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Molecular and functional evidence for early divergence of an endothelin-like system during metazoan evolution: analysis of the Cnidarian, hydra

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SUMMARY

A novel putative endothelin-converting enzyme (ECE) has been cloned from hydra, a freshwater invertebrate that belongs to the second oldest phylum of the animal kingdom. 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. To date, only a limited number of immunocytochemical studies have suggested the presence of endothelin-like peptides in invertebrates. Based on structural and functional analyses, we present evidence for a functional endothelin-like system in hydra that is involved in both muscle contraction and developmental processes. These findings indicate the broad use of endothelin systems in metazoans and also indicate that this type of signaling system arose early in evolution even before divergence of protostomes and deuterostomes.

Key words: Hydra, Endothelin-converting enzyme, Endothelins, Contraction, Morphogenesis

INTRODUCTION

Endothelins (ETs) comprise a family of 21 amino acid bicyclic isopeptides (ET1, ET2 and ET3) that are considered the most potent vasoconstrictors identified in mammals (Yanagisawa et al., 1988). Active ETs have been shown to function through transmembrane receptors coupled to a number of signal transduction pathways (Douglas and Ohlstein, 1997; Resink et al., 1990). In addition to their role in vasoconstriction, more recent studies indicate that ETs are also involved in cell proliferation, differentiation, and other developmental processes. For example, It has been shown using gene knockout technology in mice, that ETs are essential for the normal development of neural crest-derived tissues based on analysis of the ET1 (Kurihara et al., 1994), ETA receptor (Clouthier et al., 1998) and endothelin-converting enzyme (ECE; Yanagisawa et al., 1998) genes. Additional studies indicate that ETs have a crucial role in a broad range of pathological conditions (Wu et al., 1997; Nelson et al., 1997). This includes diseases associated with the cardiovascular system (Douglas and Ohlstein, 1993), such as hypertension, myocardial ischemia and diabetic vasoculopathy, as well as non-cardiovascular diseases (Rubanyi and Polokoff, 1994), such as peptic ulceration, inflammatory bowel disease and some cancers.

Biosynthesis of ETs involves a series of proteolytic steps in the processing of the initial preproendothelin protein (Yanagisawa et al., 1988). The final step in the processing of ETs is a unique proteolytic step involving the cleavage of an inactive intermediate (big ET), at the Trp21-Val22 (or Trp21-Ile22) bond, to form active ET. This step is rate limiting in the processing of ETs and is catalyzed by the metalloproteinase, ECE.

ECE belongs to a family of zinc metalloproteinases that also includes neutral endopeptidase-24.11 (NEP) and the erythrocyte cell-surface antigen, Kell. Like other family members, 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 (Xu et al., 1994). It also contains a HEXXH (His591, Glu592, X, X, His595) zinc-binding motif in the extracellular catalytic domain that is characteristic of Zincin proteinases (Bode et al., 1993). Mutational studies have established that a conserved NAYY (Asn566, Ala567, Tyr568, Tyr569) motif in ECE is important for substrate binding and unique to ECE (Sansom et al., 1998).

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 (Kohidai and Csaba, 1995; Hasegawa and Kobayashi, 1991; Montuenga et al., 1994; Bernocchi et al., 1998). In the current study, we provide evidence for the existence of a functional endothelin-like

system in hydra, a member of Cnidaria, the second oldest phylum of the animal kingdom (Field et al., 1988). As a simple freshwater metazoan, hydra is organized as a gastric tube with a mouth and tentacle ring at its head pole and a peduncle and mucous cell disc at its foot pole (Bode, 1996). Its body wall along the entire longitudinal axis is structurally reduced to a epithelio-muscular bilayer with an intervening extracellular matrix (Sarras et al., 1991). This bilayer is composed of about 20 cell types, including ectodermal and endodermal epithelio-muscular cells, and various interstitial cells (such as nerve cells, gametes and nematocysts (Bode, 1996)). Because of the reduced number of cell types, differentiated cells in hydra are multifunctional. For example, the muscular system of hydra is organized within the epithelial bilayer and consists of outer longitudinal contractile fibers in the ectodermal cells and inner circular contractile fibers in the endodermal cells. Early ultrastructural studies determined that the myonemes (actin/myosin bundles) were positioned within the basal foot processes of the ectoderm and endoderm (Slautterback, 1967; Haynes et al., 1968). These epithelial cells also function in other processes such as fluid and electrolyte transport. The cells of hydra are also in a dynamic state of proliferation and turnover. The interstitial cells arise from interstitial stem cells that reside along the gastric column (Bode, 1996). Epithelial cells continuously proliferate along the body column and migrate toward the poles, where they are eventually shed (Bode, 1996; Bode et al., 1986). Epithelial cells that are displaced into the base of tentacles or the basal disc of the foot process transdifferentiate into phenotypes specific to the head or foot regions (i.e. squamous battery cells in tentacles or columnar mucous cells in the basal disc) (Bode, 1996; Bode et al., 1986). As a result of this extensive cell turnover, hydra are in a perpetual embryonic state and therefore have an extensive regenerative capacity (Bosch, 1998). In the context of the current study, the simplified structure of hydra and its high regenerative capacity makes it an ideal model in which to study the potential role of the endothelial system in contractile processes and morphogenesis in an ancient epithelial system.

MATERIALS AND METHODS

Molecular cloning and sequence analysis

A *Hydra vulgaris* λ ZAP II 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 that encode novel hydra metalloproteinases. A cDNA fragment was obtained from PCR reactions using degenerate primers against human metalloproteinases. This PCR product was subsequently cloned into PCR II TA-cloning vector (Invitrogen, CA) for DNA sequencing. Sequence analysis indicated that the PCR product contained a 1.0 kb region that gave a conceptional translation including a distinct partial ECE catalytic domain. To obtain the full-length cDNA of hydra ECE, this PCR fragment was α^{32}-P randomly labeled to screen 1×10^6 clones of an Oligo(dT) primered UNI-ZAP cDNA library of *Hydra vulgaris*.

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.

Phylogenetic tree analysis was performed using the neighbor-joining method of Saitou and Nei (Saitou and Nei, 1987). The algorithm of this method is similar to that of Sattath and Tversky (Sattath and Tversky, 1977), but also provides the relative branch lengths in the final tree.

Northern analysis

The proteinase domain of hydra ECE spanning from 1367 bp to 2480 bp (clone ECE-A4) was one of the clones obtained from library screening. This partial clone was random primer-labeled with γ^{32}P-dCTP using a NE Blot Kit (New England BioLabs, MA) and transcribed into a riboprobe labeled with γ^{32}P-CTP. Total RNA was isolated from 2-day-old 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 7 μg *Hydra vulgaris* total RNA was hybridized overnight at 65°C with the radiolabeled probes at a concentration of 2×10^7 cpm/10 ml. The blot was washed to a final stringency of 0.1×SSC and 0.1% SDS at 65°C.

In situ hybridization

As described earlier, the proteinase domain of hydra ECE (clone ECE-A4) was used to make the RNA probe. T7 or SP6 RNA polymerases were used to make either sense or antisense digoxigenin (DIG)-labeled ribonucleotide probes (Ambion, TX).

Whole-mount in situ hybridization of hydra was performed as previously described (Grens et al., 1995). Briefly, 2-day-old starved hydra were fixed in 4% paraformaldyhyde overnight, 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).

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

Because transfection approaches have not been successfully applied to Cnidarians, we have developed a procedure to specifically test the effect of antisense oligonucleotides on head or foot regeneration in hydra. This approach uses a localized electroporation technique (LEP) in order to introduce antisense oligonucleotides into the head or foot pole of hydra. This procedure has been applied to the functional analysis of a number of hydra genes and has been described in detail previously (Yan et al., 2000a; Yan et al., 2000b; Leontovich et al., 2000). Applying the LEP procedure, we tested the hypothesis that de novo biosynthesis of hydra ECE is required for normal head or foot morphogenesis after decapitation or excision of the foot process. Based on the work of Wagner (Wagner, 1994; Wagner, 1995; Flanagan et al., 1996), a series of 20-mer oligonucleotides with phosphorothioate linkages were designed. Seven oligonucleotides were synthesized to include five antisense sequences to portions of the 5′ untranslated region (UTR), initiation site, four regions of the coding sequence, and 3′ UTR and two controls, including a sense strand of Coding-2 sequence and a mismatch (randomized sequence) oligonucleotide. Oligos used: 5′ UTR, 5′ CGCTTATTACACAGGGACTA 3′; initiation, 5′ CTTTTTACTTGCGAACTCAT 3′; Coding-1, 5′ CTAATTGAGTTTCAAACTCC 3′; Coding-2, 5′ ACCCATGTGATATCTCATGA 3′; Coding-3, 5′ CTCATGACCAACAACC 3′; Coding-4, 5′ TTTACCTTGGTCATCA 3′; 3′ UTR, 5′ ATAAAGAATATTCATTTAGG 3′; mismatch, 5′ ACTTCATC-

AGAATTGTATCG 3′; Sense control of Coding-2, 5′ TCATGA-GATATCACATGGGT 3′. Because hydra ECE is expressed in the endoderm layer of cells at the head and foot pool, LEP was performed on the inner gastric surface of these regions, following procedures previously described (Yan et al., 2000a; Yan et al., 2000b; Leontovich et al., 2000). Electroporated hydra were observed every 24 hours and the degree of regeneration was compared with mock electroporated controls. In the case of head regeneration, animals were cut in the neck region just inferior to the mouth and tentacle ring. The degree of head regeneration was monitored by (1) observing the morphology of the head process under a dissection microscope and determining the degree of tentacle eruption and hypostome formation; and (2) analyzing the cellular morphology of cells of the hypostome and tentacles using Nomarski optics. In the case of foot regeneration 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 disc cells to produce mucous by placing a glass probe against the foot pole of the regenerating hydra and monitoring cell adhesion to the probe; (3) assaying for the basal disc cell marker, peroxidase following the procedures of Hoffmeister (Hoffmeister, 1996); and (4) monitoring the cellular morphology of basal disc cells using Nomarski optics. In controls, head regeneration and foot regeneration was 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. As previously stated, control animals were treated with mismatched 20-mer thio-oligonucleotides (randomized sequence) or sense thio-oligonucleotides if a particular antisense construct was found to block morphogenesis. Control and experimental groups were statistically compared using a Chi squared test and an ANOVA test.

Enzyme immunometric assay (EIA)

The EIA kits for human ET1 and human big ET1 were purchased from Assay Designs (Ann Arbor, MI). The kit uses a polyclonal antibody to either ET1 or big ET1 immobilized on a microtiter plate to bind the ET1 or big ET1 in the sample. After a short incubation the excess sample is washed out and a polyclonal antibody to ET1 labeled with horseradish peroxidase is added. This labeled antibody binds to the ET1 or big ET1 captured on the plate. After a short incubation the excess labeled antibody is washed out and substrate is added. The substrate reacts with the labeled antibody bound to the ET1 or big ET1 captured on the plate. The color generated in a 30 minute incubation with the substrate is read at 450 nm and is directly proportional to the concentration of ET1 or big ET1 in the sample. To normalize the number of cells in each portion, DNA determination was performed using a Hoechst dye procedure (Cesarone et al., 1979). The ratio of ET1 or big ET1 to the DNA content was used for the comparison of ET1 or big ET1. The cross reactivity between ET1 and big ET1 is less than 0.1% in this assay.

For analysis of the distribution of ETs along the body axis, hydra were separated into three segments along the longitudinal axis (head, body column and foot segments). A total of 300 hydra were used for each analysis. Each segment was sonicated in 1.3 ml hydra media with a proteinase inhibitor cocktail (1 mM PMSF, 5 mM iodoacetamide, 0.2 mM TLCK, 2 mM benzamidine HCl). From this sonicate, 600 μl was used (100 μl/well) for the ET1 assay; 600 μl was used for the big ET1 assay; and the remaining 100 μl was for a DNA determination. The amount of ET1 and big ET1 (expressed in picograms) was normalized per μg DNA of each region using the microfluorometric DNA 33258 Hoechst assay (Cesarone et al., 1979).

ET contraction assay

A mutant strain of *H. magnipapilata* (Strain sf-1) that contains normal hydra epithelial cells and temperature-sensitive interstitial cells was used in this assay. The use of this hydra strain significantly reduces background contraction levels after heat shock-induced loss of nerve cells (25°C; Marcum et al., 1980). Various concentrations of human ET1, ET2 or ET3 were added to hydra media and the degree of contraction of the specimens was monitored over time using a computerized imaging system with real-time video-capture capability

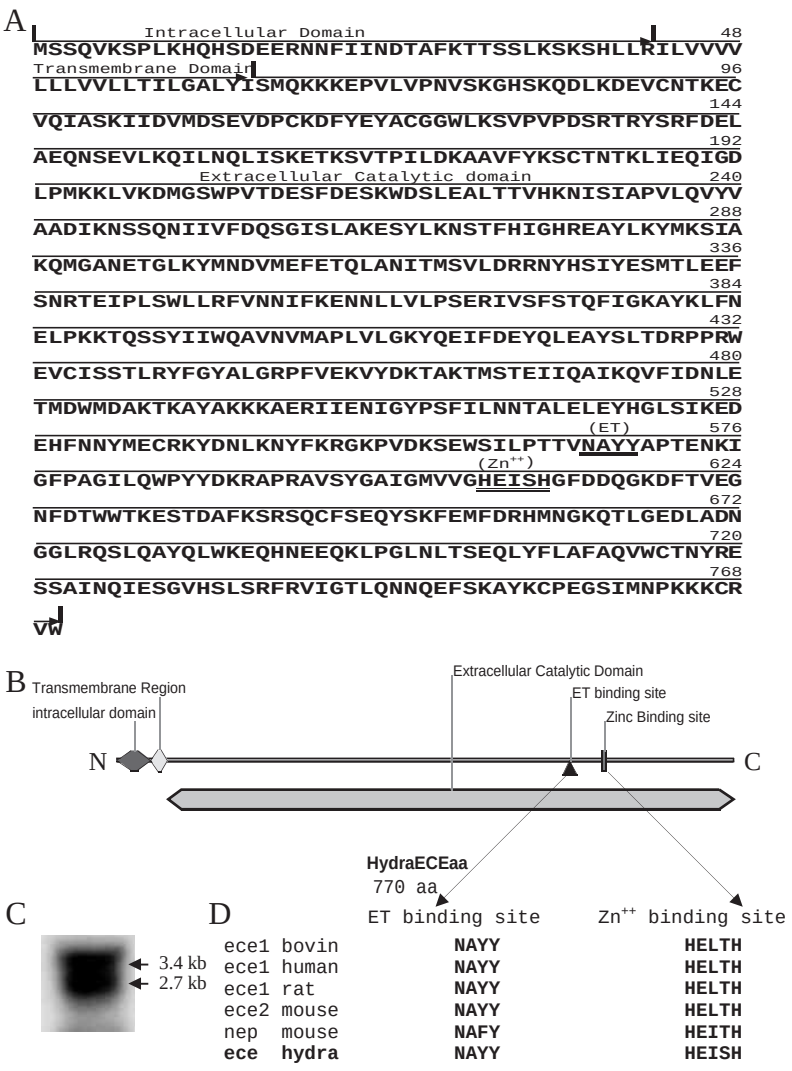

Fig. 1. Amino acid sequence analysis and northern blot analysis of ECE. (A) Amino acid sequence deduced from the hydra ECE cDNA. Domains are indicated above the sequence and delineated by vertical bars. Underlined sequences correspond to the ET-binding site and zinc-binding site. The nucleotide sequence was deposited with GenBank (Accession Number, AF162671). (B) 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. (C) Northern blot analysis indicates that there are two transcripts of hydra ECE (2.7 kb and 3.4 kb). (D) Comparison of Hydra ECE ET-binding site and zinc-binding site with vertebrate ECEs and mouse NEP.

and NIH imaging software for quantitative analysis. The length of individual polyps was continuously monitored and the percent of contraction was determined by dividing the original extended length by the contraction length at different times after addition of the peptide to the hydra media. Ten specimens were used for each peptide concentration tested and each experiment was repeated three times. Small peptides have been shown to gain access to the basolateral compartment of hydra's epithelial bilayer when such peptides are simply added to hydra media (Takahashi et al., 1997).

RESULTS

While screening cDNA libraries for hydra metalloproteinases, we obtained a putative ECE cDNA clone that encodes a polypeptide of 770 amino acids (GenBank Accession Numbers, AAD46624 and AF162671; Fig. 1A). Northern blot analysis indicated two transcripts of 2.7 kb and 3.4 kb that may be alternative splicing forms (Fig. 1C). The clone showed homology to vertebrate ECE1 and ECE2, but matched closest to ECE1 (e.g. ~35% identity and ~53% similarity to human ECE1). Accordingly, the putative hydra ECE has an overall domain structure consistent with that of vertebrate ECEs (Fig. 1A,B). As shown in Fig. 1D (right column), it has a zinc-binding site (HEXXH), which is characteristic of the general class of zincins and the characteristic ET-binding site (NAYY) of ECEs (Fig. 1D, left column). Although the cytoplasmic domain (N-terminal) is more variable, conserved patterns in the sequence can be identified (Fig. 2A). Phylogenetic tree analysis indicates that hydra ECE groups separately from all sequences shown, but branches nearest to mouse ECE1 and *Caenorhabditis elegans* ECE (Fig. 2B).

To determine if hydra contains the natural substrate for ECE, enzyme immunometric assays (EIAs) were performed using antibody to human ETs. Hydra homogenates (*H. magnipapilata*, Strain 105) contained detectable levels of immunoreactive-like big ET1 and ET1 (2.45 pg/µg DNA and 1.39 pg/µg DNA, respectively). As shown in Table 1, higher levels of both big ET1 and ET1 immunoreactive molecules were detected in the foot pole (compared to the body column or head pole) when normalized to DNA content and statistically analyzed using ANOVA.

To determine if a functional endothelin-like system exists in hydra, we tested the ability of human ETs to induce contraction in this simple metazoan. As described (Materials and Methods), experiments were performed using a strain of *H. magnipapilata* (sf-1) that has significantly reduced background contraction levels after heat shock-induced loss of nerve cells (Marcum et al., 1980). As shown in Fig. 3B, addition of human ET1 stimulated contraction of hydra at a concentration of 10^{-6} M. This contraction began by 15 minutes after addition of the peptide and a maximal contractile state was reached by 2.5 hours after addition of the peptide. Morphometric analysis of experimental groups treated with various concentrations of ET1, ET2 and ET3 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 of hydra (Fig. 3, lower panel). As indicated in the graphs shown, concentrations of 10^{-6} M and 10^{-5} M resulted in a maximal

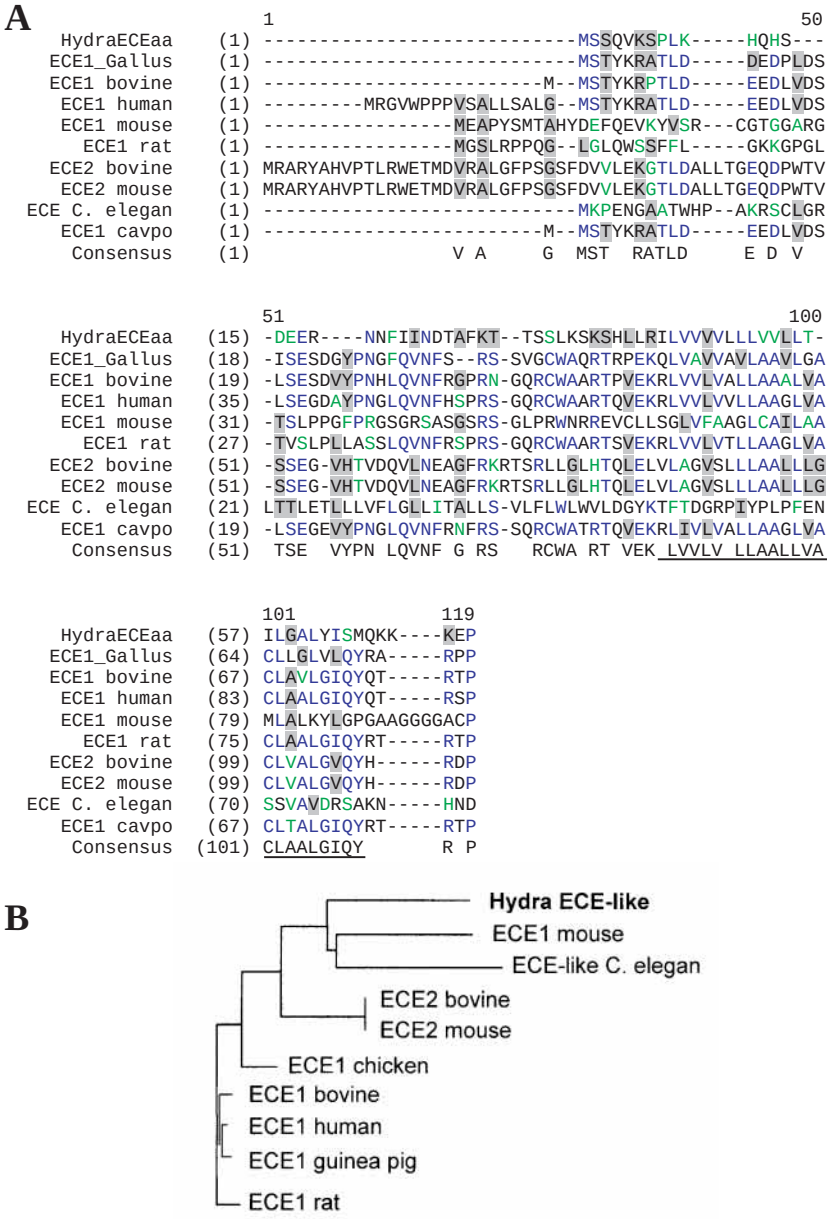

Fig. 2. Comparative sequence analysis of hydra ECE to vertebrate and invertebrate ECEs. (A) The N-terminal cytoplasmic domain of hydra ECE is shown to include the transmembrane domain (underlined) and the first five amino acids of the extracellular domain. Hydra ECE is compared with chicken (*Gallus*), human, mouse, rat and guinea pig ECE1 and with bovine ECE1; Bovine and mouse ECE2; and *C. elegans* ECE. (B) A phylogenetic tree shows the relative branch lengths of hydra ECE to ECEs of both vertebrate and invertebrate species. Shaded residues are similar to one another. Blue indicates conserved residues. Green indicates residues that have weak similarity.

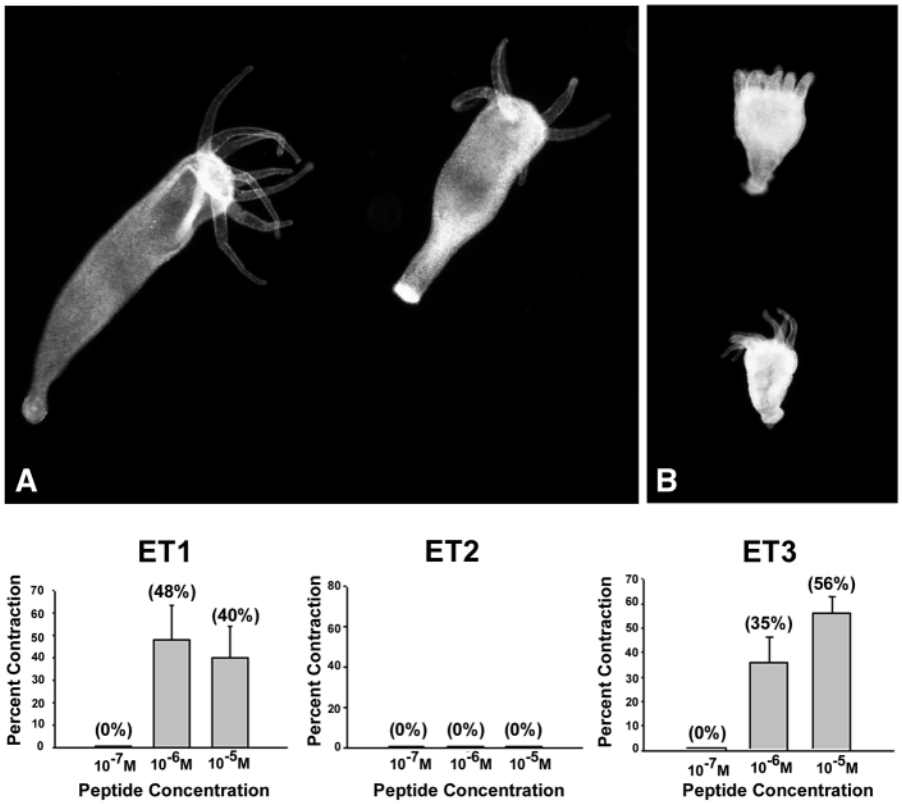

Fig. 3. Effects of human endothelins on muscle contraction in hydra. (A) Hydra before treatment with ET1. (B) Hydra 2.5 hours after addition of ET1 (10^{-6} M) to the medium. Similar results were observed with ET3, but no effect was observed with ET2. (Bottom) Dose-response analysis of human ET1 (left), ET2 (middle) and ET3 (right) on contraction of hydra polyps. As indicated, effective concentrations were in the 10^{-7} M, 10^{-6} M and $10^{-}5$ M range. Data points shown in these graphs were determined 2.5 hours after addition of the peptide. A minimum of 10 hydra were analyzed per concentration and each experiment was repeated three times. Standard deviation is indicated for each group.

contraction at 2.5 hours after addition of the peptide. In these experiments, the mean length along the longitudinal axis was reduced by 48% for groups treated with ET1 and 56% for groups treated with ET3. Contraction was not stimulated by ET2 (Fig. 3, middle graph). This negative result was consistent using ET2 peptides from different manufacturers. These ET2 peptides 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 this contraction, and therefore the nature of ET receptors in hydra cannot be ascertained from these studies. Because it has been reported that some vertebrate ECEs may function extracellularly at the cell surface, human big ET1 was also tested in hydra. Effects on contraction were inconsistent in these experiments and therefore the results were inconclusive.

Whole-mount in situ analysis (Yan et al., 2000a) 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 (Fig. 4A), but at relatively

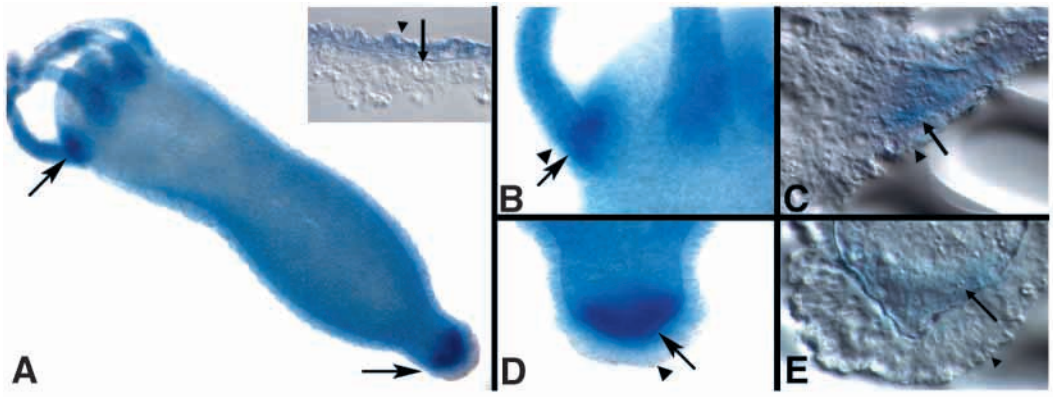

Fig. 4. Expression pattern of ECE in adult hydra. Whole-mount in situ preparations and sections of whole-mount specimens are shown. (A) Hydra ECE is expressed along the entire longitudinal axis of hydra, but at relatively higher levels at the base of tentacles (left arrow) and the basal region of foot process (right arrow). (Inset) Cross section of the body wall. The arrowhead points to ectoderm with ECE expression and the arrow points to the ECM that separates the ectoderm from the endoderm. (B) Higher magnification of head region in A. (C) DIC cross-section image of head region with an arrow pointing to endoderm with ECE expression, and an arrowhead pointing to the apical surface of ectoderm. (D) Higher magnification of foot process in A. (E) DIC cross section image of foot process with an arrow pointing to endoderm with ECE expression, and an arrowhead pointing to the apical surface of ectoderm.

Table 1. Enzyme immunometric assay for big ET1 and ET1 in hydra

	ET content (pg/μg DNA)	
Body region	Big ET1	ET1
Head pole	1.85	1.27
Gastric tube	1.56	1.21
Foot pole	6.77*	2.23*

*Significant difference at $P \leq 0.05$.

higher levels at the base of tentacles and the basal region of the foot process (Fig. 4A,B,D). The expression at the head and foot poles was localized to endodermal cells, while expression along the gastric column was associated with the ectoderm. Cross sectioning of hydra after whole-mount in situ hybridization confirmed the distribution of ECE in the endoderm of the head and foot regions (Fig. 3C,E) and the ectoderm of the body column (Fig. 4A, inset). The expression of ECE along the entire body column is consistent with the effect of ETs on hydra muscle contraction.

As indicated, gene knockout experiments have established that the endothelin system is crucially involved with developmental processes in mice (Kurihara et al., 1994; Clouthier et al., 1998; Yanagisawa et al., 1998). Because the base of tentacles and the basal region of the foot process are regions where cell transdifferentiation occurs, high expression of ECE at these positions implies that the gene for ECE may also be involved in developmental processes in hydra. 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. Decapitation was performed just inferior to the head pole, while the foot was excised superior to the basal disc at approximately the middle of peduncle or just superior to the peduncle in some experiments. During head morphogenesis, ECE was not detected until after eruption of tentacles occurred at approximately 48 hours following decapitation (Fig. 5A-D,F). High expression levels of ECE were also associated with erupting tentacles of developing buds as shown in Fig. 5E. In contrast, ECE expression was detected within 3 hours of foot regeneration (Fig. 5H) and this expression pattern was maintained throughout the entire regenerative process (Fig. 5I,J) for all experiments. These data indicate that ECE expression occurs early during foot regeneration, but late during head regeneration (after tentacle eruption has already occurred).

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-oligonucleotide 20 mers to ECE were introduced into the endoderm of the foot pole immediately after excision of the foot process. Antisense oligonucleotides were introduced into the endoderm using the localized electroporation technique described previously (Yan et al., 2000a; Leontovich et al., 2000; Yan et al., 2000b). As shown in Fig. 6, antisense to the Coding-2, Coding-3 or Coding-4 regions blocked foot regeneration (79%, 65% and 65%, respectively), but no inhibition was observed with a number of other antisense oligonucleotides designed against various regions of ECE mRNA (see Fig. 6 for a description of the thio-oligonucleotides used in these experiments). Controls using sense oligonucleotides or mismatch oligonucleotides with randomized sequence also had no inhibitory affect (sense controls for the Coding-2 region are shown in Fig. 6). To determine if the blocking effect of the Coding-2 region was due to a general blocking of all mRNAs, we monitored the appearance of some proteins known to be essential to foot regeneration (e.g. laminin) and found that their expression was not altered in these experiments (data not shown). Monitoring of the experimental groups following the initial block indicated that inhibition of foot regeneration using the Coding-2, Coding-3 or Coding-4 thio-oligonucleotides was reversible (data not shown). Recovery from block using the

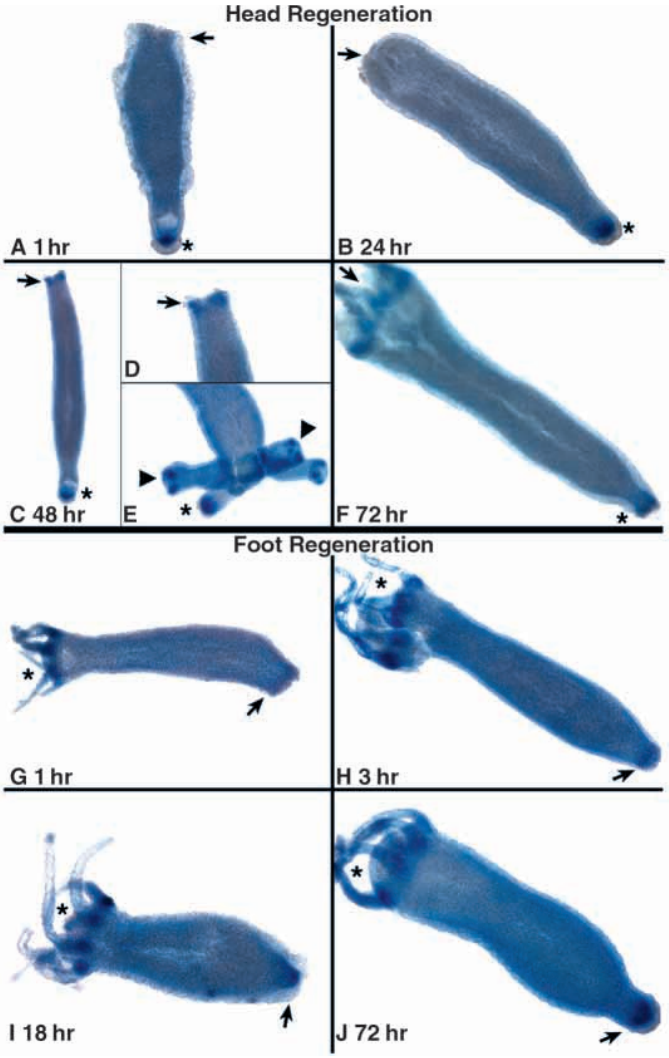

Fig. 5. In situ whole-mount analysis of ECE expression during head and foot regeneration. (A-F) Expression patterns of ECE during head regeneration. Asterisks, foot poles; arrows, regenerating head poles. In situ hybridization is shown for the following time points after decapitation: (A) 1 hour, (B) 24 hours, (C) 48 hours and (F) 72 hours. (D) Higher magnification of C. (E) Expression levels of ECE associated with erupting tentacles of developing buds (arrowheads). (G-J) Expression patterns of ECE during foot regeneration. Asterisks, head poles; arrows, regenerating foot poles. In situ hybridization is shown for the following time points after excision of the foot pole: (G) 1 hour, (H) 3 hours, (I) 18 hours and (J) 72 hours.

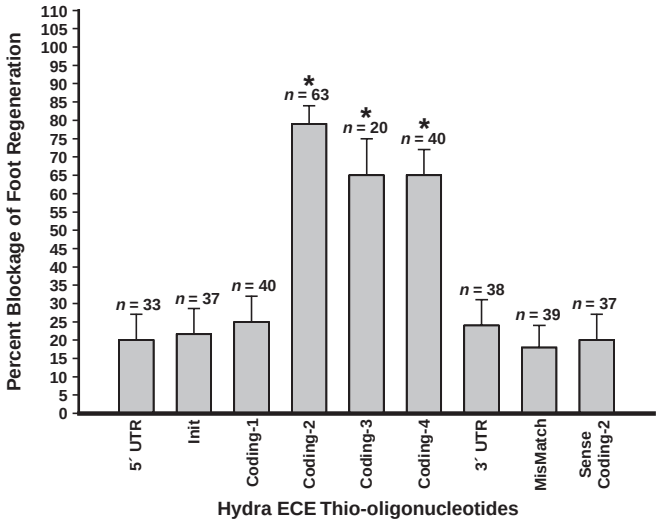

Fig. 6. Effects of antisense thio-oligonucleotides to hydra ECE on foot regeneration. Asterisk shows significant a difference when compared with sense or mismatch controls using the χ^2 analysis. *n*, number of hydra analyzed per group.

Coding-2, Coding-3 or Coding-4 thio-oligonucleotides indicates that the blocking effect was not the result of general toxicity of the oligos. Consistent with the late appearance of ECE during head regeneration (at the time when tentacles first appear), antisense experiments after decapitation had no effect on tentacle evagination and head regeneration proceeded normally (data not shown). In head regeneration experiments, antisense oligonucleotides were introduced into the endoderm at the time of decapitation or at 24 hours following decapitation. The 24 hour time point was used to effect hydra mRNA that may be expressed later in the head regeneration process, when compared with that observed during foot regeneration. Introduction of thio-oligonucleotides at the time zero or 24 hour time point had no inhibitory effect on tentacle evagination. An important limitation regarding these experiments stems from the lack of an antibody to hydra ECE. In previously studies where antibodies were available to the protein of interest (Deutzmann, et al., 2000, Yan et al., 2000a, Yan et al., 2000b,), we were able to document that the antisense thio-oligonucleotides did block translation of the protein. Two additional studies involving hydra laminin α-chain and β-chain also show that this antisense approach specifically blocks protein translation (M. P. S., unpublished). Because no antibody was available to monitor translation of hydra ECE, additional experiments were performed to determine if the antisense experiments were specific for the targeted mRNA. These studies incorporated the use of additional antisense thio-oligonucleotides to the 5′ and 3′ flanking regions of the Coding-2 region; the first oligo to give significant blocking effects. The rationale for these experiments was based on the premise that inhibition by multiple thio-oligonucleotides to a single mRNA sequence reduces the possibility that the effect is due to nonspecific effects on other mRNA species. As indicated above (and shown in Fig. 6), block was observed with both antisense thio-oligonucleotides (Coding-3 and Coding-4) that flanked the Coding-2 region.

DISCUSSION

Although putative, analysis of the sequence of this hydra metalloproteinase indicates that the protein should be categorized as an ECE 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 on from mutational studies with vertebrate ECE that have shown that replacement of Tyr^{568} in ECE to phyenylalanine reduces the V_{max}/K_m of ECE for the conversion of big ET to ET by 18-fold (Sansom et al., 1998). 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 (Shimada et al., 1996). Recent studies with solubilized recombinant ECE1 however, dispute the need for a dimer form of the enzyme (Korth et al., 1997). In addition, potential ECE clones in *C. elegans* (GenBank Accession Number, Q22523) and *Drosophila* (GenBank Accession Number, AAD34741) 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 monomer form.

Although previous studies have proposed an evolutionary relationship between the sarafotoxins of reptiles and mammalian ETs (Landan et al., 1991), analysis of non-chordates has been mainly limited to immuncytochemical studies (Kohidai and Csaba, 1995; Hasegawa and Kobayashi, 1991; Montuenga et al., 1994; Bernocchi et al., 1998). In functional studies by Kohidai and Csaba (Kohidai and Csaba, 1995), human ET1 has also been shown to induce physiological changes in *Tetrahymena*; however, as with our studies, these effects were not reported to be blocked by ET-receptor antagonists. Our contraction studies indicated that human ET1 and ET3 were effective at a concentration of 10^{-6} M, while ET2 had no effect at this or higher concentrations. These results are unexpected, as ET1 and ET2 are considered to be more potent vasoconstrictors that ET3 in vertebrates. The active form of each of these three peptides has 21 amino acids. ET1 and ET2 vary by two amino acids, whereas ET3 has four additional amino acid substitutions. In this regard, Landan et al. have analyzed the evolutionary relation of ETs and suggest from their work that ET3 is the more ancient form of the ET peptides (Landan et al., 1991). The effective concentration range for ET1 and ET3 is consistent with reports for endogenous hydra contractile peptides such as Hym-54 (Takahashi et al., 1997); however, it is one log higher than that reported for the effective concentration of ETs needed to induce contraction in human tissues under in vitro conditions (10^{-6} M in hydra versus 10^{-7} M in human tissue; Yap et al., 2000). It should be noted however, that because these peptides must gain access to the basolateral compartment of the hydra epithelial bilayer, we do not know the actual concentration of peptide that accumulates between the cells. It could in fact, be lower than the concentration in the medium. In any case, this later observation, coupled with the fact that ET-receptor antagonists were not effective in blocking ET-induced contraction of hydra, suggests that there are ligand and/or receptor differences in this peptide system in hydra when compared with vertebrates. Therefore, even though hydra ECE shows strong structural similarity to

vertebrate ECE, we feel it is best to propose this system as an endothelin-like system in hydra. The ability to classify this system in hydra as a bona fide endothelin system will require (1) cloning and/or isolation of a hydra endothelin (precursor and active form); and (2) biochemical analysis of expressed hydra ECE to determine if it can convert a hydra big ET to active ET. Given these qualifications, the structural and functional studies presented in the current study do indicate that a functional endothelin-like system diverged early during metazoan evolution. The existence of an endothelin-like system in invertebrates is also supported by putative *C. elegans* ECE fragments that are reported in the EST GenBank (Accession Number C52919). Similar to functions observed in higher chordates, the hydra endothelin-like system is involved in muscle contraction and developmental processes. These processes are associated with epithelio-muscular cells in hydra, as no distinct smooth muscle type cell or neural crest type cell exists in this simple metazoan. It should be noted, however, that previous ultrastructural studies on hydra have suggested that the epithelio-muscular cells of this simple invertebrate have some characteristics of a smooth muscle-like cell (Chapman, 1974). The antisense studies indicate a direct role of ECE in early phases of foot regeneration. The fact that (1) ECE is expressed late in head regeneration (following eruption of tentacles); and (2) antisense experiments during head regeneration could not block tentacle morphogenesis; implies that in contrast to foot regeneration, ECE is involved in events downstream of the initial morphogenetic signals occurring during head regeneration. The events are probably associated with specific aspects of tentacle development that occur subsequent to the initial eruption of the tentacles.

The early divergence of important receptor systems, signaling molecules, and crucial metalloproteinases is not unique to components of the endothelin-like system in hydra. For example, other studies have shown the presence in hydra of an insulin-like receptor (Steele et al., 1996); ligands and receptor systems of the Wnt pathway (Hobmayer et al., 2000; Minobe et al., 2000); homeobox genes (Gauchat et al., 2000); various ECM components, such as laminin (Sarras et al., 1994), fibrillar collagen (Duetzmann, et al., 2000) and collagen type IV (Fowler et al., 2000); matrix metalloproteinases (Leontovich et al., 2000); and astacin class metalloproteinases (Kumpfmuller et al., 1999; Yan et al., 1995; Yan et al., 2000a; Yan et al., 2000b). Together, these findings suggest that the endothelin system is widely used by a broad spectrum of metazoans; they also indicate that this signaling system arose early in evolution even before divergence of protostomes and deuterostomes.

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REFERENCES

Bernocchi, G., Vignola, C., Scherini, E, Necchi, D and Pisu, M. B. (1998). Bioactive peptides and serotonin immunocytochemistry in the cerebral ganglia of hibernating helix aspersa. *J. Exp. Zool.* **280**, 354-367.

Bode, H., Dunne, J., Heimfeld, S., Huang, L., Javois, L., Koizumi, O., Westerfield, J. and Yaross, M. (1986). Transdifferentiation occurs continuously in adult hydra. *Curr. Top. Dev. Biol.* **20**, 257-280.

Bode, H. R. (1996). The interstitial cell lineage of hydra: a stem cell system that arose early in evolution. *J. Cell Sci.* **109**, 1155-1164.

Bode, W., Gomis, R. F. and Stockler, W. (1993). Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'. *FEBS Lett.* **331**, 134-140.

Bosch, T. C. G. (1998). In *Cellular and Molecular Basis of Regeneration: From Invertebrates to Humans* (ed. O. Ferrete, P. and J. Geraudie), pp. 111-134. New York: Wiley.

Cesarone, C. F., Bolognesi, C. and Santi, L. (1979). Improved microfluorometric DNA determination in biological material using 33258 Hoechst. *Anal. Biochem.* **100**, 188-197.

Chapman, D. M. (1974). In *Coelenterate Biology* (ed. L. Muscatine and H. M. Lenhoff), pp. 2-48. New York: Academic Press

Clouthier, D. E., Hosoda, K., Richardson, J. A., Williams, S. C., Yanagisawa, H., Kuwaki, T., Kumada, M., Hammer, R. E. and Yanagisawa, M. (1998). Cranial and cardiac neural crest defects in endothelin-A receptor-deficient mice. *Development* **125**, 813-824.

Deutzmann, R., Fowler, S., Zhang, X., Boone, K., Dexter, S., Boot-Handford, R. P., Rachel, P., Sarras, M. P. Jr (2000). Molecular, Biochemical, and Functional Analysis of a Novel and Developmentally Important Fibrillar Collagen (Hcol-I) in Hydra. *Development* **127**, 4669-4680.

Douglas, S. A. and Ohlstein, E. H. (1993). Endothelin-1 modulates vascular smooth muscle structure and vasomotion-Implication in cardiovescular pathophysiology. *Drug Dev. Res.* 108-122.

Douglas, S. A. and Ohlstein, E. H. (1997). Signal transduction mechanisms mediating the vascular actions of endothelin. *J. Vasc. Res.* **34**, 152-164.

Field, K. G., Olsen, G. J., Lane, D. J., Giovannoni, S. J., Ghiselin, M. T., Raff, E. C., Pace, N. R. and Raff, R. A. (1988). Molecular phylogeny of the animal kingdom. *Science* **239**, 748-753.

Flanagan, W. M., Kothavale, A. and Wagner, R. W. (1996). Effects of oligonucleotide length, mismatches and mRNA levels on C-5 propyne-modified antisense potency. *Nucleic Acids Res.* **24**, 2936-2941.

Fowler, S. . Jose, S., Zhang, X., Deutzmann, R., Sarras, M. P. Jr and Boot-Handford, R. (2000). Characterization of Hydra type IV collagen: Type IV collagen is essential for head regeneration and its expression is up-regulated upon exposure to glucose. *J. Biol. Chem.* **275**, 39589-39599.

Gauchat, D., Mazet, F., Berney, C., Schummer, M., Kreger, S., Pawlowski, J. and Galliot, B. (2000). Evolution of Antp-class genes and differential expression of Hydra Hox/paraHox genes in anterior patterning. *Proc. Natl. Acad. Sci. USA* **97**, 4493-4498.

Grens, A., Mason, E., Marsh, J. L. and Bode, H. R. (1995). Evolutionary conservation of a cell fate specification gene: the Hydra achaete-scute homolog has proneural activity in Drosophila. *Development* **121**, 4027-4035.

Hasegawa, K and Kobayashi, H. (1991) Immunohistochemical localization of endothelin-1 in the nervous system of the earthworm Eisenia foetida. *Gen. Comp. Endocrinol.* **81**, 433-441

Haynes, J. F., Burnett, A. L. and Davis, LE. (1968) Histological and ultrastructural study of the muscular and nervous systems of hydra. *J. Exp. Zool.* **167**, 253-294.

Hobmayer, B., Rentzxch, F., Kuhn, K., Happel, C. M., Cramer von Laue, C., Snyder, P., Rothbacher, U. and Holstein, T. W. (2000). WNT signaling molecules act in axis formation in the diplobastic metazoan Hydra. *Nature* **407**, 186-189.

Hoffmeister, S. A. (1996). Isolation and characterization of two new morphogenetically active peptides from Hydra vulgaris. *Development* **122**, 1941-1948.

Kohidai, L. and Csaba, G. (1995) Effects of the mammalian vasoconstrictor peptide, endothelin-1, on Tetrahymena pyriformis GL, and the immunocytological detection of endogenous endothelin-like activity. *Comp. Biochem. Physiol* **111**, 311-316.

Korth, P., Egidy, G., Parnot, C., LeMoullec, J. M., Corvol, P. and Pinet, F. (1997). Construction, expression and characterization of a soluble form of human endothelin-converting-enzyme-1. *FEBS Lett.* **417**, 365-370.

Kumpfmuller, G., Rybakine, V., Takahashi, T., Fujisawa, T., Bosch, T. C. G. (1999). Identification of an astacin matrix metalloproteinase as target gene for Hydra foot activation peptides. *Dev. Genes Evol.* **209**, 601-607.

Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W. H., Kamada, N. et al. (1994). Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature* **368**, 703-710.

Landan, G., Bdolah, A., Wollberg, Z., Kochva, E. and Graur, D. (1991). The evolutionary history of the sarafotoxin/endothelin/endothelin-like superfamily. *J.Cardiovasc.Pharmacol*. **17**, Suppl. 7, S517-S519

Leontovich, A. A., Zhang, J., Shimokawa, K., Nagase, H. and Sarras, M. P. Jr (2000). A novel hydra matrix metalloproteinase (hmmp) functions in extracellular matrix degradation, morphogenesis, and the maintenance of differentiated cells in the foot process. *Development* **127**, 907-920

Marcum, B. A., Fujisawa, T. and Sugiyama, T. (1980). *Developmental and Cellular Biology of Coelenterates*. Amsterdam: Elsevier.

Minobe, S., Fei, K., Yan, L., Sarras, M. P. Jr and Werle, M. J. (2000). Identification and characterization of the epithelial polarity receptor, 'Frizzled' in Hydra vulgaris. *Dev. Genes Evol.* **210**, 507-511.

Montuenga, L. M., Prado, M. A., Springall, D. R., Polak, J. M. and Sesma, P. (1994) Endothelin-like immunoreactivity in midgut endocrine cells of the desert locust, Locusta migratoria. *Gene. Comp. Endocrinol.* **93**, 9-20.

Nelson, J. B., Lee, W. H., Nguyen, S. H., Jarrard, D. F., Brooks, J. D., Magnuson, S. R., Opgenorth, T. J., Nelson, W. G. and Bova, G. S. (1997). Methylation of the 5′ CpG island of the endothelin B receptor gene is common in human prostate cancer. *Cancer Res.* **57**, 35-37.

Resink, T. J., Scott, B. T. and Buhler, F. R. (1990). Activation of multiple signal transduction pathways by endothelin in cultured human vascular smooth muscle cells. *Eur. J. Biochem.* **189**, 415-421.

Rubanyi, G. M. and Polokoff, M. A. (1994). Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol. Rev.* **46**, 325-415.

Saitou, N., and Nei, M. (1987). Reconstructing phylogenetic trees. *Mol. Biol. Evol*. **4**, 406-425.

Sansom, C. E., Hoang, M. V. and Turner, A. J. (1998). Molecular modelling and site-directed mutagenesis of the active site of endothelin-converting enzyme. *Prot. Eng.* **11**, 1235-1241.

Sarras, M. P. Jr, Madden, M. E., Zhang, X. M., Gunwar, S., Huff, J. K. and Hudson, B. G. (1991). Extracellular matrix (mesoglea) of Hydra vulgaris. I. Isolation and characterization. *Dev. Biol.* **148**, 481-494.

Sarras, M. P. Jr, Yan, L., Grens, A., Zhang, X., Agbas, A., Huff, J. K., St, J. P. and Abrahamson, D. R. (1994). Cloning and biological function of laminin in Hydra vulgaris. *Dev. Biol.* **164**, 312-324.

Sattath, S. and Tversky, A. (1977). Additive similarity trees. *Psychometrika* **42**, 319-345.

Shimada, K., Takahashi, M., Turner, A. J. and Tanzawa, K. (1996). Rat endothelin-converting enzyme-1 forms a dimer through Cys412 with a similar catalytic mechanism and a distinct substrate binding mechanism compared with neutral endopeptidase-24.11. *Biochem. J.* **315**, 863-867.

Slauterback, D. B. (1967). The cnidoblast-musculoepithelial cell complex in the tentacles of hydra. *Zeitsehrift fur Zellforschung* **79**, 296-318.

Steele, R. E., Lieu, P., Mai, N. H., Shenk, M. A. and Sarras, M. P. Jr (1996). Response to insulin and the expression pattern of a gene encoding an insulin receptor homologue suggest a role for an insulin-like molecule in regulating growth and patterning in Hydra. *Dev. Genes Evol*. **206**, 247-259.

Takahashi, T., Muneoka, Y., Lohmann, J., Lopez-de, H. M., Solleder, G., Bosch, T. C., David, C. N., Bode, H. R., Koizumi, O., Shimizu, H., Hatta, M., Fujisawa, T. and Sugiyama, T. (1997). Systematic isolation of peptide signal molecules regulating development in hydra: LWamide and PW families. *Proc. Natl. Acad. Sci. USA* **94**, 1241-1246.

Wagner, R. W. (1994). Gene inhibition using antisense oligodeoxynucleotides. *Nature* **372**, 333-335.

Wagner, R. W. (1995). The state of the art in antisense research. *Nat. Med.* **1**, 1116-1118.

Wu, W. J., Chiou, W. J., Dickinson, R. and Opgenorth, T. J. (1997). Endothelin attenuates apoptosis in human smooth muscle cells. *Biochem. J.* **328**, 733-737.

Xu, D., Emoto, N., Giaid, A., Slaughter, C., Kaw, S., deWit, D. and Yanagisawa, M. (1994). ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell* **78**, 473-485.

Yan, L., Pollock, G. H., Nagase, H. and Sarras, M. P. Jr (1995). A 25.7×10^3 M_r hydra metalloproteinase (HMP1), a member of the astacin family, localizes to the extracellular matrix of Hydra vulgaris in a head-specific manner and has a developmental function. *Development* **121**, 1591-1602.

Yan, L., Fei, K., Zhang, J., Dexter, S. and Sarras, M. P. Jr (2000a). Identification and characterization of hydra metalloproteinase 2 (hmp2): a meprin-like astacin metalloproteinase that functions in foot morphorgenesis. *Development* **127**, 129-141

Yan, L., Leontovich, A. A., Fei, K. and Sarras, M. P. Jr (2000b). Hydra metalloproteinase 1: a secreted astacin metalloproteinase whose apical axis expression is differentially regulated during head regeneration. *Dev. Biol.* **219**, 115-128.

Yanagisawa, H., Yanagisawa, M., Kapur, R. P., Richardson, J. A., Williams, S. C., Clouthier, D. E., de, W. D., Emoto, N. and Hammer, R. E. (1998). Dual genetic pathways of endothelin-mediated intercellular signaling revealed by targeted disruption of endothelin converting enzyme-1 gene. *Development* **125**, 825-836.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**, 411-415.

Yap, E. Y., Battistini, B. and McKay, K. O. (2000). Contraction to big endothelin-1, big endothelin-2 and big endothelin-3, and endothelin-converting enzyme inhibition in human isolated bronchi. *Br. J. Pharmacol.* **129**, 170-176.