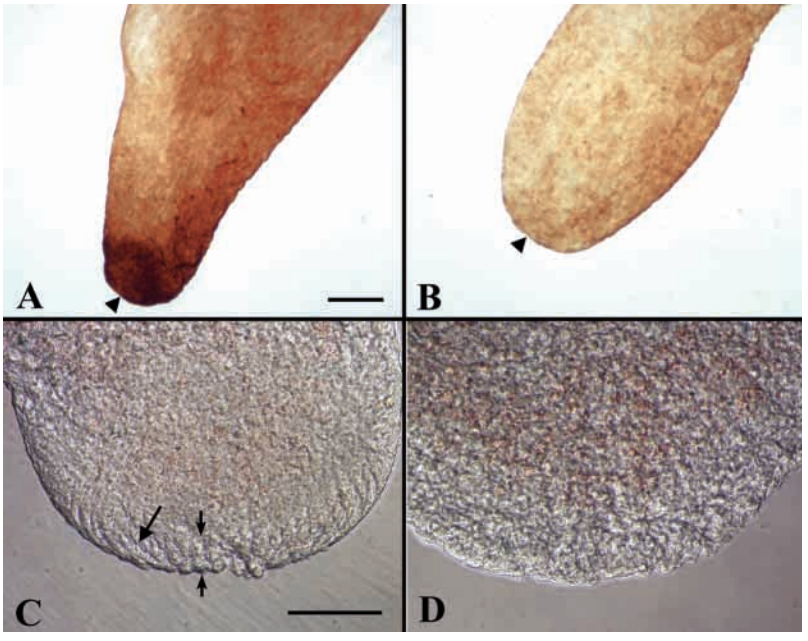

Fig. 8. Effect of antisense to HMP2 mRNA on the morphology and differentiation markers of basal disk cells of the foot process. The foot process of adult hydra is shown at 48-72 hours following initiation of foot regeneration and introduction of HMP2 antisense or control thio-oligo constructs into the endodermal cells of the basal pole. (A) The basal disk cell peroxidase activity of a control animal in which 5′ UTR sense construct was introduced into the basal pole; (B) an animal in which 5′ UTR antisense construct was introduced into the basal pole. The arrowhead shows the base of the basal pole. (C) The foot process of a control animal in which 5′ UTR sense construct was introduced into the basal pole; (D) an animal in which 5′ UTR antisense construct was introduced into the basal pole. The arrows show the apical and basal extent of the basal disc cells in the control specimen and the longer arrow shows one of an array of well-oriented basal cells that compose the ectodermal layer. In controls, the basal disk cells were functional and produced a mucous secretion that allowed the animals to stick to a glass probe or the substratum of a culture dish. In contrast, the ectodermal layer of antisense-treated specimens was not well defined or oriented and the animals were not able to stick to glass probes or the substratum of culture dishes. Scale bar in A, 200 μm, applies to A and B; scale bar in C, 100 μm, applies to C and D.

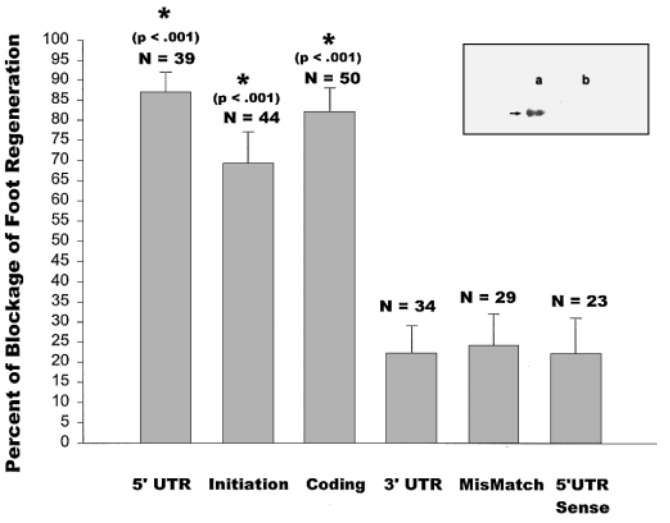

Fig. 9. Effect of antisense to HMP2 mRNA on foot regeneration. Localized electroporation (LEP) was performed with antisense thio-oligo constructs as described in the Materials and Methods section to determine if HMP2 was involved in foot regeneration. Antisense thio-oligo 20-mer constructs were designed against regions of the HMP2 mRNA as indicated in the treatment groups headings. N = number of animals per treatment group. Based on an ANOVA analysis, the starred groups (*) were statistically different from the control groups using a "*P* value" of less than 0.001. Controls included Mismatch constructs (randomized sequence) and sense constructs (5′ UTR sense construct shown). Thio-oligo 20-mer constructs included (written in the 5′ to 3′ direction):
5′ UTR, ATTTCTAAAATATAAGCACC;
Initiation, ATCAAAACCAGTTTCGTCAT;
Coding, CTGGACGATTTTGCTCATGT; 3′
UTR, AGTCATTTAACATTTAACAT;
Mismatch, ACTTGACAATACGCATTATT; 5′ UTR
Sense, GGTGCTTATATTTTAGAAAT. Inset: [^{35}S]methionine-labeled proteins were imunoprecipitated using HMP2 antibodies. A decrease in the 56 kDa band (arrow) corresponding to putative HMP2 was observed at 24 hours following antisense treatment (b) as compared to sense control (a).

5.0), which is very close to that of the subunit of meprin A, 5.7 (Jiang et al., 1992). In addition, most of the residues that have been previously shown to be essential for protein secondary and tertiary structure, including four cysteine residues (position 110, 131, 150 and 260 in HMP2) that are presumed to form two internal disulfide bonds, are well conserved (Bode et al., 1992; Stocker et al., 1993, 1995). Therefore, HMP2 possesses not only a conserved primary protein sequence, but also potential secondary and tertiary structures that are characteristic for members of the astacin metalloproteinase family.

Additional evidence for an astacin-type classification of HMP2 comes from a comparison of the domain structure of this hydra metalloproteinase to specific members of the astacin group to include meprins and BMP1-like molecules. Such a comparison revealed that HMP2 most closely resembles the structure of meprins, a subgroup of astacin metalloproteinases. As shown in Figs 2, 3A, both HMP2 and meprins contain a MAM domain (Beckmann and Bork, 1993) at the carboxyl terminus of the proteinase domain. The MAM domain was first defined in receptor protein tyrosine phosphatase κ (RPTPκ) (Jiang et al., 1993). The MAM domain was also identified in the extracellular domain of a variety of proteins with different functions, namely meprins, membrane-bound proteinases involved in processing biologically active polypeptide growth factors (Corbeil et al., 1992; Jiang et al., 1992; Johnson and Hersh, 1992; Gorbea et al., 1993), a *Xenopus* A5 protein that is a candidate for neuronal recognition (Takagi et al., 1991), and RPTPs, cell-surface receptors (Gebbink et al., 1991; Jiang et al., 1993). Recently, it has been further demonstrated that the MAM domain of RPTPs is essential for homophilic cell-cell interaction and helps determining the specificity of these interactions (Zondag et al., 1995). In addition, unlike other astacin proteinases, such as huBMP1, *Drosophila tolloid*, sea urchin SpAN and BP10, neither HMP2 nor meprins contains a COOH-terminal CUB domain (Bork and Beckmann, 1993). Taken in concert, these sequence analysis results support the proposal that HMP2 is a meprin-like member of the astacin-class of metalloproteinases.

Finally, immunological studies also suggested a structural similarity between HMP2 and meprins. In these studies, anti-HMP2-MAP antibodies reacted with three hydra protein products in the western blot analysis. The estimated size of the smallest major protein product fits well with the calculated molecular mass of HMP2. The same protein product was also detected using the polyclonal antibodies raised against purified mouse meprin A, further supporting the idea that HMP2 is homologous to meprin A. It was also noticed that two other protein products with higher molecular masses were detected by both antibodies. It is possible that these protein products represent some of the higher molecular weight proteolytic activities observed previously in the zymography analysis (Yan et al., 1995). Alternatively, they may be related to other proteins that also contain the MAM domain.

The conserved primary structure, similar potential secondary and tertiary structure, the common domain structure, and the detection of a hydra protein product with a molecular mass close to that of HMP2 by both anti-HMP2-MAP and anti-mouse meprin A antibodies, strongly suggest that HMP2 and meprins are closely related members of the astacin metalloproteinase family.

HMP2, as an early form of non-mammalian meprin-like astacin metalloproteinases, suggests a conserved function for this group of proteinases

Currently, meprin-like astacin proteinases, a group of cell-surface metalloproteinases, have been identified from mammals and possibly from one species of jellyfish (Pan et al., 1998). The mature proteinases are homo/heterodimers composed of α/β subunits that have been separately cloned from mouse, rat and human tissues (Jiang et al., 1992; Gorbea et al., 1993; Johnson and Hersh, 1992; Dumermuth et al., 1993; Corbeil et al., 1992). Different combinations of α/β subunits result in different forms of mature proteinases that are either located to the cell surface through a membrane-anchoring domain of the β subunit (Gorbea et al., 1991; Marchand et al., 1994), or secreted into the extracellular environment (Beynon et al., 1996). Although their physiological substrates are still unknown, meprin-like astacin metalloproteinases have been proposed to function in both adult and developing systems by processing biologically active polypeptides, such as bradykinin, insulin B chain, glucagon, azocasein (Butler et al.,

1987; Wolz et al., 1991), transforming growth factor alpha (TGFα; Choudry and Kenny, 1991) and parathyroid hormone (PTH; Yamaguchi et al., 1994). Identification of HMP2 provides evidence that meprin-like astacin metalloproteinases containing the MAM domain also exist in non-mammalian animals. The fact that hydra is one of the earliest divergent multicellular animals further implies the importance of these proteinases in essential biological processes.

Functional studies indicate that HMP2 has a role in foot morphogenesis in hydra

Several members of the astacin metalloproteinase family, represented by the *Drosophila* astacin, *tolloid*, have been shown to function in morphogenesis during embryonic development (Sarras, 1996). *Tolloid* was first identified as a *Drosophila* homolog of human BMP1 that plays a role in dorsoventral patterning (Shimell et al., 1991). Genetic studies have indicated a functional interaction between *tolloid* and *decapentaplegic* (*dpp)*, a *Drosophila* TGFβ. Mutations in *tolloid* and *dpp* result in quantitatively similar defects in cell differentiation and morphogenesis of the dorsal part of the *Drosophila* embryo (Shimell et al., 1991; Ferguson and Anderson, 1991, 1992a,b; Finelli et al., 1994). More recent studies have established that *tolloid* regulates *dpp* activity by degrading SOG (a chordin-like protein), which binds to *dpp* and represses *dpp* activity. Therefore, by degrading SOG, *tolloid* releases *dpp* protein from the latent complex with SOG (Marques et al., 1997). Similar de-repression mechanisms related to TGFβ activity have been shown for homologues of *Tolloid* in zebrafish (Blader et al., 1997), *Xenopus* (Piccolo et al., 1997) and sea urchin (Wardle et al., 1999). To date, however, no meprin-like astacin has been implicated in morphogenetic processes.

In the current study, we have utilized the Cnidarian, *Hydra vulgaris*, to investigate the potential role of a meprin-like astacin proteinase in morphogenesis. The functional antisense studies described in this study indicate that HMP2 is involved in processes related to foot morphogenesis; however, the precise mechanisms that account for this process are unknown at this time. Recent studies do point to a number of potential signaling molecules associated with the pattern formation in hydra and specifically with the control of foot regeneration in this simple metazoan. For example, considerable evidence now exists indicating that peptides affect patterning and epithelial morphogenesis in Cnidarians. Peptides belonging to the LWamide family have been isolated from Cnidarians and have been shown to induce morphogenetic processes in sea anemone (Leitz et al., 1994) and hydra (Takahaski et al., 1997). Another Cnidarian peptide, head activator, has been shown to be involved in a variety of developmental processes in hydra such as head regeneration and budding (Schaller, 1973; Javois and Tombe, 1991; Hobmeyer et al., 1997). Most recently, three additional peptides named pedibin, pedin (Hoffmeister, 1996) and Hym-346 (Grens et al., 1999) have all been tied to signaling pathways affecting foot regeneration in hydra. The localization of such peptides as pedibin matches well with the expression pattern of HMP2. While only correlative, this does provide a foundation for further experimentation to tie the action of HMP2 to peptide processing pathways. In addition, the work of Grimmelikhuijzen's laboratory (Mitgutsch et al., 1999) indicates that preprohormones exist in hydra, thus establishing the requirement for activating enzyme systems in this organism. Therefore, the existence of biologically active peptides associated with foot morphogenesis in hydra opens up the possibility that these peptides may be regulated by metalloproteinases as has been reported in other developmental systems such as *Drosophila* (Marques et al., 1997), sea urchin (Wardle et al., 1999), *Xenopus* (Piccolo et al., 1997) and zebrafish (Blader et al., 1997). This regulation could involve (1) activation through extracellular processing of a pro-peptide (McMahon et al., 1991), (2) release of a latent peptide from the ECM (Taipale et al., 1994), or (3) modulation of activity through proteolysis of inhibitory binding proteins such as seen in the *dpp*/SOG (Marques et al., 1997) or BMP4/chordin (Blader et al., 1997) signaling complexes. Such regulatory mechanisms are consistent with previously published data showing that meprin-class metalloproteinases can modulate the activity of biological peptides such as bradykinin, insulin B chain, glucagon, collagen and TGFα (Butler et al., 1987; Choudry and Kenny, 1991; Wolz et al., 1991; Wolz, and Bond, 1995). The fact that in situ hybridization and immunofluorescence indicated that HMP2 protein was not only expressed at the foot pole, but also as far apically as the base of the tentacles suggests that HMP2 may be multifunctional as has been proposed for another Cnidarian astacin, PMP1, first identified in jellyfish (Pan et al., 1998). Additional functions could include a role in head morphogenesis or pathways related to the processing of molecules associated with feeding and digestion such as mucins that are secreted into the gastric cavity at the apical pole region. Additional mechanistic studies with HMP2 will depend on the successful expression of this metalloproteinase for in vitro and in vivo analysis.

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