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REVIEW

Structure, expression, and developmental function of early divergent forms of metalloproteinases in Hydra

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ABSTRACT

Metalloproteinases have a critical role in a broad spectrum of cellular processes ranging from the breakdown of extracellular matrix to the processing of signal transduction-related proteins. These hydrolytic functions underlie a variety of mechanisms related to developmental processes as well as disease states. Structural analysis of metalloproteinases from both invertebrate and vertebrate species indicates that these enzymes are highly conserved and arose early during metazoan evolution. In this regard, studies from various laboratories have reported that a number of classes of metalloproteinases are found in hydra, a member of Cnidaria, the second oldest of existing animal phyla. These studies demonstrate that the hydra genome contains at least three classes of metalloproteinases to include members of the 1) astacin class, 2) matrix metalloproteinase class, and 3) neprilysin class. Functional studies indicate that these metalloproteinases play diverse and important roles in hydra morphogenesis and cell differentiation as well as specialized functions in adult polyps. This article will review the structure, expression, and function of these metalloproteinases in hydra.

Key words: *Hydra, metalloproteinases, development, astacin, matrix metalloproteinases, endothelin.*

INTRODUCTION

As a member of the Phylum, Cnidaria, hydra arose early during metazoan evolution before divergence of the protosome and deuterostome branches. Its body plan is organized as a gastric tube with a mouth and ring of tentacles at the head pole and a peduncle and basal disk at the foot pole. The entire body wall of hydra is organized as a simple epithelial bilayer with an intervening extracellular matrix (ECM). Previous studies have established that hydra ECM has a similar molecular composition to that of vertebrate species[1-5] and functional studies have established that cell-ECM interactions are critical to developmental processes in hydra[2-10].The organism has about 20 different cell types that are distributed along the longitudinal axis in a specific pattern[11], [12]. 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[13]. In the case of the ectoderm, for example, as cells are displaced into the

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tentacles or the basal disk, they trans-differentiate into battery cells and basal disk cells, respectively [11]. Battery cells and basal disk cells are eventually shed from the body column when they reach the tip of the tentacles or central portion of the base of the foot pole. As a consequence of this extensive cell turnover, hydra are highly regenerative[11],[12]. This high regenerative capacity allows one to analyze developmental processes using a variety of molecular and cell biological approaches.

Numerous studies involving both invertebrate and vertebrate organisms have established that members of the metalloproteinase superfamily have a critical role in a broad spectrum of developmental processes[15]. Through studies designed to analyze the role of cell-ECM interactions in hydra morphogenesis and cell differentiation, our laboratory has identified a number of metalloproteinases that have been found to play an important role in a variety of processes related to hydra development. These hydra metalloproteinases fall into three classes that include: 1) astacin-class metalloproteinases (e.g. Hydra metalloproteinase-1, HMP-1; and Hydra metalloproteinase-2, HMP-2), 2) matrix metalloproteinases (e.g. Hydra MMP, HMMP), and 3) neprilysin-class metalloproteinases (e.g. Hydra endothelin converting enzyme, HECE). The following review will summarize our current understanding of the structure of these three classes of metalloproteinases in hydra as well as their expression patterns and their role in morphogenesis and cell differentiation in this simple metazoan.

Hydra Metalloproteinases (HMPs) of the astacin class

To date, three astacin-class metalloproteinases, HMP-1, HMP-2, and a foot pole related HMP (Farm1) have been identified in hydra [16-19]. Because more functional studies were performed with HMP-1 and HMP-2, these metalloproteinases will be the focus of this section.

The first hydra metalloproteinase (HMP-1) was i solated based on its enzymatic activity in cleaving g elatin as a substrate[18]. HMP-1 was originally det ected as a gelatinase activity with a molecular mass o f 25-29×10^3 that localized to the upper body of th e organism. By applying a series of biochemical c hromatography procedures, this enzymatic activit y was successfully purified from cell extracts of H ydra vulgaris. Purified HMP-1 migrated as a sing le band with a molecular mass of approximately 2 5.7×10^3 that retained its gelatinase activity. N-te rminal sequence results suggested that HMP-1 w as not a classic matrix metalloproteinase, but rath er an astacin metalloproteinase family member[2 0]. This was further confirmed when the full-len gth HMP-1 cDNA was cloned (GenBank Accessi on number U22380) and sequenced[16].

Based on the amino acid sequence deduced fr om cDNA, HMP-1 is composed of 285 amino acid s with a predicted molecular mass of 32.7×10^3. T he domain structure of HMP-1 resembles that of other astacin family members (Fig 1A).An N-ter minal hydrophbic region of 21 residues with the c haracteristics of a putative signal sequence sugge sts that HMP-1 is a secreted proteinase. A 30-res idue prodomain was identified based on its homo logy to the same region of Podocoryne metallopro teinase-1 (PMP-1)[21]. The existence of the pro domain was further supported by the N-terminal sequence of purified HMP-1, which suggested a proteolytic cleavage of secreted HMP-1 between Phe(51) and Lys(52). The processed HMP-1 wou ld have a predicted molecular mass of 27×10^3, fit ting well with the size of purified HMP-1. Matur e HMP-1 has a relatively simple structure consist ing of a well-conserved astacin domain followed by a Cys-rich domain that was also identified in P MP-1 as a toxin homology (TH) domain[21]. A zi nc-binding motif and a Met-turn, both characteri stics of astacin proteinases were also well conserv ed in HMP-1 (Fig 1A)[20].

A cDNA probe corresponding to the HMP-1 astacin proteinase domain was generated to screen a Hydra vulgaris cDNA library in order to identify any additional hydra astacin metalloproteinases. This led to the successful cloning of HMP-2[17]. The putative protein sequence deduced from HMP-2 cDNA (GenBank accession number AF101400200) contains 496 residues. Like HMP-1, a 21-residue hydrophobic region at

the N-terminus represents a cleavable signal sequence, suggesting that HMP-2 is also a secreted proteinase (Fig 1B). A well conserved RXXR signature sequence predicts a proteolytic processing between R (63) and A (64)[18]. Mature HMP-2 protein consists of an astacin proteinase domain, a MAM domain[23], and a cys-rich domain (Fig 1B). The proteinase domain of HMP-2 contains a zinc-binding motif that is conserved in all astacin metalloproteinases. The MAM domain is also present in the extracellular segment of many cell surface proteins with diverse functions. These proteins include proteinases like meprin α[24] and β[25], receptors involved in neuronal recognition and angiogenesis such as neuropilin-1 and 2[26], cell adhesion proteins like zonadhesin[27],[28], and receptor tyrosine phos-

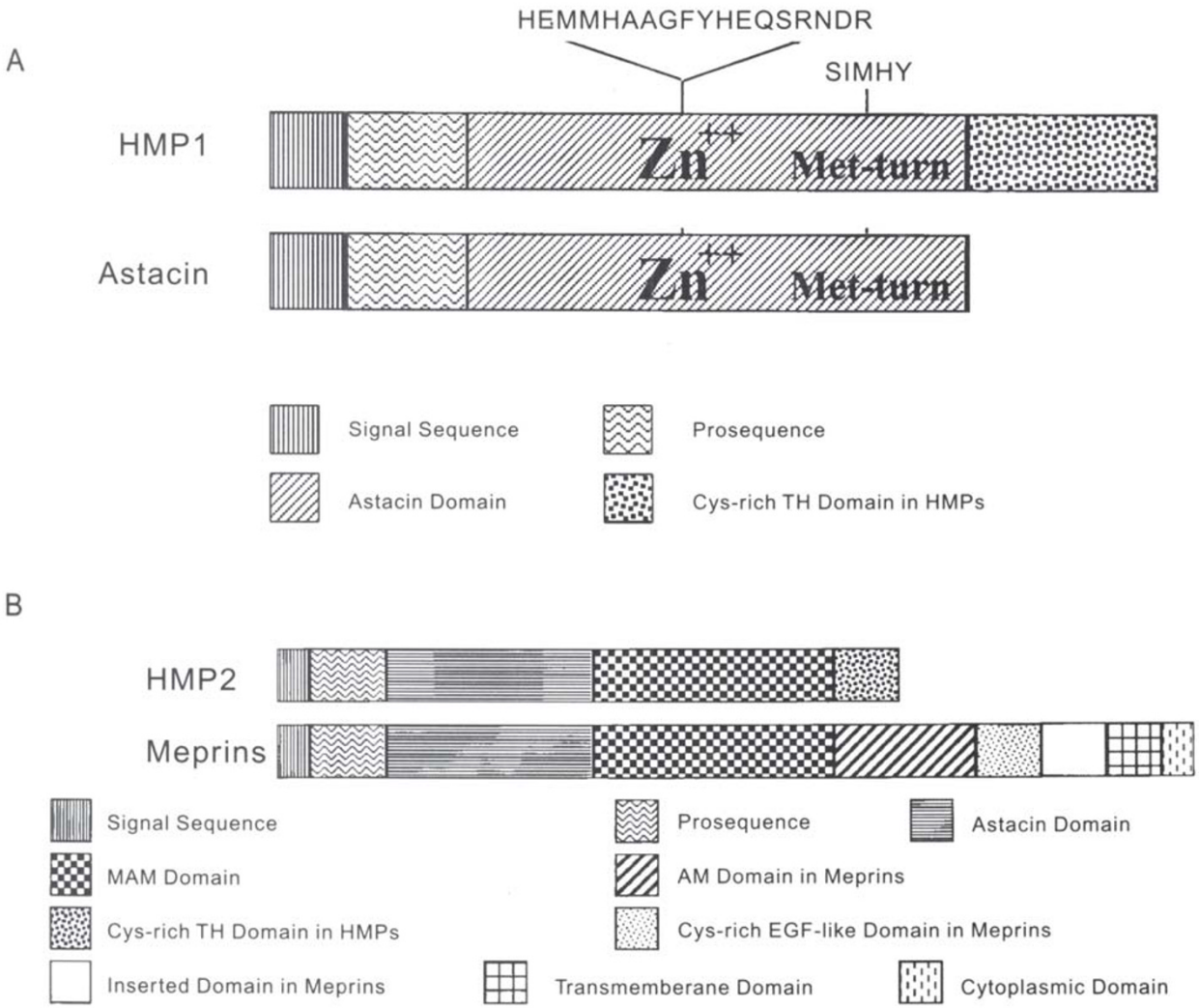


Fig 1. Domain structure of Hydra Metalloproteinase 1 (HMP-1) and Hydra Metalloproteinase 2 (HMP-2) Domain structure of HMP-1 and HMP-2 compared to astacin and meprin. The NH2-terminus of HMP-1and HMP-2 are both composed of a short signal sequence and a prosequence. Post-translationally, the proteolytic cleavage of the pro-sequences releases mature proteinases. In the case of HMP-1 this consists of an astacin metalloproteinase domain with conserved residues in astacin signature and Met-turn shown in bold, and a Cys-rich toxin homology (TH) domain. In the case of HMP-2, the predicted mature proteinase consists of an astacin domain, MAM domain, and Cys-rich TH domain. No transmembrane domain or cytoplasmic domains were found in HMP-1 or HMP-2, suggesting that these two proteinases are likely 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.

phatase mu-like molecules[29].The C-terminal cys-rich domain of HMP-2 is homologous to the TH toxin domain in HMP-1[16] and PMP-1[21].

To study the function of these two novel hydra astacin metalloproteinases, the expression pattern of HMP-1 and HMP-2 was determined by in situ hybridization and immunohistochemistry[16-18]. Interestingly, these two enzymes are expressed in two opposite gradients within the endoderm cell layer (Fig 4). HMP-1 mRNA is expressed at highest levels at the head pole while HMP-2 mRNA is expressed at highest levels at the foot pole. At the protein level, HMP-1 is synthesized in endodermal cells in the upper body column as revealed by the cytoplasmic immuno-staining of these cells. However, once these cells migrate into the tentacle region, HMP-1 protein is released into the extracellular space, as suggested by the disappearance of the cytoplasmic staining of tentacle cells and the concomitant appearance of HMP-1 staining in the extracellular matrix[16]. On the other hand, HMP-2 protein is detected at a high level in endodermal cells of the foot pole. HMP-2 positive cells localize mainly in the lower body column and are excluded from either tentacles of the head pole or the basal disk of the foot pole[17].

The gradient expression pattern of HMP-1 and HMP-2 and their reciprocal distribution pattern (HMP-1 highest at the head pole versus HMP-2 highest at the foot pole) suggested that these two enzymes are likely involved in pattern formation. This hypothesis was further supported by the finding that other astacin family members such as tolloid[30-34] play a proactive role in morphogenesis[8]. To study the potential function of these two hydra astacin metalloproteinases in morphogenesis, advantage was taken of the high regenerative capacity of hydra to determine the detailed expression pattern of HMP-1 and HMP-2 during head and foot regeneration, respectively[16], [17]. In summary, these studies indicated that at 2 h after decapitation, HMP-1 mRNA is only expressed by a ring of cells along the cut edge. At 12 h, the number of HMP-1 positive cells increased and these cells began to migrate to the upper body column of the regenerating animal. At 36 h, a large population of endodermal cells of the upper body column expressed high levels of HMP-1. This was accompanied by a transient expression of HMP-1 by some ectodermal cells in the very apical pole of the regenerating animal. By 48 hours, the HMP-1 expression gradient was re-established and was followed by head regeneration with functional tentacles observed by 72 h. In contrast, HMP-2 expression was up-regulated by foot excision and this expression persisted throughout foot regeneration. Interestingly, both HMP-1 and HMP-2 expression was restricted within clear boundary zones during these regeneration processes. For example, HMP-1 was only expressed by endodermal cells in the upper body column, but not by any cells in the tentacles. In contrast, HMP-2 expression was restricted to the lower body column and not by any cells in the basal disk. These lines of evidence further suggest that HMP-1 and HMP-2 are involved in regulating head and foot regeneration, respectively.

The role of hydra astacin metalloproteinases in controlling morphogenesis was directly studied using neutralizing antibodies and antisense thio-oligonucleotides [16-18]. A neutralizing antibody raised against HMP-1 was shown to inhibit head regeneration in a reversible fashion[18]. In addition, this antibody was capable of blocking tentacle battery cell differentiation as shown by the progressive loss over time of annexin XII staining in the tentacle of antibody- treated animals[18]. The involvement of HMP-1 in hydra head regeneration was further confirmed by antisense treatment in which HMP-1 protein expression was specifically inhibited by the introduction of antisense thio-oligonucleotides to the head pole of hydra. Subsequently, head regeneration was significantly inhibited as judged by hypostome and tentacle formation[16]. On the other hand, when HMP-2 antisense oligonucleotides were introduced locally to the foot pole, hydra foot regeneration was significantly inhibited[17]. This was monitored by the loss of peroxidase staining as a marker for basal disk cells, a flattening of the basal disk cells, and the inability of basal disk cells to stick to the substratum due to discontinued mucous secretion [17].

The mechanism(s) underlying the control of morphogenesis by HMP-1 and HMP-2 in hydra are unclear. Based on studies of homologous proteins in other species, it has been proposed that HMP-1 and HMP-2 execute their regulatory functions through

multiple pathways. These regulatory pathways may involve generation of growth factor gradients like their distantly homologous counterparts in Drosophila[35], Zebrafish[36], Xenopus [37], and sea urchin[38]. Alternatively, HMP-1 and HMP-2 could function by releasing biologically active peptides via proteolytic processing of precursor proteins[39],[40]. Interestingly, the expression of a recently identified third hydra astacin metalloproteinase, foot activator responsive matrix metalloprotease (Farm1), is regulated by such peptides[19]. This suggests a reciprocal regulatory mechanism between proteinases and peptides. Lastly, it is also possible that HMP-1 and HMP-2 could act directly on extracellular matrix (ECM) proteins and effect morphogenesis by cleavage and modification of ECM structure or mechanics[41], [43]. Given the capacity of HMP-1 and HMP-2 in cleaving gelatin, a denatured collagen molecule, one could envision that these proteinases play an active role in modifying basement membrane structure either alone or together with other proteinases, such as hydra matrix metalloproteinse (HMMP)[44].

Matrix Metalloproteinase (MMP) in hydra

Studies of extracellular matrix in hydra have established that it has a similar molecular composition to that seen in vertebrate species[1-4] and functional studies have established that cell-ECM interactions are critical to developmental processes in hydra[2-10],[45]. Hydra ECM is in a constant state of turnover[6]; 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[46]) led to the logical conclusion that an important aspect of cell-ECM interactions in hydra likely involves MMPs.

The number of MMP families has grown to over 25 with each type containing members from a broad spectrum of vertebrate species[47-50]. In contrast, relatively few MMPs have been identified in invertebrates. These invertebrate MMPs include envelysin from sea urchin[51-53], a Drosophila MMP [54]; and at least three separate MMPs from Caenorhabditis elegans[55]. Additional putative MMPs have been reported in plants such as soybean (Glycine max)[56], Arabidopsis thaliana[57], [58], and green alga [gamete lytic enzyme][59]. Recent identification of MMP in hydra therefore adds a new member to the MMP family of metalloendopeptidases[44].

In studies by Leontovich et al[44], a full-length clone (1642bp) of a hydra MMP (HMMP) was obtained from a cDNA library (GenBank accession number AF162688). Analysis of the deduced amino acid sequence indicated that HMMP had a 34% identity with human MMP-3 and contained a signal

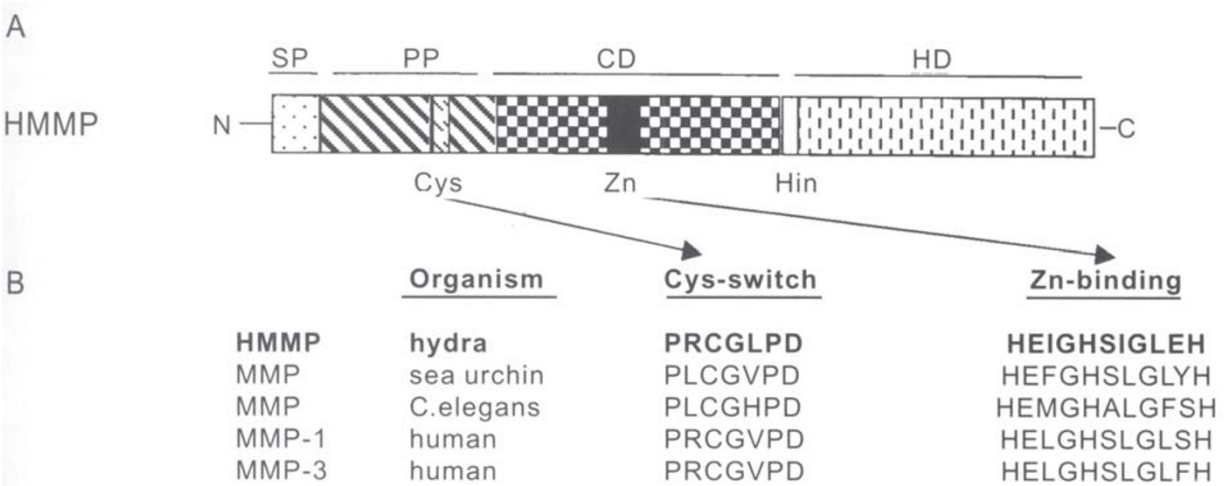


Fig 2. Domain structure of hydra matrix metalloproteinase (HMMP) (A) Diagram of the domain structure of HMMP protein. SP, signal peptide; PP, pro-peptide; CD, catalytic domain; HD, hemopexin domain; Cys, cysteine switch region; Zn, Zn- binding region; Hin, Hinge region between CD and HD. (B) Comparison of HMMP Cysteine switch and Zn-binding regions to that of vertebrate and invertebrate MMPs.

peptide, a pro-domain, a catalytic domain, a hinge region, and a C-terminal hemopexin domain as shown in Fig 2. The calculated mass of the protein was 55.4×10^3. Northern blot analysis revealed that two HMMP mRNA transcripts could be identified with sizes of 1.6 kb and 1.9 kb.

Comparison of the deduced amino acid sequence of HMMP with other MMPs shows that HMMP has an overall 30-35% identity with known MMPs. The least identical region is the C-terminal hemopexin domain (23-38%). A detailed analysis of the HMMP sequence indicates that this enzyme has many similarities to vertebrate MMPs but it also contains a number of unique features. A possible furin cleavage site for activation of the pro-enzyme intracellularly exists at amino acid residues R^{101} R^{102} -Y^{103}. The sequences of two critical regions, the Cys-switch and catalytic Zn^{++}-binding region, are well conserved in HMMP. 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. 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[60]. This region in vertebrate MMPs typically contains four or more proline residues[61]; although it should be noted that some vertebrate MMPs completely lack a hinge region[47], [48]. In addition, two cysteines commonly found in the hemopexin domain of MMPs are substituted with methionine (Met^{288}) and phenylalanine (Phe^{481}) in HMMP. This is of particular interest in regard to the highly conserved cysteines that are normally found at the N-terminus of the hemopexin domain of vertebrate MMPs. Another region of variation in HMMP is the cysteine-switch region where a leucine residue (Leu89) substitutes for the valine residue typically seen in many vertebrate MMPs. A similar substitution has been reported for MMP-19 in humans[62] and MMP-7 in felines. Variations in this region are more common among MMPs as reflected by the divergence of this region in the three MMPs of C. elegans[55], the MMP of soybean leaf, 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[63]. These cysteines are conserved in the hemopexin domain of all vertebrate and invertebrate MMPs that have been previously analyzed.

Recombinant HMMP expressed in E. coli and folded to an active state [44] was able to cleave not only gelatin, transferrin, and synthetic fluorogenic substrates, but also hydra extracellular matrix. Recombinant HMMP has its maximal activity at about pH 6.5-7.5; a typical optimal pH for known MMPs [64]. The fact that the activity of recombinant HMMP was blocked by a specific MMP inhibitor such as recombinant human TIMP-1[65] and by synthetic MMP inhibitors such as GM6001[66] and Matlistatin provided strong evidence that this enzyme had characteristics reflective of bonafide MMPs. Recombinant HMMP was also able to cleave hydra laminin as monitored by Western blot analysis and analysis of SDS-PAGE profiles indicated that HMMP was able to degrade Hcol-1 (hydra fibrillar collagen 1). 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 are not obvious, but may be related to the structure of HMMP's 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[67-69].

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 4). The expression pattern for the hydra ECM component, laminin mirrored what was observed for HMMP suggesting that high levels of ECM turnover occur at regions of cell transdifferentiation (Fig 4). The expression of HMMP was monitored during foot regeneration using both Northern blot and whole mount in situ hybridization techniques[44]. These analyses indicated that the normally high expression level of HMMP was lost between 1-3 h after excision of the foot process, but then progressively increased in the endoderm by 4 to 10 h of foot

regeneration. Similar patterns were observed during head regeneration[9].

The effects of GM6001 and recombinant human TIMP-1 were analyzed during foot regeneration. At concentrations known to inhibit recombinant HMMP, both GM6001 and recombinant human TIMP-1 blocked foot regeneration. 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, it was demonstrated that foot regeneration was significantly blocked by a number of antisense thio-oligonucleotides, but was unaffected by sense or control oligonucelotides[44]. This effect was reversible, in that hydra recovered from blockage when observed 5-7 d from the initial time of inhibition. Similar results were observed during head regeneration[9]. In adult polyps (non-regenerating), basal disk cells of hydra treated with GM6001 or TIMP-1 failed to produce normal amounts of mucous, and had significant reductions in the expression of peroxidase activity, one of their differentiation markers. When the inhibitors were removed, differentiated basal disk cells reformed within 2 days indicating that the effect was reversible as observed with foot regeneration. These studies indicate that HMMP is important to both morphogenesis and to cell differentiation processes in hydra.

Endothelin converting enzyme (ECE) in hydra

Endothelin Converting Enzyme belongs to the neprilysin family of zinc metalloproteinases that includes: neutral endopeptidase-24.11 (NEP), Endothelin Converting Enzyme (ECE) and the erythrocyte cell-surface antigen-Kell. ECE is a type II integral membrane protein consisting of 1) a short N-terminal cytoplasmic domain, 2) a single transmembrane domain, and 3) a large extracellular domain that includes the active catalytic site of the enzyme comprising the C-terminal end of the enzyme. The protein contains ten highly conserved cysteine residues and ten N-linked glycosylation sites that help to stabilize the protein[70]. It also contains a HEXXH (His^{591}, Glu^{592}, X, X, His^{595}) zinc-binding motif in the extracellular catalytic domain that is characteristic of Zincin proteinases[71]. Mutational studies have established that a conserved NAYY (Asn^{566}, Ala^{567}, Tyr^{568}, Tyr^{569}) motif in ECE is important for substrate binding and unique to ECE [72].

As an integral component of the endothelin system, vertebrate ECE functions in the activation of endothelin (ET) peptides. Vertebrate ETs are 1) the most potent vasoconstrictors known in mammals and 2) function as essential signaling ligands during development of tissues derived from neural crest cells. Biosynthesis of ETs involves a series of proteolytic steps in the processing of the initial preproendothelin protein [73]. The final step in the processing of ETs is a unique proteolytic step involving the cleavage of an inactive intermediate (bigET), at the Trp^{21}-Va^{22} (or Trp^{21}-Ile^{22}) bond, to form active ET. This step is rate-limiting in the processing of ETs and is catalyzed by the metalloproteinase, ECE.

To date, the endothelin system has mainly been investigated in higher chordates such as mammals. A limited number of studies with invertebrates using antibodies to vertebrate endothelins have suggested the presence of these peptides in non-vertebrate organisms[74-77]. Our laboratory has provided evidence for the existence of a functional endothelin-like system in hydra.

The putative ECE clone obtained from a hydra cDNA library encodes for a polypeptide of 770 amino acids (GenBank accession number AF162671). Northern blot analysis indicated two mRNAs of 2.7 kb and 3.4 kb that may be alternative splicing forms. The clone showed homology to vertebrate ECE-1 and ECE-2, but matched closest to ECE-1 (e.g. $\sim$ 35% identity and $\sim$ 53% similarity to human ECE-1). Accordingly, the putative hydra ECE has an overall domain structure consistent with that of vertebrate ECEs (Fig 3A). As shown in Figure 3C (right column), it has a zinc-binding site (HEXXH) characteristic of the general class of zincins and the characteristic ET binding site (NAYY) of ECEs (Fig 3B, left column). While the cytoplasmic domain (N-terminal) is more variable, conserved patterns in the sequence can be identified. Phylogenetic tree analysis indicated that hydra ECE grouped separately from all sequences analyzed, but branched nearest to mouse ECE1 and C. elegans ECE. Although putative, analysis of the sequence of this hydra metalloproteinase indicates that the protein should

be categorized as an endothelin converting enzyme homolog. For example, the first tyrosine residue in the ET-binding domain distinguishes this hydra ECE gene from being classified as a NEP, which has a NAFY motif in place of NAYY. This follows from mutational studies with vertebrate ECE demonstrating that replacement of tyrosine[568] in ECE to phyenylalanine reduces the Vmax/Km of ECE for the conversion of bigET to ET by 18 fold[72]. Interestingly, hydra ECE lacks a cysteine residue at position 412. This cysteine has been reported to be important for dimerization of ECE at cell membranes; a process proposed to be required for optimal activity of the enzyme[78]. Recent studies with solubilized recombinant ECE-1 however, dispute the need for a dimeric form of the enzyme[79]. In addition, potential ECE clones in C. elegans (GenBank accession number gi:6136663) and Drosophila (GenBank accession number gi:4972692) also lack this cysteine. The lack of a cysteine residue in position 412 of hydra ECE indicates that early divergent forms of this enzyme may have existed in monomeric form.

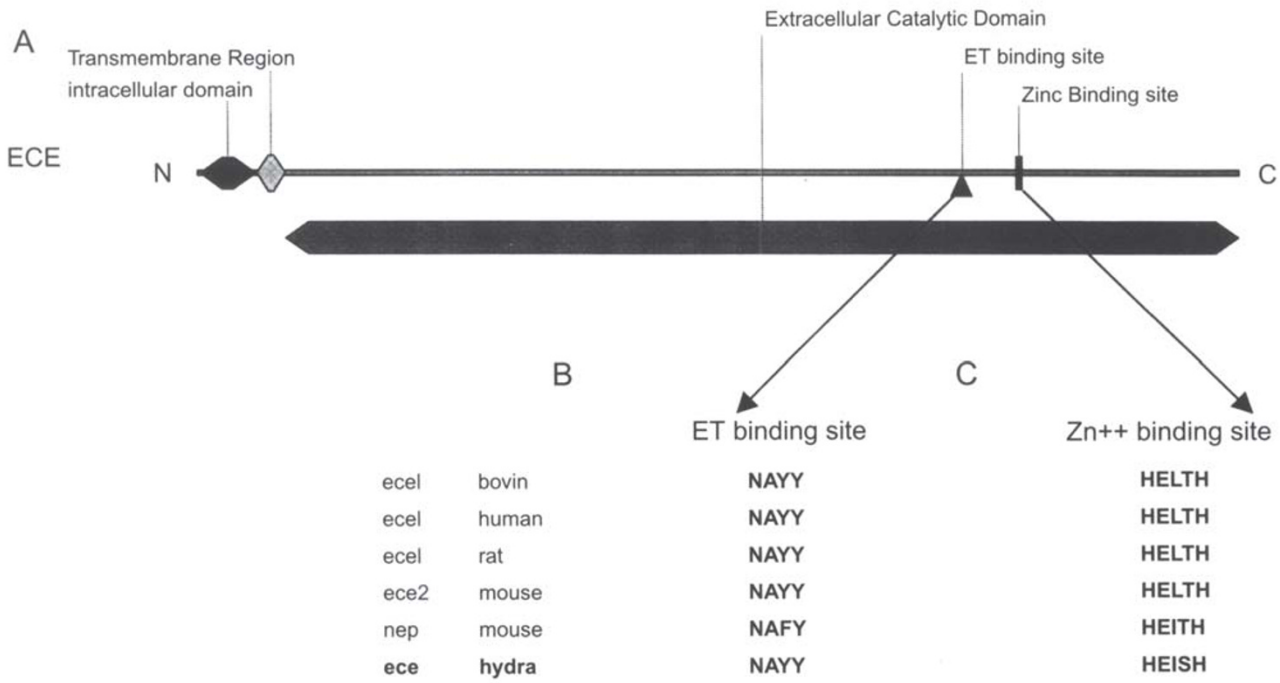


Fig 3. Domain structure of hydra endothelin converting enzyme (ECE) **(A)** Diagram of the domain structure of Hydra ECE. The protein consists of a short N-terminal intracellular domain, a transmembrane domain, and a large extracellular catalytic domain that includes the ET binding site and the Zinc binding site. Comparison of Hydra ECE ET binding site **(B)** and Zinc binding site **(C)** with vertebrate ECEs and mouse NEP.

Enzyme Immunometric Assays (EIAs) using antibody to human ETs showed that hydra contains the natural substrate for ECE. Hydra homogenates (H. magnipapilata: Strain 105) were shown to contain detectable levels of immuno-reactive bigET-1 and ET-1. Higher levels of both bigET-1 and ET-1-immunoreactive molecules were also detected in the foot pole as compared to the body column or head pole when normalized to DNA content and statistically analyzed using ANOVA. The significance of this distribution pattern is unknown, but as will be discussed, initiation of foot regeneration requires translation of ECE while initiation of head regeneration does not.

The existence of a functional endothelin-like system in hydra was suggested by the finding that human ET's could induce contraction in this simple metazoan. In this regard, muscle contraction experiments were performed using a strain of H. magnipapilata (Strain sf-1) that has significantly reduced background contraction levels following heat shock-induced loss of nerve cells[80]. Addition

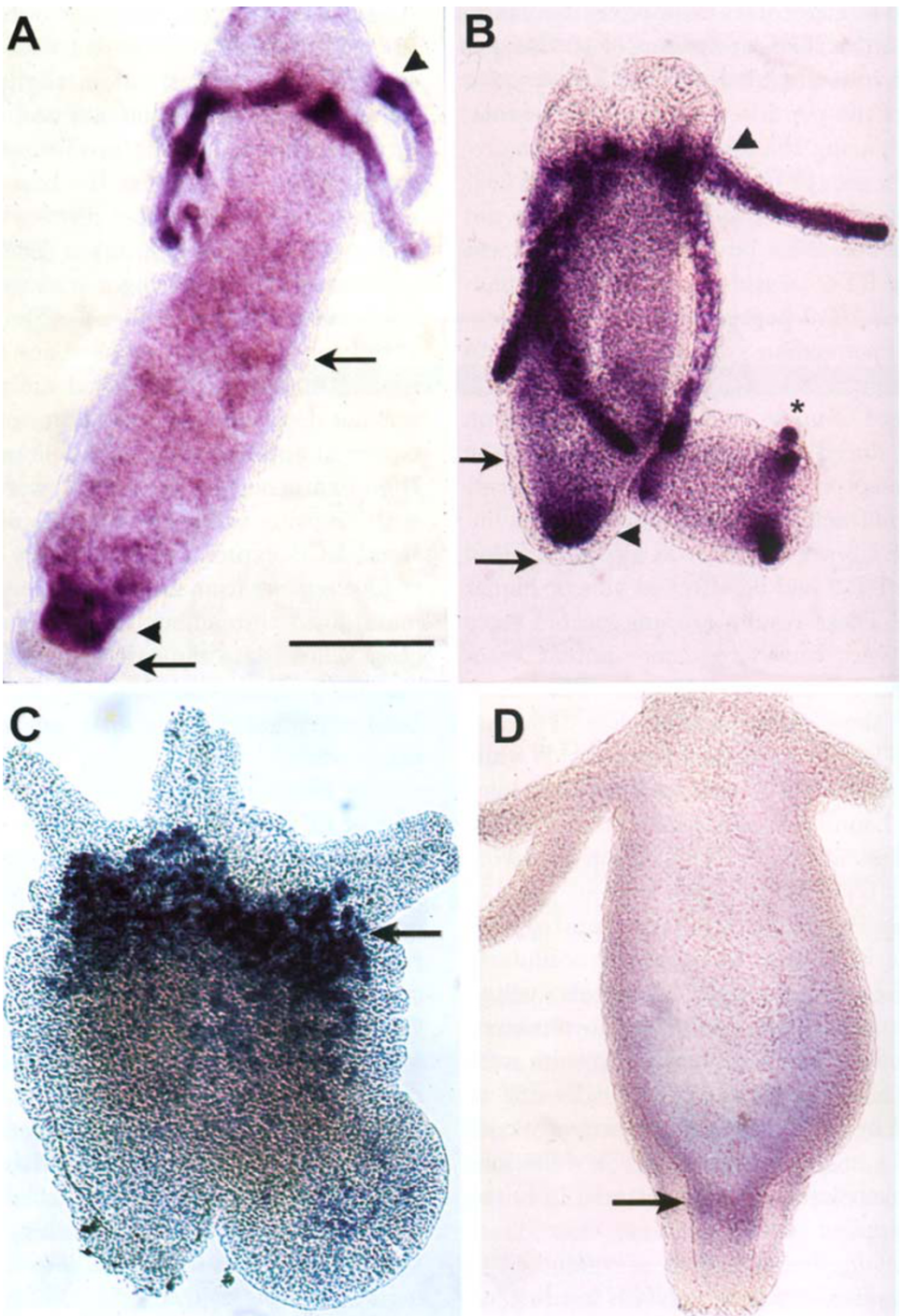
of human ET-1 stimulated contraction of hydra at a concentration of 10^{-6}M. This contraction began by 15 min after addition of the peptide and a maximal contractile state was reached by 2.5 h. Morphometric analysis of experimental groups treated with various concentrations of ET-1, ET-2 and ET-3 indicated that human endothelins used at a concentration range of 10^{-7}M (not effective), 10^{-6}M (effective), and 10^{-5}M (effective) could induce contraction along the longitudinal axis of hydra. Concentrations of 10^{-6}M and 10^{-5}M resulted in a maximal contraction at 2.5 h after addition of the peptide. In these experiments, the mean length along the longitudinal axis was reduced by 48% for groups treated with ET-1 and 56% for groups treated with ET-3. Contraction was not stimulated by ET-2. This later negative result was consistent using ET-2 peptides from different manufacturers and all ET-2 peptides tested were biologically active in mammalian systems. Antagonists to vertebrate ET receptors such as BQ-123 and BQ-788 (at concentrations of up to 10^{-5}M) had no effect on ET-1 or ET-3-induced contraction and therefore the nature of ET receptors in hydra could not be ascertained. These contraction studies indicated that human ET-1 and ET-3 were effective at a concentration of 10^{-6}M while ET-2 had no effect at this or higher concentrations. These results are unexpected since ET-1 and ET-2 are considered more potent vasoconstrictors than ET-3 in vertebrates. The active form of each of these three peptides has 21 amino acids. ET-1 and ET-2 vary by two amino acids while ET-3 has four additional amino acid substitutions. In this regard, Landan et al[81] have analyzed the evolutionary relationship of ETs and suggest from their work that ET-3 is the more ancient form of the ET peptides. Because it has been reported that some vertebrate ECE may function extracellularly on the cell surface, human bigET-1 was also tested in hydra. Effects on contraction were inconsistent in these experiments and therefore the results were inconclusive. This negative result might be due to 1) the fact that hydra ECE does not properly convert human ETs or 2) that hydra ECE functions intracellular and therefore was sequestered from the extracellularly applied bigET.

Whole mount in situ analysis was conducted to determine the expression pattern of ECE in adult hydra. Expression of ECE mRNA was observed along the entire longitudinal axis of hydra, but at relatively higher levels at the base of tentacles and the basal region of the foot process (Fig 4). The expression at the head and foot poles was localized to endodermal cells, while expression along the gastric column was associated with the ectoderm (Fig 4). This has also been confirmed by analysis of cross sections obtained from hydra whole mount in situ hybridization preparations (Fig 4). The expression of ECE along the entire body column is consistent with the effect of ETs on hydra muscle contraction. Higher expression levels at the cell transdifferentiation regions provided further evidence for the involvement of ETs in hydra development because the base of tentacles and the basal region of the foot process are regions where active cell transdifferentiation occurs. To study the expression of ECE during morphogenetic events, the head and foot poles were excised and in situ analysis at different time points of head and foot regeneration was performed. During head morphogenesis, ECE was not detected until after eruption of tentacles occurred at approximately 48 h following decapitation. High expression levels of ECE were also associated with erupting tentacles of developing buds. In contrast, ECE expression was readily detected by 3 h of foot regeneration and this expression pattern was maintained throughout the entire regenerative process. These data indicate that ECE expression occurs early during foot regeneration, but late during head regeneration only after tentacle eruption has taken place.

Because the temporal and spatial expression pattern of ECE correlated with the initial stages of foot regeneration, experiments were performed to determine if ECE is required for this morphogenetic process. Antisense thio- olligonucleotides to the coding region (referred to as Coding-2, 3, or 4 regions in the original article[82]) blocked foot regeneration (79%, 65% and 65%, respectively), but no inhibition was observed with a number of other antisense oligonucleotides designed against various other regions of ECE mRNA. Controls using sense oligonucleotides or mismatch oligonucleotides with randomized sequence also had no inhibitory effect. Monitoring of the experimental groups following the initial blockage indicated that inhibition of foot regeneration using the Coding-2, 3, or 4 thio-oligo-

nucleotides was reversible. However, consistent with the late appearance of ECE during head regeneration (at the time when tentacles first appear), antisense experiments following decapitation had no effect on tentacle evagination and head regeneration proceeded normally. In head regeneration experiments, antisense oligonucleotides were introduced into the endoderm at the time of decapitation or at 24 h following decapitation. The 24 h time point was used to effect hydra mRNA that may be expressed later in the head regeneration process as compared to that observed during foot regeneration. Introduction of thio- oligonucleotides at time zero (T0) or at 24 h following decapitation had no inhibitory effect on tentacle evagination[82].

In summary, these findings suggest that the



endothelin system is widely used by a broad spectrum of metazoans and also indicate that this signaling system arose early in evolution even before divergence of protostomes and deuterostomes. Therefore, as with vertebrates, the hydra endothelin-like system is involved in both muscle contraction and morphogenetic processes.

Summary of the structure, expression pattern, and function of metalloproteinases in hydra

Studies with hydra indicate that metalloproteinases from a wide variety of families within the superfamily of metalloproteinases diverged early during metazoan evolution. These hydra metalloproteinases have a well-conserved overall domain structure to that of their invertebrate and vertebrate counterparts. A common theme for all

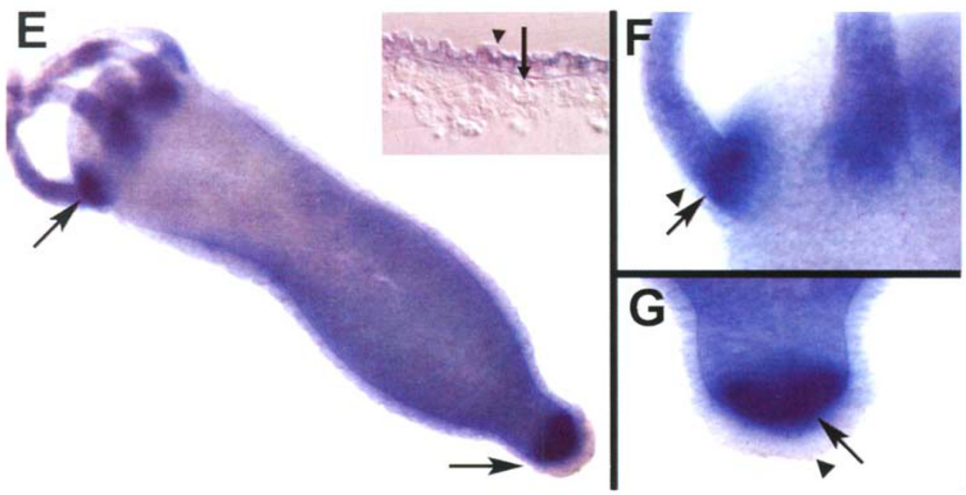


Fig 4. Whole mount in situ hybridization of hydra laminin and hydra metalloproteinases in adult polyps (Laminin b1 chain, **A**; HMMP, **B**; HMP-1, **C**; HMP-2, **D**; ECE, **E-G**). Localization of HMMP **(B)** and laminin mRNA **(A)** indicated expression in the endoderm along the entire longitudinal axis; however the arrowheads indicate that areas of strongest mRNA expression correspond to cell differentiation zones (see text) (arrows point to the outer edge of ectoderm and the asterisk indicates forming tentacles in buds). Whole mount In situ hybridization of HMP-1 in adult hydra is shown in **(C)** and indicates that HMP-1 is differentially expressed in the endoderm along the longitudinal axis with the highest levels of expression at the apical pole (arrow). No HMP-1 mRNA was localized in either the tentacles or the foot process. The arrow also indicates the position of the ECM that separates the ectoderm from the endoderm. Whole mount in situ hybridization of HMP-2 mRNA is shown in **(D)** and shows that HMP-2 mRNA is expressed by the endoderm cells with the highest levels of expression in the lower part of the body column (region by the arrow). The highest level of HMP-2 mRNA expression correlates to a cell trans-differentiation zone at the junction between the body column and the foot process of hydra (peduncle and basal disk). Arrow points to the region of high expression and indicates the position of the ECM that separates the outer ectodermal layer from the inner endodermal layer. Whole mount in situ preparations of ECE in adult polyps and sections of whole mount specimens are shown in **(E. F. G)**. Hydra ECE is expressed along the entire longitudinal axis of hydra, but at relatively higher levels at the base of tentacles (arrow) and the basal region of foot process (arrow) **(E)**. Inset of E shows a cross section of the body wall showing that along the body column, ECE is expressed in ectoderm. Arrowhead points to ectoderm with ECE expression and the arrow points to the ECM that separates the ectoderm from the endoderm. Higher magnification of the head pole **(F)** and foot pole **(G)**. Arrow points to the endoderm with ECE expression and the arrowhead points to the apical surface of the ectoderm. Bar in A = 500 μ m (applies to A-G).

these three classes of metalloproteinases in hydra is that they are up-regulated during head and/or foot regeneration and this up- regulation is associated with both morphogenetic and cell differentiation processes. In the case of many of these metalloproteinases, high levels of expression is associated with regions of cell-transdifferentiation such as the base of the tentacles and just superior to the basal disk region. This was most clearly shown for HMP-1 and HMMP which when inhibited, resulted in a loss of differentiation markers for battery cells and basal disk cells, respectively. While the mechanisms of action of these metalloproteinases are not well understood in hydra, it is likely that they may involve unmasking and/or processing of signaling molecules in the basolateral cell compartment or associated with the extracellular matrix. Current studies are underway to clarify these underlying mechanisms of action.

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