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SCIENCE DIRECT

Gene 334 (2004) 133–143

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

www.elsevier.com/locate/gene

Ependymin, a gene involved in regeneration and neuroplasticity in vertebrates, is overexpressed during regeneration in the echinoderm *Holothuria glaberrima*

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Received 27 October 2003; received in revised form 25 February 2004; accepted 18 March 2004
Available online 7 May 2004
Received by R. Di Lauro

Abstract

We report the characterization of an ependymin-related gene (*EpenHg*) from a regenerating intestine cDNA library of the sea cucumber *Holothuria glaberrima*. This finding is remarkable because no ependymin sequence has ever been reported from invertebrates. Database comparisons of the conceptual translation of the *EpenHg* gene reveal 63% similarity (47% identity) with mammalian ependymin-related proteins (MERPs) and close relationship with the frog and piscine ependymins. We also report the partial sequences of ependymin representatives from another species of sea cucumber and from a sea urchin species. Conventional and real-time reverse transcriptase polymerase chain reaction (RT-PCRs) show that the gene is expressed in several echinoderm tissues, including esophagus, mesenteries, gonads, respiratory trees, hemal system, tentacles and body wall. Moreover, the ependymin product in the intestine is overexpressed during sea cucumber intestinal regeneration. The discovery of ependymins in echinoderms, a group well known for their regenerative capacities, can give us an insight on the evolution and roles of ependymin molecules.

Keywords: Invertebrate; Sea cucumber; Extracellular matrix; Learning and memory; Development

1. Introduction

Ependymin, a secretory glycoprotein that is the predominant protein in the cerebrospinal fluid (CSF) of many teleost fish, was initially identified in the ependymal zone of goldfish brain (Shashoua, 1977; Hoffmann and Schwarz, 1996). Subsequently, the ependymins of several other fishes have been localized (Rother et al., 1995) and their proteins and gene sequences characterized (Orti and Meyer, 1996). More recently, genes belonging to a family of ependymin-related proteins have been identified in the frog *Xenopus laevis* and in mammals (i.e., human, monkey and mouse) (Apostolopoulos et al., 2001; Nimmrich et al., 2001). Since, until now, ependymins had only been found in vertebrate species, they were proposed to be vertebrate-specific molecules that define the evolution of the chordate nervous system (Landers et al., 2001; Venter et al., 2001; Ponting and Russell, 2002).

Abbreviations: *EpenHg*, *Holothuria glaberrima* ependymin-related gene; EST, expressed sequence tag; *EpenHm*, *Holothuria mexicana* ependymin-related EST; *EpenLv*, *Lytechinus variegatus* ependymin-related EST; MERP, mammalian ependymin-related protein; UCC1, upregulated in colon cancer 1; dpe, days post-evisceration; ECM, extracellular matrix; ENS, enteric nervous system; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; aa, amino acid(s); bp, base pair(s); MW, molecular weight; ORF, open reading frame; UTR, untranslated region; BLAST, an algorithm for sequence comparison; S.E.M., standard error of the mean; S.D., standard deviation.

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doi:10.1016/j.gene.2004.03.023

Whereas the physiological roles of ependymins have not been clearly elucidated, they are known to undergo enhanced expression during neuroplasticity in memory consolidation (Shashoua, 1991; Rother et al., 1995; Pradel et al., 1999), optic nerve regeneration (Schmidt and Shashoua, 1988) and cold exposure (Tang et al., 1999). Extensive evidence classifies the ependymins as important molecules of the extracellular matrix (ECM) responding to calcium levels (Shashoua, 1991; Ganss and Hoffmann, 1993; Pradel et al., 1999). The ependymins have also been postulated as a new class of antiadhesive molecules, playing a key role in establishing specific cell contacts during neural regeneration, differentiation and cell migration (Hoffmann and Schwarz, 1996; Nimmrich et al., 2001). Additionally, short peptides derived from the goldfish ependymins have been shown recently to work as neurotrophic factors by activating the AP-1 transcription factor regulating neuronal cell survival, proliferation and axon guidance (Shashoua et al., 2001; Adams et al., 2003).

Here, we report the characterization of an echinoderm ependymin-related gene (*EpenHg*) in the sea cucumber *Holothuria glaberrima*, and the partial sequences of the representatives from another species of sea cucumber *Holothuria mexicana* (*EpenHm*) and from the sea urchin *Lytechinus variegatus* (*EpenLv*). These are the first invertebrate members of the family of ependymin-related genes, and their discovery rules out the possibility that ependymins represent markers of the chordate lineage as previously suggested (Ponting and Russell, 2002). Moreover, the echinoderms comprise a group of animals that show amazing regenerative capacities and are phylogenetically related to chordates (Hyman, 1955).

Sea cucumbers exposed to adverse stimuli respond by ejecting most of their internal organs. This evisceration process is followed by a period of regeneration during which the ejected organs are replaced. The intestinal system is the first organ to be regenerated (García-Arrarás and Greenberg, 2001). Here, we also show that the *EpenHg* gene is overexpressed in *H. glaberrima* during intestinal regeneration. Previous studies in our laboratory have shown regeneration of the enteric nervous system (ENS) (García-Arrarás et al., 1999) and a role of the ECM in the formation of the new intestine (Quiñones et al., 2002). The identification of ependymin-related genes in invertebrate animals undergoing a complex process of regeneration that involves ECM remodelation, cell proliferation, migration, differentiation and neuroplasticity can give new insights on the evolution of ependymin molecules and their functions in regeneration-related processes.

2. Materials and methods

2.1. Animals

Adult sea cucumbers *H. glaberrima*, *H. mexicana* and sea urchins *L. variegatus* specimens were collected in surrounding water of Puerto Rico and maintained in seawater aquaria at 22–24 °C. Evisceration of sea cucumbers was induced by KCl 0.35 M injections (2 ml) into the coelomic cavity. Prior to the dissections, animals were anesthetized by placement in ice cold water for 1 h. The intestines of non-eviscerated and regenerating animals at 3, 5, 7, 14, 21 and 28 days post-evisceration (dpe) were dissected in RNAse-free conditions. The samples were stabilized and stored in RNAlater (Ambion, Austin, TX) until use. Tissues such as esophagus, mesenteries, gonads, tentacles, hemal system, body wall and respiratory trees collected from either non-eviscerated sea cucumbers or sea urchins were obtained using the same procedures.

2.2. Isolation and sequencing of the EpenHg gene

The *EpenHg* clone was obtained by mass excision and random sequencing of clones from a *H. glaberrima* cDNA library from the early stage of intestinal regeneration (5–7dpe). This is a unidirectional library made in our laboratory using Stratagene's Uni-Zap kit (Stratagene, La Jolla, CA), whose construction has been described previously (Méndez et al., 2000). The initial and several confirmatory sequence reactions of the *EpenHg* clone were obtained by using forward and reverse primers, using the DYEnamic ET Dye Terminator Kit as described by the manufacturer (APBiotech:Amersham, Piscataway, NJ) and read on a MegaBACE DNA Analysis system (Molecular Dynamics/APBiotech:Amersham). Contig assembly and sequence edition were done using Sequencher v 3.1.1 (GeneCodes, Ann Arbor, MI).

2.3. Characterization of invertebrate ependymin-related genes with bioinformatics, phylogeny and hydropathy profiles

Sequences of piscine ependymins and other ependymin-related proteins were obtained from the GeneBank database (NCBI). Sequences were aligned with the software CLUSTALX v.1.81 using the BLOSUM30 matrix. GeneDoc v.2.6.002 was used for multiple sequence alignment edition and shading. The phylogenetic unrooted tree was generated using TreeView v.1.6.1 based on the alignments from the CLUSTALX package. The neighbor-joining method followed by bootstrapping was used for tree construction. The more probable open reading frame (ORF) for each cDNA clone characterized was determined using the Sixframe option on Biology Workbench 3.2. For the accurate determination of the presence and location of the signal peptide cleavage site in the amino acid sequences, the web based SignalP V2.0.b2 prediction software was run using neural networks and hidden Markov models trained on eukaryotes. Hydropathic profiles were obtained in the ProtScale utility, selecting the Kyte–Doolittle method of calculating hydrophilicity over a window length of 9. Other tools for protein sequence analysis (prediction of cysteine bond and the

N-glycosylation sites) were run on the PredictProtein server.

2.4. cDNA synthesis and analysis by conventional RT-PCR

Total RNA from tissue was extracted using the RNAqueous-4PCR kit following the recommendations of the manufacturer (Ambion) and treated with 4 U of DNase 1 (RNAse-free) included in the kit. cDNA was synthesized from 2 μg of total RNA using random primers and the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA) following the manufacturer's guidelines. The success of the cDNA synthesis was evaluated by a 25-μl conventional reverse transcriptase polymerase chain reaction (RT-PCR) using 5% of the cDNA synthesis reaction (1 μl) for co-amplification of the internal standard cytochrome *b* (161 base pairs [bp]) (isolated in our laboratory), and an *EpenHg* sector (437 bp) delimited by the forward primer EpenHgF1: (5′-CGG GGA TCA ATG TTT CAC C-3′) and reverse primer EpenHgR: (5′-GGA GTG TAG ATG CCA ATC CAG C-3′). The RT-PCR reaction was done in saturating conditions (35 cycles, 58 °C annealing temperature). Negative RT-PCR controls included reactions containing all reagents and template except the reverse transcriptase enzyme to verify that there was no genomic amplification and a reaction without templates to verify the absence of reagent contaminants. No amplified fragments corresponding to genomic DNA and/or reactant contaminants were detected in the conventional RT-PCRs.

2.5. Isolation of other echinoderm ependymin-related ESTs

Primers designed from the sequence of the *H. glaberrima* (*EpenHg*) ependymin-related gene were used to amplify other echinoderm ependymin-related expressed sequence tags (ESTs). RNA extractions, cDNA syntheses and reverse transcriptase PCR (RT-PCR) reactions were done as described in Section 2.4. Primers used were EpenHgF1 and EpenHgR for *H. mexicana*, and EpenHgF1 and qEpenHgR (see primer sequence in Section 2.7) for *L. variegatus*. The amplified ESTs were cloned into pCR4-TOPO TA cloning vector (Invitrogen) following the manufacturer's recommendations and sequenced (both strands) as described in Section 2.2.

2.6. Expression analysis by Northern blot

For size determination of the echinoderm ependymin transcripts, total RNA (from 1 to 10 μg) along with 4 μg of a RNA size marker (Millennium Markers-Formamide, Ambion) were electrophoresed on a 1% denaturing agarose gel and transferred overnight to nylon Hybond-N+ membrane (APBiotech:Amersham) by capillary blotting. The RNA was UV cross-linked to the blot and prehybridized for 45 min in hybridization buffer at 55 °C. A DNA probe from an *EpenHg* sector (299 bp) delimited by the forward primer EpenHgF2: (5′-GGC ATA GGT ATG AGG AAG AGG G-3′), and the reverse primer EpenHgR was labeled with alkaline phosphatase by using the Alk-phos direct labeling system, as per the manufacturer's protocol (APBiotech:Amersham). Hybridization at 55 °C, blot washes and chemiluminescent signal detection with CDP-Star (APBiotech:Amersham) were conducted without modification according to instructions provided by the supplier.

2.7. Expression analysis by real-time RT-PCR

The differential gene expression of *EpenHg* was semi-quantitatively determined by the real-time RT-PCR technique using the SYBR Green1 Dye Method. Primers amplifying an *EpenHg* sector (165 bp) were designed for optimal performance using web-based tools, particularly the Whitehead Institute's Primer3 program, the Oligo Plot option in the Qiagen's Oligo Toolkit and the Zuker's DNA mfold server. The real-time RT-PCR reaction was done in triplicate following the recommendations of the manufacturer (QuantiTect SYBR Green PCR kit, Valencia, CA) with the following modifications: 1μl of a 1:4 dilution of the cDNA synthesized as described in Section 2.4 was used as template in a 25-μl reaction containing 12.5 μl of 2× QuantiTect SYBR Green PCR Master Mix, 0.3 μM of primer forward qEpenHgF: (5′-GCA AAA CCA CAC CAT TCC T-3′), 0.3 μM of primer reverse qEpenHgR: (5′-CAG CAA CCA CCA TTC TCT GTT-3′), and RNase-free water. For the relative quantification of *EpenHg*, 0.4 μM Universal 18S PCR primer pair and 0.6 μM 18S PCR competimers™ (4:6 ratio) were used as reference for normalization (Quantu*mRNA* 18S Internal Standards, Ambion). Three standard curves were generated from six serial dilutions of a sample amplified in parallel with either the qEpenHg primers or the 18S standards. The platform used was the iCycler IQ Detection System (Bio Rad, Hercules, CA). Activation was performed in a 15-min, 95 °C incubation step, followed by 40 cycles of amplification with denaturation, 15 s at 95 °C, and 1 min annealing at 58 °C. The specificity of the amplified products was assessed by a standard melting protocol. The starting quantity of *EpenHg* and *18S* were calculated using the resulting Ct (threshold cycle) values of the samples and the corresponding standard curve. Quantitation was done by determining the ratio between the calculated starting quantity of *EpenHg* for a specific regeneration stage and that of the *18S* gene in the same sample. Arbitrary units were used to illustrate the *EpenHg*'s gene expression (mean ± S.E.M.) calculated from triplicate determinations (at least five animals per stage were sampled). Negative controls included reactions containing all reagents except template, and reactions containing all reagents and DNA template to verify that there was no genomic amplification of *EpenHg* or *18S*. The amplicon size was confirmed by agarose gel electrophoresis of the PCR products from each primer pair. The same real-time RT-PCR procedure was used to detect the expression of the sea cucumber

ependymin-related genes (*EpenHg* and *EpenHm*) in several tissues from non-regenerating animals. In this case, the expression values (mean ± S.D.) were calculated from triplicate measurements of one sample.

3. Results and discussion

3.1. Identification and sequence analysis of the EpenHg gene

The ependymin clone was isolated from a cDNA library of *H. glaberrima* intestine regenerating. This clone of 1449-bp length features an ORF of 234 amino acids (aa) preceded by a consensus Kozak sequence (Kozak, 1995) (Fig. 1). No TATA box is observed within the 41-bp 5′ untranslated region (UTR), nor is a canonical polyadenylation signal present in the 655-bp 3′ UTR. However, two potential polyadenylation signals, ATTAAA and AATACA (Beaudoing et al., 2000), are found 81 and 27 bp before the poly(A) tail, respectively. A lack of canonical poly(A) addition signals has also been observed in the 3′ UTRs of other echinoderms (Loguercio-Polosa et al., 1999). The sequence analysis of the conceptual translation of the ependymin clone reveals 63% similarity (47% identity) with mammalian ependymin-related proteins MERP-1/upregulated in colon cancer 1 (UCC1) (accession no. AY027862 and AJ250475, respectively) (Apostolopoulos et al., 2001; Nimmrich et al., 2001), close relationship (30% similarity, 40% identity) with the frog *X. laevis* (accession no. BG553216.1) and 40–46% similarity, 24–32% identity with the piscine ependymins (Orti and Meyer, 1996). No significant homology with non-ependymin proteins is found. With some modifications, possibly related to the echinoderm lineage, this clone has the hallmarks of ependymin proteins, such as the unique four conserved cysteines at positions 31, 102, 165 and 206, computationally predicted to form disulfide bonds and postulated to be crucial in dimeric interactions. Also found are the invariable residues (D^{59}, L^{107}, P^{117}, G^{129}, and W^{140}), whose distribution is strictly conserved in all ependymin sequences analyzed so far. Thus, with the characterization of the *EpenHg* transcript, we have named this clone the *H. glaberrima* ependymin-related gene (*EpenHg*), and its sequence has been submitted to the GenBank database with the accession no. AY383544 (Fig. 1). The finding of an ependymin-related sequence expressed in the sea cucumber is remarkable because no ependymin sequence has ever been reported from inverte-

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                                                        M  N  K  L  I  L  L
1       aattcggcacgaggtaacgttagctcgacgaaagaagaacgatgaacaaacttattcttc  60
          S  V  V  V  A  V  A  T  G↓I  N  V  S  P  S  T  G  V  E  Q
61      tctctgtcgtcgttgccgtggcaacggggatcaatgtttcaccttcaactggcgtcgagc  120
          K  A  P [C] Q  T  A  E  Q  W  E  G  R  A  S  E  W [D] H  V
121     agaaggcaccctgccagactgcggaacagtgggagggacgtgccagtgaatgggaccatg  180
          Q  G  R  N  N  R  F  L  I  S  F  D  G  I  G  M  R  K  R  V
181     ttcagggtagaaacaaccgatttctgatcagtttcgatggcataggtatgaggaagaggg  240
          I  E  E  K  K  S  F  M  P  G  R  R  F  Y  E  Y  I  M  E  Y
241     tgattgaggagaagaagtcgtttatgcctggacgaagattttacgagtacatcatggaat  300
          K  T  N  K  M  Y  T  I  N  M  N  L  G  T [C] T  V  S  T [L]
301     acaagaccaacaaaatgtataccataaacatgaacttgggcacgtgcaccgtctcaacac  360
          K  Q  P  W  Q  N  H  T  I [P] S  D  A  T  L  E  D  E  Y  E
361     tgaagcaaccatggcaaaaccacaccattccttccgatgccaccctagaagacgaatatg  420
          M [G] D  P  G  S  G  L  N  V  Q  E [W] S  D  R  L  P  A  R
421     agatgggtgatcctggttctggcctcaatgtccaggagtggtctgacagactccccgcca  480
          R  S  E  S  W  I  G  I  Y  T  P  K  T  E  N  G  G [C] W  P
481     ggagaagcgagagctggattggcatctacactcctaaaacagagaatggtggttgctggc  540
          V  V  E  V  F  T  D  D  T  T  D  P  P  V  S  L  T  T  R  F
541     ctgtggtggaagttttcacagacgacactaccgatcctcccgtttccttgacaacgcgtt  600
          F  D  I  T  P  G  I  K  N  M  S  V  F  I  P  P  P  S [C] N
601     tctttgacatcacgcctgggataaagaacatgtcagtcttcattcctccccttcctgta  660
          Q  N  V  E  I  V  V  E  N  V  E  N  S  G  P  I  Y  T  F  Y
661     atcagaacgtggagatcgtagtggaaaacgtggagaatagtggccccatttatacttttt  720
          N  Y  I  K  S  W  F  *
721     acaactacatcaagtcctggttttagttttgtcaattttcatcccatcaacattcatcaa  780
781     acagaaatgacgtacgcatgaccttctgcttggtgttactcatgtgcctggcaaagcgat  840
841     cattaacaaaaaagtctaaatgaatttatgtgaagcaaatagatttcttgtaggtgcag  900
901     atttacgttagaagaacgttaatctgtttatggtatcaagcaacacttttaataggtcg  960
961     cgtccaacccgtcctctaagactttcttttaaaatcaagtatttgaagggagagtaaata  1020
1021    gacttgcaacatgtaccgcatctcctatgttttgtgatagaataataattatcacagtgc  1080
1081    atttttctgcaagccacgaattgttttcattaatattcatttccatagctatagcaccaa  1140
1141    ttaagtgctgcccacttgttcatttccccgatttcactgattgaagggtagtgaatacct  1200
1201    acaggtaagtgagagattccacgaatattccatttttcgaatattgtgctttaatcaaag  1260
1261    cgtctgaaaatttgtaatatgccttccacatgaatatgttatgaggataactattgtaat  1320
1321    attaaaaatgtaccaactagcttccatgctagcaaacatgctcaaccgtttgaaaatacа  1380
1381    actgcagaaaattgcaatgttaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa  1440
1441    aaaaaaaaa  1449
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Fig. 1. Nucleotide sequence (1449 bp) of the *H. glaberrima* ependymin-related gene (*EpenHg*; accession no. AY383544) aligned with its predicted amino acid sequence (single-letter code). A consensus Kozak sequence (italic-underlined) and 41-bp 5′ UTR region precede the open reading frame (234 aa). The amino acids characteristic of ependymin proteins are boxed. The predicted cleavage site for signal peptidase is indicated by a vertical arrow. The putative N-glycosylation sites and polyadenylation signals are underlined. The stop codon is indicated by an asterisk.

brates. As a matter of fact, because of the previous failure to detect invertebrate representatives, the ependymins were cataloged as 1 of 94 vertebrate-specific protein domain families and 1 of 17 families associated with the evolution of the chordate nervous system (Landers et al., 2001; Venter et al., 2001; Ponting and Russell, 2002). Our results suggest that ependymins might really be a deuterostome-specific protein.

An in-depth analysis of *EpenHg* shows a signal peptide after the glycine residue at position ATG^{16}-IN. This signal is typical of secreted proteins and is highly predicted to be cleaved by signal peptidase releasing a 218-amino-acid peptide. Also, the hydropathic profile of the conceptual translation of *EpenHg* (Fig. 2) is consistent with the previous observation on ependymins proteins as predominantly hydrophilic proteins without transmembrane domains. The assumption of EpenHg as a secretory glycoprotein is also supported by the presence of two predicted N-glycosylation sites (PredictProtein server) at positions N^{113}H^{114}T^{115} and N^{196}M^{197}S^{198}. N-glysosylation sites are important for the proposed function of ependymin. They can serve as anchorage positions for different oligosaccharides such as glucuronic acid (Shashoua et al., 1986; Schmidt and Schachner, 1998). In addition, it has been suggested that residues of sialic acid attached to the N-glysosylation sites confer the ability to alter ependymin tertiary structure in the presence of calcium and allow its interaction with other ECM components (Ganss and Hoffmann, 1993; Hoffmann and Schwarz, 1996). The position of the two conserved N-glycosylation sites is strictly conserved among the piscine ependymins. Alternative glycosylation positions are also found to be strictly conserved in the mammalian and frog-related genes. We have found that in the *EpenHg* clone, the localization of putative N-glycosylation sites differ from those of vertebrate species. If these putative N-glysosylation sites are eventually shown to be glycosylated, then they will serve as characteristics unique to the echinoderm ependymins.

3.2. Identification and sequence analysis of other echinoderm ependymin-related ESTs

We have cloned the partial sequences (ESTs) of the ependymin-related gene from two other echinoderm species; the sea cucumber *H. mexicana* and the sea urchin *L. variegatus* using primers that amplify the most conserved region of ependymin genes. The 338-bp clone (*EpenHm*) from *H. mexicana* differs in seven nucleotide substitutions (transitions) from the *H. glaberrima* species, but only one of these causes a nonconserved change at the amino acid level (M^{92} in *EpenHg* is changed to T in *EpenHm*). In the 414-bp cloned region of (*EpenLv*) from *L. variegatus*, 10 nucleotide substitutions are also transitions, 2 of them produce amino acid changes (K^{88}, and M^{92} in *EpenHg* are changed by R and T, respectively, in *EpenLv*). The partial sequences of these echinoderm ependymin-related clones have been sub-

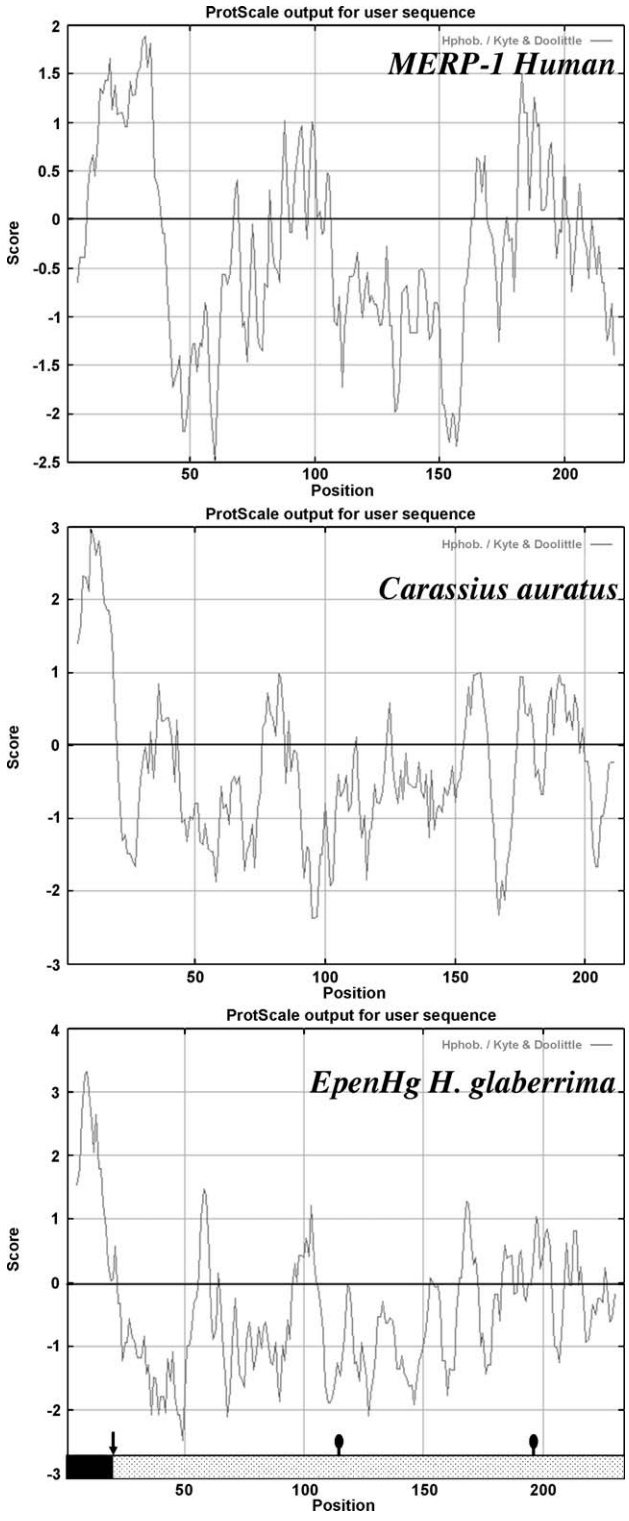
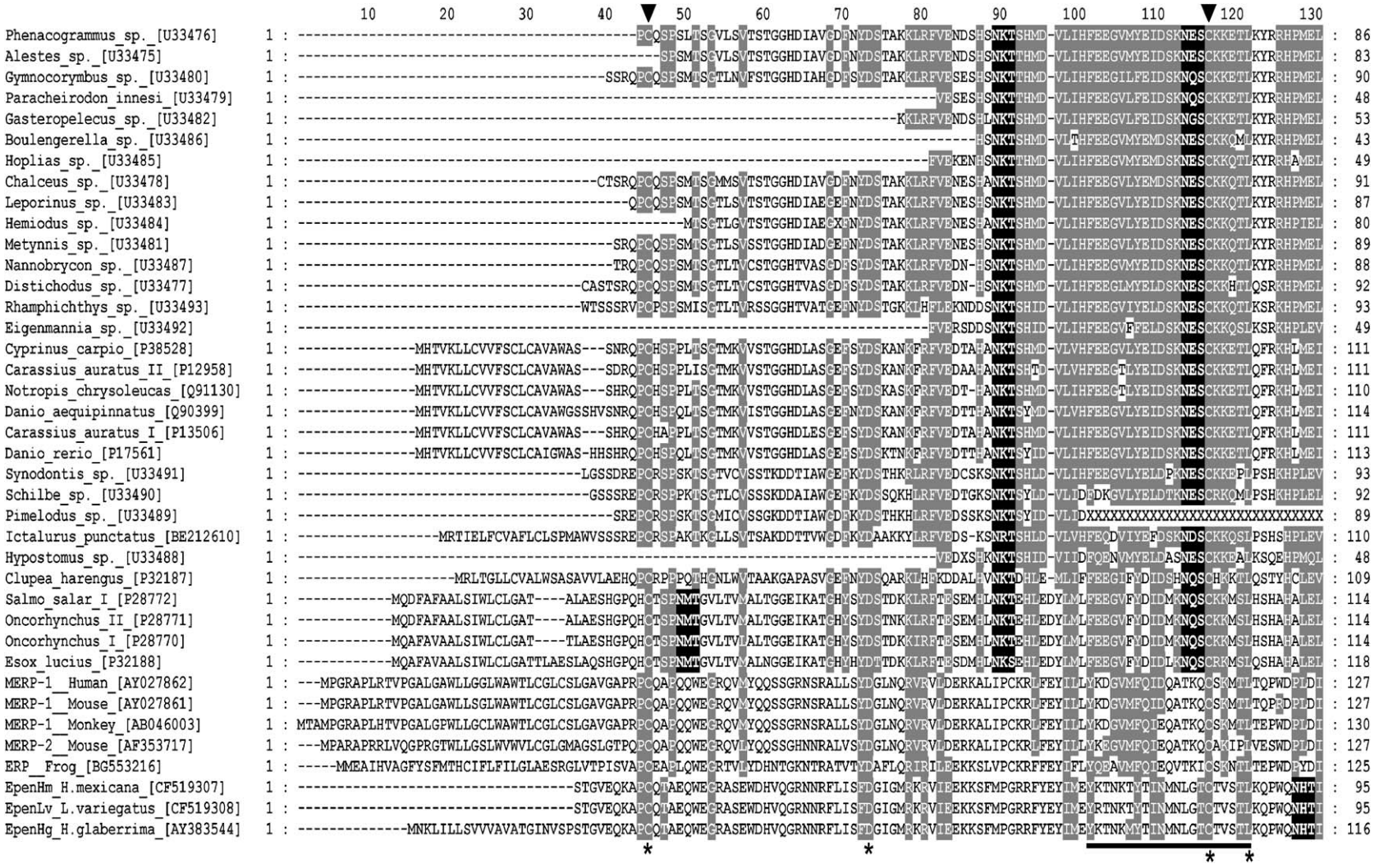


Fig. 2. Kyte and Doolitle hydropathic profile comparison between human MERP, goldfish ependymin and the echinoderm *EpenHg* gene. The vertical scale represents the hydropathic score for each amino acid, and the horizontal scale shows the amino acid sequence. Scores above zero are considered hydrophobic, while those below are considered hydrophillic. The diagram below the hydropathy profile of *EpenHg* shows the highly hydrophobic N-terminal signal peptide sequence black shaded, the predicted cleavage site indicated by an arrow and the potential N-glycosylation sites.


10 20 30 40 50 60 70 80 90 100 110 120 130
Phenacogrammus_sp._[U33476] 1 : -PCQSPSLMSCVLSVTSTGGHDIAVCDENYDSTAKKLRFVENDSHSNKTSHMD-VLIHFEEGVMYEIDSKNESCKKETLKYRRHPMEL : 86
Alestes_sp._[U33475] 1 : -SPSMMSCVLSVTSTGGHDIAVCDENYDSTAKKLRFVENDSHSNKTTHMD-VLIHFEEGVMYEIDSKNESCKKETLKYRRHPMEL : 83
Gymnocorymbus_sp._[U33480] 1 : -SSRQPCQSPSMMSCTLNVFSTGGHDIAHCDESYDSTAKKLRFVESESHSNKTTHMD-VLIHFEEGILFEIDSKNQSCKKETLKYRRHPMEL : 90
Paracheirodon_innesi_[U33479] 1 : -VESESHSNKTTHMD-VLIHFEEGVLFEIDSKNQSCKKETLKYRRHPMEL : 48
Gasteropelecus_sp._[U33482] 1 : -KKLRFVENDSHLNKTSHMD-VLIHFEEGVLFEIDSKNGSCKKETLKYRRHPMEL : 53
Boulengerella_sp._[U33486] 1 : -HSNKTSHMD-VLTHFEEGVMYEMDSKNESCKKQMLKYRRHPMEL : 43
Hoplias_sp._[U33485] 1 : -FVEKENHSNKTTHMD-VLIHFEEGVMYEMDSKNESCKKQTLKYRRHAMEL : 49
Chalceus_sp._[U33478] 1 : -CTSRQPCQSPSMMSCMMSVTSTGGHDIAVCDENYDSTAKKLRFVENESHANKTSHMD-VLIHFEEGVLYEMDSKNESCKKQTLKYRRHPMEL : 91
Leporinus_sp._[U33483] 1 : -QPCQSPSMMSCTLSVTSTGGHDIAECDENYDSTAKKLRFVENESHSNKTSHMD-VLIHFEEGVLYEIDSKNESCKKQTLKYRRHPMEL : 87
Hemiodus_sp._[U33484] 1 : -MMSCTLGVTSTGGHDIAECXENYDSTAKKLRFVENESHANKTSHMD-VLIHFEEGVLYEIDSKNESCKKQTLKYRRHPIEL : 80
Metynnis_sp._[U33481] 1 : -SRQPCQSPSMMSCTLSVSSTGGHDIADCDENYDSTAKKLRFVENESHSNKTSHMD-VLIHFEEGVLYEIDSKNESCKKQTLKYRRHPMEL : 89
Nannobrycon_sp._[U33487] 1 : -TRQPCQSPSMMSCTLTVCSTGGHTVASCDESYDSTAKKLRFVEDN-HSNKTSHMD-VLIHFEEGVMYEIDSKNESCKKQTLKYRRHPMEL : 88
Distichodus_sp._[U33477] 1 : -CASTSRQPCQSPSMMSCTLTVCSTGGHTVASCDESYDSTAKKLRFVEDN-HSNKTSHMD-VLIHFEEGLMYELDSKNESCKKHTLQSRKHPMEL : 92
Rhamphichthys_sp._[U33493] 1 : -WTSSSRVPCPSPSMISCTLTVRSSGGHTVATCEENYDSTGKKLHFLEKNDDSNKTSHID-VLIHFEEGVIYELDSKNESCKKQTLKSRKHPMEL : 93
Eigenmannia_sp._[U33492] 1 : -FVERSDDSNKTSHID-VLIHFEEGVFFELDSKNESCKKQSLKSRKHPLEV : 49
Cyprinus_carpio_[P38528] 1 : -MHTVKLLCVVFSCLCAVAWAS---SNRQPCHSPPLMSCTMKVVSTGGHDLASCEESYDSKANKFRFVEDTAHANKTSHMD-VLVHFEEGVLYEIDSKNESCKKETLQFRKHLMEI : 111
Carassius_auratus_II_[P12958] 1 : -MHTVKLLCVVFSCLCAVAWAS---SDRQPCHSPPLISCTMKVVSTGGHDLASCEESYDSKANKFRFVEDAAHANKTSHTD-VLVHFEECTLYEIDSKNESCKKETLQFRKHLMEI : 111
Notropis_chrysoleucas_[Q91130] 1 : -MHTVKLLCVVFSCLCAVAWAS---SHRQPCHSPPLMSCTMKVVSTGGHDLASCEESYDSKASKFRFVEDT-HANKTSHMD-VLIHFEECTLYEIDSKNESCKKETLQFRKHLMEI : 110
Danio_aequipinnatus_[Q90399] 1 : -MHTVKLLCVVFSCLCAVAWGSSHVSNRQPCHSPQLMSCTMKVISTGGHDLASCEENYDSKANKFRFVEDTTHANKTSYMD-VLVHFEEGVLYEIDSKNESCKKETLQFRKHLMEI : 114
Carassius_auratus_I_[P13506] 1 : -MHTVKLLCVVFSCLCAVAWAS---SHRQPCHAPPLMSCTMKVVSTGGHDLESCEESYDSKANKFRFVEDTAHANKTSHMD-VLIHFEEGVLYEIDSKNESCKKETLQFRKHLMEI : 111
Danio_rerio_[P17561] 1 : -MHTVKLLCVVFSCLCAIGWAS-HHSHRQPCHSPQLMSCTMKVVSTGGHDLASCEESYDSKTNKFRFVEDTTHANKTSYID-VLIHFEEGVLYEIDSKNESCKKETLQFRKHLMEI : 113
Synodontis_sp._[U33491] 1 : -LGSSDREPCRSPSKMSCTVCVSSTKDDTIAWCEEKYDSTHKRLRFVEDCSKSNKTSHLD-VLIHFEEGVLYELDPKNESCKKEPLPSHKHPLEV : 93
Schilbe_sp._[U33490] 1 : -GSSSREPCRSPPKMSCTLCVSSSKDDAIAWCEEKYDSSQKHLRFVEDTGKSNKTSYLD-VLIDPDKGVLYELDTKNESCRKQMLPSHKHPLEL : 92
Pimelodus_sp._[U33489] 1 : -SREPCRSPSKMSCMICVSSGKDDTIAWCDEKYDSTHKHLRFVEDSSKSNKTSYID-VLIDXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX : 89
Ictalurus_punctatus_[BE212610] 1 : -MRTIELFCVAFLCLSPMAWVSSSREPCRSPAKMKCLLSVTSAKDDTTVWCDEKYDAAKKYLRFVEDS-KSNRTSHLD-VLVHFEQDVIYEFDSKNDSCKKQSLPSHSHPLEV : 110
Hypostomus_sp._[U33488] 1 : -VEDXSHKNKTSHID-VIIDFQENVMYELDASNESCKKEALKSQEHPMQL : 48
Clupea_harengus_[P32187] 1 : -MRLTGLLCVALWSASAVVLAEHQPCRPEPQLHCNLWVTAAKGAPASVCEENYDSQARKLHEKDDALHVNKTDHLE-MLIFFEEGIFYDIDSHNQSCHKKTLQSTYHCLEV : 109
Salmo_salar_I_[P28772] 1 : -MQDFAFAALSIWLCLGAT----ALAESHGPQHCTSPNMTGVLTVVALTGGEIKATCHMSYDSTDKKLRFTESEMHLNKTEHLEDYLMLFEEGVFYDIDMKNQSCKKMSIHSHAHALEL : 114
Oncorhynchus_II_[P28771] 1 : -MQDFAFAALSIWLCLGAT----ALAESHGPQHCTSPNMTGVLTVVALTGGEIKATCHMSYDSTNKKLRFTESEMHLNKTEHLEDYLMLFEEGVFYDIDMKNQSCKKMSIHSHAHALEL : 114
Oncorhynchus_I_[P28770] 1 : -MQAFAVAALSIWLCLGAT----TLAESHGPQHCTSPNMTGVLTVVALTGGEIKATCHMSYDSTDKKIRFTESEMHLNKTEHLEDYLMLFEEGVFYDIDMKNQSCKKMSIHSHAHALEL : 114
Esox_lucius_[P32188] 1 : -MQAFAVAALSIWLCLGATTLAESLAQSHGPQHCTSPNMTGVLTVVALNGGEIKATCHMHYDTTDKKLRFTESDMHLNKSEHLEDYLMLFEEGVFYDIDLKNQSCRKMSLQSHAHALEL : 118
MERP-1__Human_[AY027862] 1 : ---MPGRAPLRTVPGALGAWLLGGLWAWTLCGLCSLGAVGAPRPCQAPQQWECRQVVYQQSSGRNSRALLSYDGLNQRVRVVDERKALIPCKRLFEYILLMKDGVMFQIDQATKQCSKMTLTQPWDPLDI : 127
MERP-1__Mouse_[AY027861] 1 : ---MPGRAPLRTVPGALGAWLLSGLWAWTLCGLCSLGAVGAPRPCQAPQQWECRQVVYQQSSGRNSRALLSYDGLNQRVRVVDERKALIPCKRLFEYILLMKDGVMFQIDQATKQCSKMTLTQPSDPLDI : 127
MERP-1__Monkey_[AB046003] 1 : MTAMPGRAPLHTVPGALGPWLLGCLWAWTLCGLCSLGAVGAPRPCQAPQQWECRQVVYQQSSGRNSRALLSYDGLNQRVRVVDERKALIPCKRLFEYILLMKDGVMFQIEQATKQCSKMTLTEPWDPLDI : 130
MERP-2__Mouse_[AF353717] 1 : ---MPARAPRRLVQGPRGTWLLGSLWVWVLCGLGMAGSLGTPQPCQAPQQWECRQVIYQQSSGHNNRALVSYDGLNQRVRVVDERKALIPCKRLFEYILLMKEGVMFQIEQATKQCAKIPLVESWDPLDI : 127
ERP__Frog_[BG553216] 1 : -----MMEAIHVAGFYSFMTHCIFLFILGLAESRGLVTPISVAPCEAPLQWECRTVIYDHNTGKNTRATVTYDAFLQRIRILEEKKSLVPCKRFFEYFLFLKQEAVMFQIEQVTKICSKNTLTEPWDEYDH : 125
EpenHm_H.mexicana_[CF519307] 1 : -STGVEQKAPCQLAEQWECRASEWDHVQGRNNRFLISFDGIGMRKRVIEEKKSFMPGRRFYEYIMEMKTNKTYTINMNLGTCTVSTLKQPWQNHTI : 95
EpenLv_L.variegatus_[CF519308] 1 : -STGVEQKAPCQLAEQWECRASEWDHVQGRNNRFLISFDGIGMRKRVIEEKKSFMPGRRFYEYIMEMYRTNKTYTINMNLGTCTVSTLKQPWQNHTI : 95
EpenHg_H.glaberrima_[AY383544] 1 : -MNKLILLSVVVAVATGINVSPSTGVEQKAPCQLAEQWECRASEWDHVQGRNNRFLISFDGIGMRKRVIEEKKSFMPGRRFYEYIMEMKTNKMYTINMNLGTCTVSTLKQPWQNHTI : 116

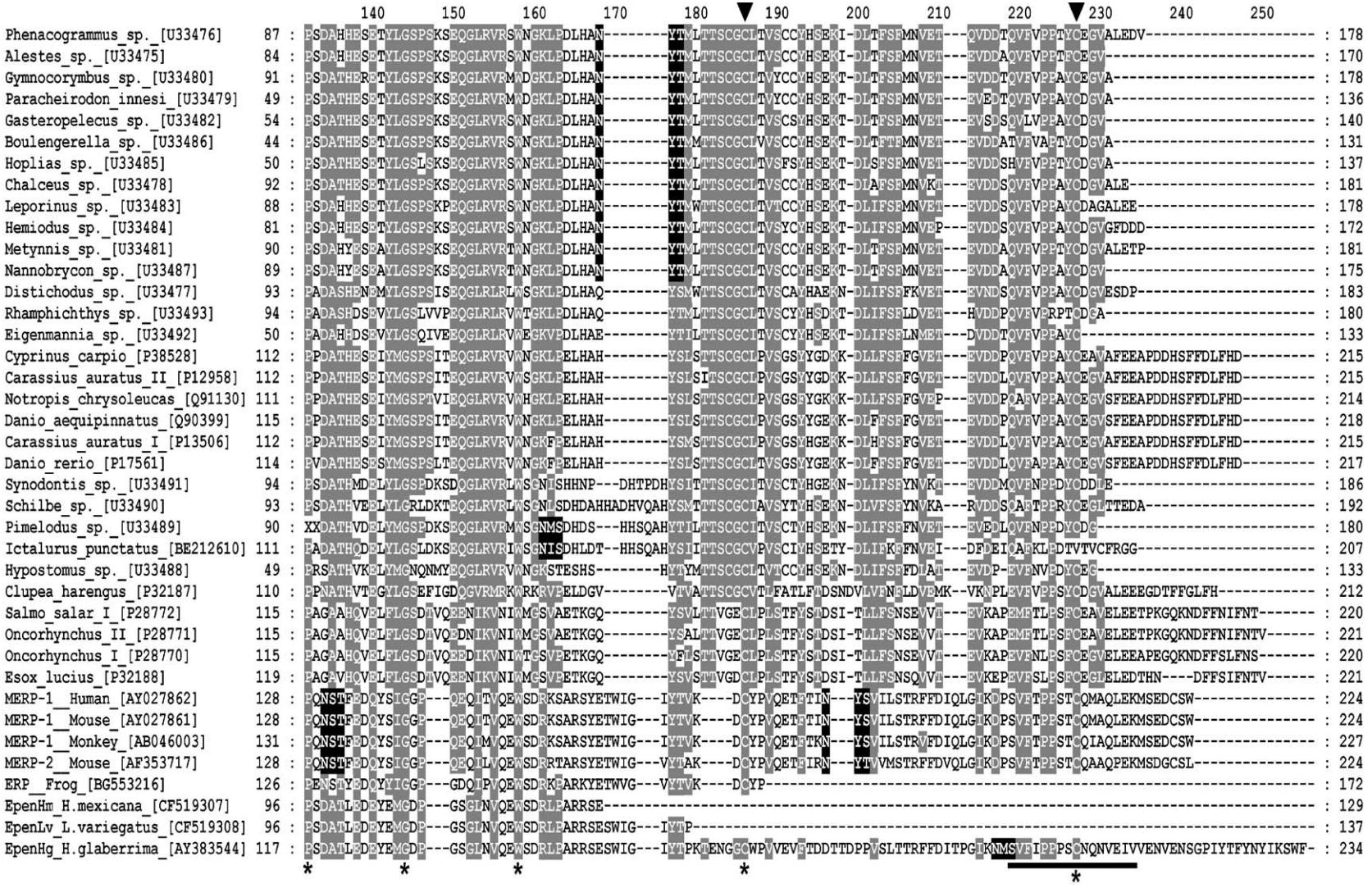


Fig. 3. Amino acid alignment of the echinoderm and vertebrate ependymin-related sequences. The echinoderm ependymins (*EpenHg*, *EpenHm* and *EpenLv*) were compared with the known vertebrate ependymins (fish, frog and mammals). The accession number for each sequence is presented in brackets next to each name. The numbers at both sides are the amino acid position of each sequence. Gray-shaded areas represent strongly conserved residues. Diagnostic cysteines are indicated by arrowheads above the sequences (▼). Potential N-glycosylation sites are black shaded. Asterisks (*) denote invariable residues, while the residues included in the ependymin consensus pattern are underlined.

mitted to the dbEST database with the accession numbers: *EpenHm* (CF519307) and *EpenLv* (CF519308).

3.3. Alignment comparison and phylogeny of ependymin proteins

The alignment and sequence comparison among different species (Fig. 3) shows the echinoderm ependymins to be more similar to frog and mammalian ependymins. Examples of that similarity are the sequences perfectly conserved $Q^{36}WEGR^{40}$, and $V^{137}QEWSDR^{143}$, and the highly conserved R^{66}VIEEKKSFMPGRRFYEYIMEYK^{88}, and $E^{150}SWIGIYT^{157}$ (numbers with reference to the *EpenHg* sequence). Previously, two consensus patterns that include all the ependymin proteins known from vertebrates (Apostolopoulos et al., 2001) were identified. By including the echinoderm sequences, we have updated the ependymin consensus pattern including all the vertebrate and the invertebrate sequences known so far: [Y^{87}F]–[KREQ]–[DETQ]–[NAG]–X–[FLIMT]–[YF]–[DETQ]–[LIM]–[NED]–X–X–[LNT]–[KEQG]–[TISQ]–C–X–[VK]–X–[TSMP]–L^{107}, and [S^{198}EQ]–[VAM]–F–X–[PL]–P–[PASTD]–[SYFT]–C–[DENQ]–[GAMIQ]–[NALV]–X–X–[DEGI]–[DEKGV^{213}].

The phylogenetic analysis (Fig. 4) of all known ependymin sequences shows that the echinoderm sequences form a clade in close relationship with the frog and the mammalian ependymin-related sequences, while piscine ependymins are clustered together. Our results correlate with an extensive evolutionary study made on fish ependymins (Orti and Meyer, 1996) and with a recently published work that included the frog and mammalian proteins (Apostolopoulos et al., 2001). It is intriguing that the whole alignment of the conceptual translation of *EpenHg*, *EpenHm* and *EpenLv* seems to be more alike to the mammalian and frog ependymins than to the piscine representatives suggesting that evolutionary changes have occurred in the teleost lineage that have not occurred in higher vertebrates.

3.4. Expression analysis with Northern blot and real-time RT-PCR

The *EpenHg* gene is expressed as a single transcript in *H. glaberrima* as shown by a single band of ~1500 bp in Northern blot analysis (Fig. 5), a size corresponding approximately to the whole *EpenHg* clone (1449 bp). The *EpenHg* mRNA is expressed in the non-eviscerated digestive tract, as well as in the regenerating intestine (7, 14, 21,

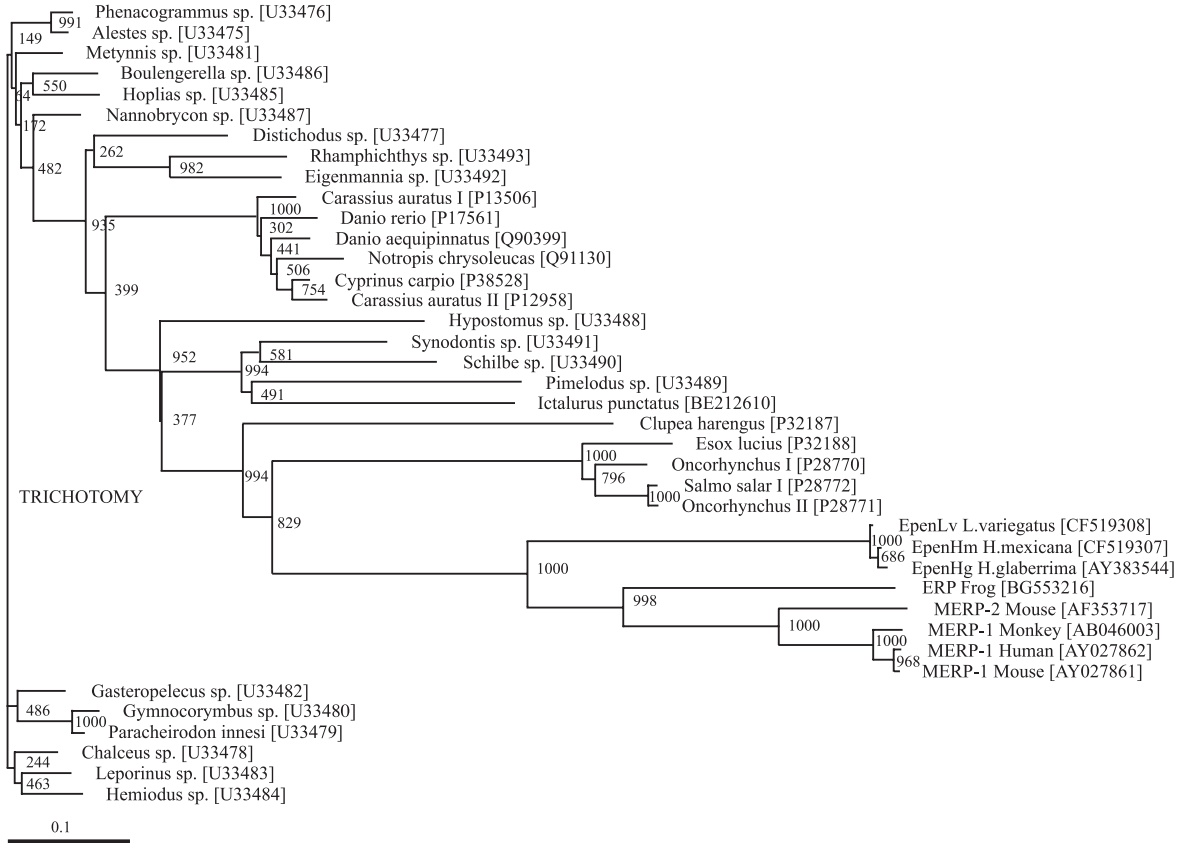


Fig. 4. Phylogenetic relationship of all ependymin and ependymin-related sequences known so far. Unrooted tree generated using a sequence distance method (neighbor-joining) from Clustal X alignments. The numbers on the nodes are percentages of 1000 bootstrap replicates supporting the topology shown. The echinoderm ependymin-related sequences clearly form a clade in close relationship with the frog and mammalian ependymin-related sequences, while piscine ependymins are clustered together. The scale indicates expected nucleotide substitutions per site.

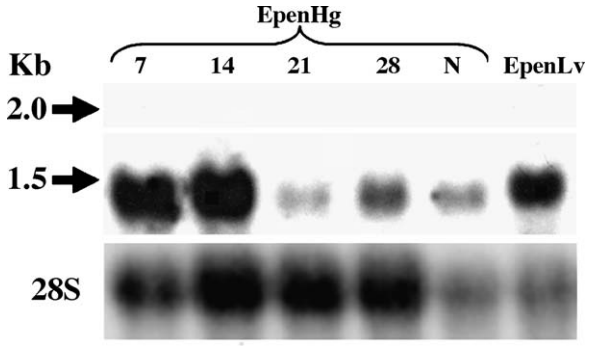


Fig. 5. Nonisotopic Northern analysis of the *EpenHg* and *EpenLv* genes. These ependymin-related genes are expressed as a single transcript of approximately 1.5 kb, corresponding to the whole *EpenHg* clone length (1449 bp). The *EpenHg* mRNA is expressed in the non-eviscerated digestive tract (N), as well as in the regenerating intestine (7, 14, 21 and 28 dpe). As described in Materials and methods and evidenced by the 28S bands, unequal amounts of intestine RNA from *H. glaberrima* and *L. variegatus* were probed with a cDNA probe from an *EpenHg* sector. Molecular weight standards are indicated on left.

and 28 dpe). Moreover, a single band of approximately the same size is observed in *L. variegatus*, implying that the similarity among the echinoderm ependymin-related genes observed in the cloned ESTs is consistent through the entire molecule. Although Northern blots were made without loading equal amounts of RNA (as can be seen by change in the 28S band), they do suggest an over-expression of the transcript during early stages of regeneration. To determine the relative expression of ependymin during the regeneration process, we have used real-time RT-PCR where the *EpenHg* expression is normalized to the endogenous standard 18S ribosomal subunit. *EpenHg* expression was indeed shown to be enhanced during intestinal regeneration. Our quantitative real-time RT-PCR results show two peaks, at 7 days post-evisceration (dpe) and at 28 dpe (Fig. 6).

EpenHg expression was also detected in other tissues (i.e., esophagus, mesenteries gonads, tentacles, body wall and respiratory trees) by conventional and real-time RT-PCRs (Fig. 7). Similarly, *EpenLv* is found in the intestine, esophagus and gonads, and *EpenHm* in the intestine, gonads and hemal system. Contrary to the widespread ependymin expression in echinoderm tissues, the fish ependymin expression appears to be restricted to the brain (Tang et al., 1999). Two possible explanations can account for the differential expression of ependymins found between our echinoderm and the piscine species. First, that different isoforms are present in fishes but only one has been identified using antibodies. In fact, two genes for ependymin-related proteins have been found in mouse (Apostolopoulos et al., 2001). This would be just another case where a gene present in the invertebrates has undergone duplication and changes during the evolution of vertebrates. The second possibility is that *EpenHg* expression is also restricted to the nervous tissue in echinoderms. It is known that nerve cells are present within the coelomic epithelia of most, if not all, echinoderm viscera (Hyman, 1955), and various types of neurons can be found within the enteric system of *H. glaberrima* (García-Arrarás et al., 2001). Therefore, the option exists that some or all of these neuronal elements are expressing the *EpenHg* mRNA detected by PCR. Ongoing experiments in our laboratory that aim to localize *EpenHg* mRNA or its protein will serve to determine the similarities or differences in expression between echinoderms and vertebrates.

Although it is premature to assign a putative role to *EpenHg* during intestinal regeneration, some analogies can be made with the proposed ependymin's roles in vertebrate systems. Utmost, the gene appears to be involved in regeneration, as it is over-expressed during the regenerative process. The enhanced expression observed at 7 dpe correlates with what has been reported for the mammalian

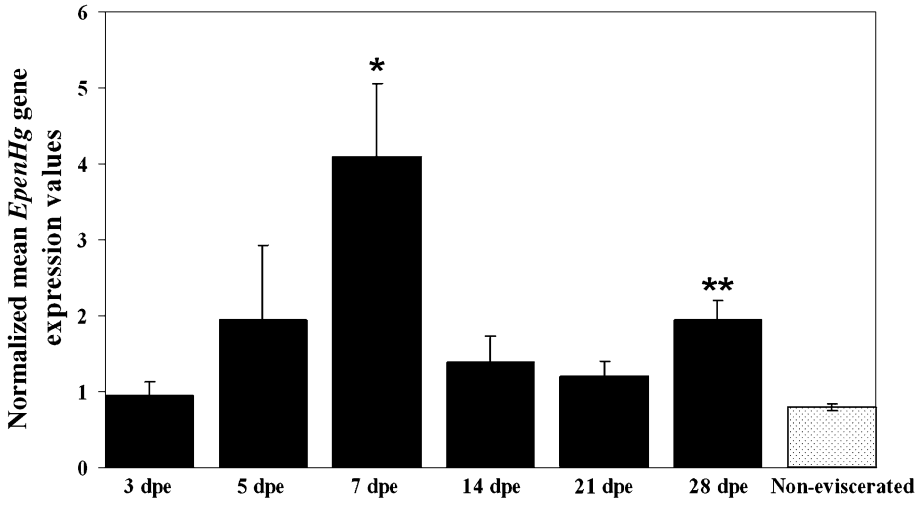


Fig. 6. Expression profile of *EpenHg*'s mRNA during intestinal regeneration. Real-time RT-PCR for relative quantitation using the 18S ribosomal gene as standard for normalization. Compared to non-eviscerated animals, there is significant overexpression at 7 and 28 dpe (*$p < 0.05$, **$p < 0.005$; two-tailed t test). Representative correlation coefficients (0.996 average) were obtained for standard curves. Each bar is the average of at least five independent experiments made in triplicate ± S.E.M.

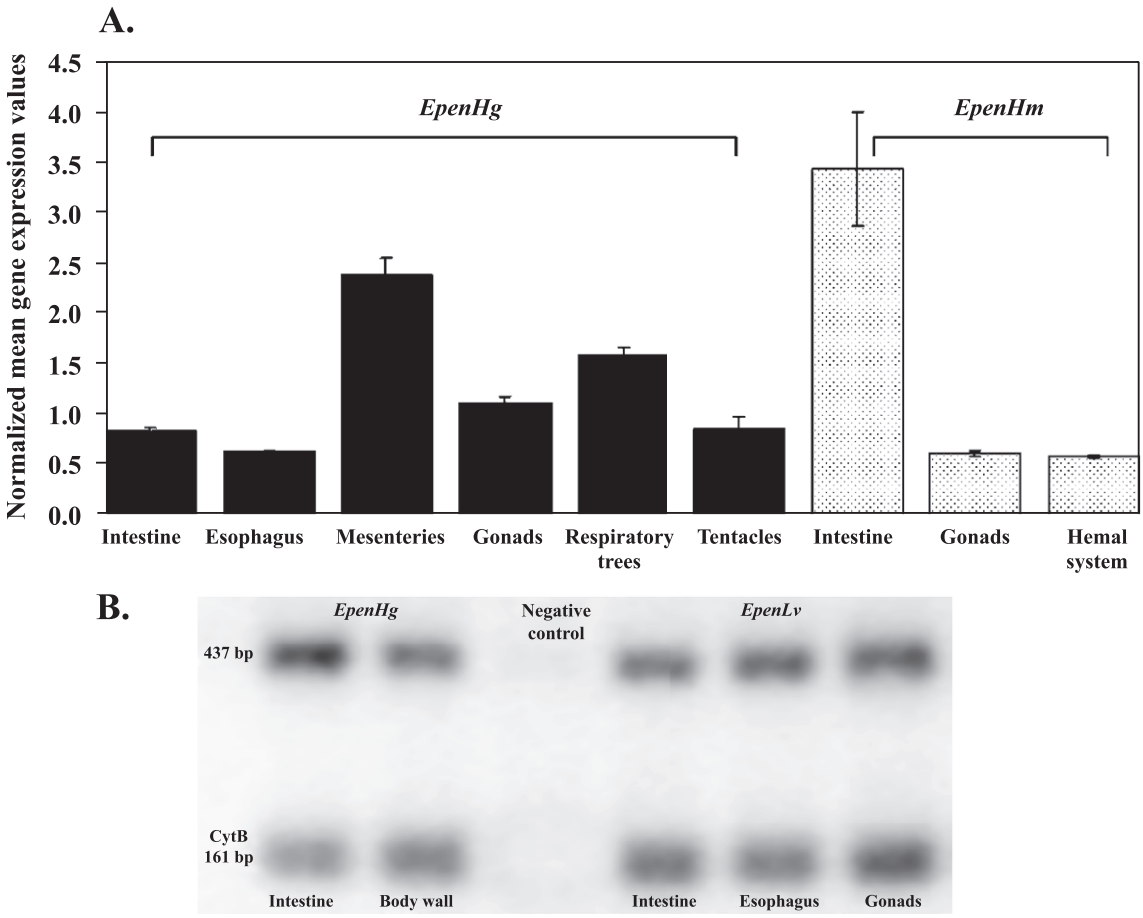


Fig. 7. Expression detection of ependymin-related genes in several echinoderm tissues. (A) Normalized real-time RT-PCR detection of *EpenHg* gene expression (black columns) and *EpenHm* (white dotted). Each bar is the average triplicate measures of one same sample ± S.D. (B) Co-amplification by conventional RT-PCR of *EpenHg* (left) or *EpenLv* (right) with the internal standard cytochrome *b* (CytB, 161 bp).

ependymin-related MERP1 mRNA in hematopoietic stem/progenitor cells (Gregorio-King et al., 2002). In these cells, there is an increased expression of the gene prior to proliferation and differentiation. In this context, previous experiments from our laboratory have shown that at 7 dpe, there is little cell division in the regenerating structure, and few differentiated cells can be found (García-Arrarás et al., 1998). It is at this stage that the initial steps in formation of the enteric nervous system occur. Therefore, *EpenHg* might be one of the molecules that promotes the repair, elongation and reconnection of fibers or the formation of new neurons. A similar role as a neurotrophic factor and inductor of neurite sprouting has been proposed for the goldfish ependymin protein (Shashoua et al., 2001; Adams et al., 2003). Finally, the enhanced expression of *EpenHg* during late regeneration stages (28 dpe) may suggest its involvement in the steps of organ growth and belated sharpening of neural connections. This is similar to the putative function of this protein family in the sharpening of the regenerating retinotectal projection in goldfish, where they are expressed possibly as substrate for axonal outgrowth (Schmidt and Shashoua, 1988; Schmidt and Schachner, 1998; Schmidt et al., 1991). However, because of the intrinsic complexity of the generation of a new organ, such as occurs in the sea cucumber, it is possible to consider additional roles for *EpenHg* that lie beyond nervous system associated roles. In fact, the mammalian ependymin-related genes were found to be expressed in non-nervous tissues and to be associated with developmental processes (Nimmrich et al., 2001; Gregorio-King et al., 2002). Future experiments studying the expression of echinoderm ependymins will help in the elucidation of these issues.

4. Conclusions

We have characterized an ependymin-related gene from the sea cucumber *H. glaberrima* and cloned related ependymin ESTs from two other echinoderms. To the best of our knowledge, these are the first ependymin-related sequences reported from invertebrates. Our sequence analyses show that the echinoderm ependymin-related sequences are more similar to the frog and mammalian sequences than to fish ependymin sequences. Spatial expression studies show that the echinoderm ependymins are expressed in multiple tissues. Moreover, the *H. glaberrima* ependymin gene is

overexpressed during the regeneration of the digestive tract, strengthening the idea that ependymins are important molecules for regenerative processes.

Acknowledgements

We are grateful Dr. Pablo Vivas-Mejía of the Center for Molecular, Developmental and Behavioral Neurosciences (CMDBN-UPR), for help in the real-time RT-PCR technique. This study was supported by NSF-IBN (0110692), NIH-MBRS (S06GM08102), and the University of Puerto Rico. We also acknowledge partial support from NIH-RCMI (RRO-3641-01).

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