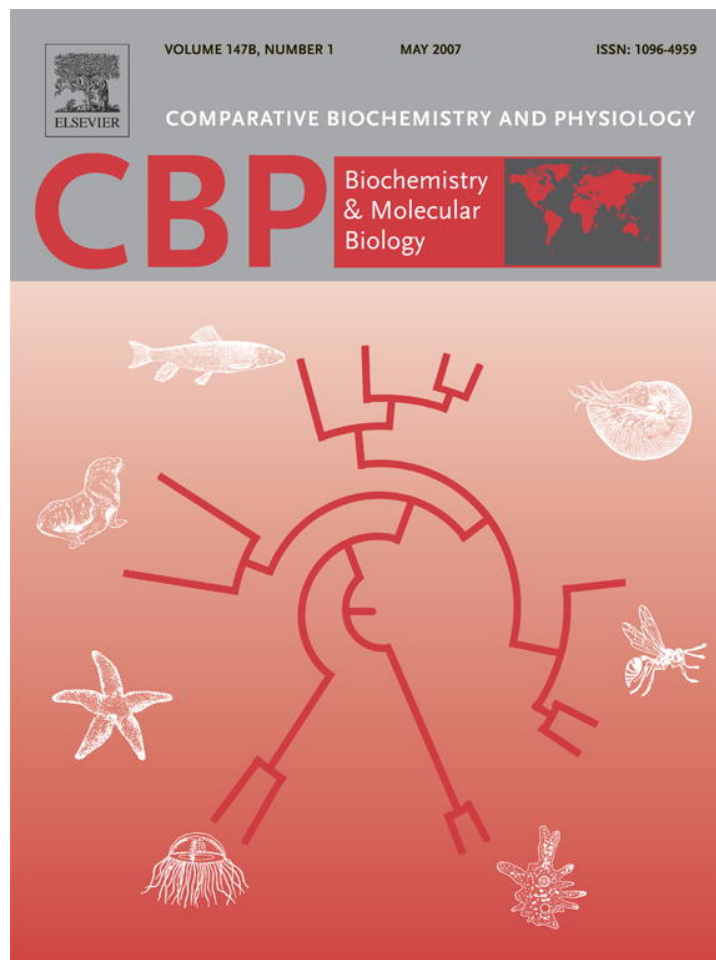


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Hypoxia-inducible factor, gsHIF, of the grass shrimp *Palaemonetes pugio*: Molecular characterization and response to hypoxia

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Abstract

Hypoxia-inducible factor 1 α (HIF-1 α) is a key transcription factor that controls a variety of cellular and systemic homeostatic responses to hypoxic stress. Expression and function of HIF-1 α have not been studied in crustaceans, which experience wide fluctuations of oxygen tensions in their aquatic environment. Here we show that an HIF-1 α homolog, gsHIF, is present in the hypoxia-tolerant grass shrimp *Palaemonetes pugio*. Using RT-PCR and 3' and 5'RACE, we cloned a full-length gsHIF cDNA (3822 bp) with an open reading frame encoding a 1057 amino acid protein. Similar to vertebrate HIF-1 α , gsHIF has one basic helix–loop–helix (bHLH) domain, two PAS domains, an oxygen-dependent degradation domain (ODD) with two proline hydroxylation motifs, and a C-terminal transactivation domain (C-TAD) with an asparagine hydroxylation motif. In addition to these conserved sequences, gsHIF has a unique 230 amino acid sequence (aa 790–1020) not found in any vertebrate HIF proteins. Phylogenetic analysis indicates that grass shrimp and vertebrate HIFs belong to distinct clades within the HIF protein family. Expression analysis shows that gsHIF is constitutively expressed under normoxic (7.5 ppm DO), moderate (2.5 ppm DO) and severe (1.5 ppm DO) hypoxic conditions. In addition to gsHIF, we cloned a fragment of a second bHLH-PAS transcription factor from the grass shrimp, which had one bHLH and two PAS domains, and an overall 68% amino acid sequence homology with *Apis mellifera* trachealess protein.

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Keywords: Gene expression; Hypoxia; Hypoxia-inducible factor; *Palaemonetes pugio*; Phylogenetic classification; Trachealess protein

1. Introduction

All organisms possess mechanisms to maintain oxygen homeostasis which are essential for survival. In a state of hypoxia, when oxygen demand exceeds supply, a physiological response is mounted which increases the capacity of blood to carry oxygen to tissues, and alters cellular metabolism, such as facilitating ATP production by anaerobic glycolysis. The hypoxia-inducible factor (HIF), conserved during evolution from worms to flies to vertebrates, is central to adaptation to low oxygen availability (Semenza, 1998). HIF regulates the transcription of many genes involved in control of cellular and short-term and long-term systemic responses to hypoxia, including glycolysis, erythropoiesis, breathing, vasodilatation, and angiogenesis. HIF controls oxygen homeostasis during embryonic development and postnatal life in physiological

processes and also in pathophysiological processes such as tumor growth and metastasis (Ryan et al., 1998).

The discovery of HIF was enabled by the identification of a minimal hypoxia-responsive element (HRE), A/(G)CGTG, in the 5' enhancer region of the erythropoietin gene. Subsequent analysis identified HIF as a phosphorylation-dependent protein which binds the major groove of DNA under hypoxic conditions (Bracken et al., 2003). HIF is a heterodimer consisting of one of four hypoxia-regulated α -subunits (HIF-1 α , HIF-2 α , HIF-3 α , and HIF-4 α) and the oxygen-insensitive HIF-1 β subunit. The latter is a constitutive nuclear protein which also serves as a binding partner (so-called Arnt or aryl hydrocarbon receptor nuclear translator) of the dioxin/aryl hydrocarbon receptor (DR/AhR) and hence participates in the cellular response to environmental toxins. In addition, HIF-1 β /Arnt is required in multiple signaling pathways (Berra et al., 2001, 2003).

In mammals, HIF belongs to a class of transcription factors termed the basic helix–loop–helix/Per-Arnt-Sim (bHLH/PAS)

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proteins, characterized by two conserved domains, bHLH and PAS. The PAS domain was named after the first three proteins in which it was identified: Per (period circadian protein), Arnt (Ah receptor nuclear translocator protein) and Sim (single-minded protein). All known members of bHLH/PAS family function as dimers. The bHLH domain is involved in DNA binding and dimerization, and the PAS domain in target gene specificity, transactivation, and dimerization. The bHLH signature domain consists of approximately 60 amino acids with two functionally distinct regions. The basic region, located at the N-terminal end of the domain, is involved in DNA binding and consists of 15 amino acids with a high number of basic residues. The HLH region, at the C-terminal end, functions as a dimerization domain and is constituted mainly of hydrophobic residues that form two amphipathic helices separated by a loop region of variable sequence and length. The PAS domain encompasses 200–300 amino acids containing two loosely conserved hydrophobic regions of approximately 50 amino acids, designated PAS-A and PAS-B. This domain forms a secondary dimerization interface between family members in addition to other roles, such as ligand and chaperone binding in the dioxin receptor (DR) (Isaacs et al., 2002). Despite of not directly binding DNA, the PAS domain has also been reported to confer target gene specificity to the *Drosophila* protein Trachealeless (trh) and single-minded (Sim). The functions played by PAS in HIF still remain unknown (Berra et al., 2003; Hansson et al., 2002).

Although oxygen availability regulates multiple steps in HIF-mediated transcriptional activation, the dominant control mechanism occurs through HIF-1 α . In normoxia, HIF-1 α subunit is constitutively synthesized, and two proline residues in the so-called HIF oxygen-dependent degradation domain (ODD) are hydroxylated by HIF prolyl hydroxylase. This modification targets HIF for rapid degradation by the ubiquitin-proteasome pathway, resulting in a half-life of less than 5 min (Erez et al., 2004). Under hypoxic conditions, the proline residues are unmodified and degradation of HIF-1 α is blocked, allowing it to accumulate within the nucleus where, upon binding to HIF-1 β , it recognizes HREs within the promoters of hypoxia-responsive target genes (Bruick, 2003; Huang et al., 1998; Masson et al., 2001). In addition to proline hydroxylation, an asparagine residue in the C-terminal transactivation domain (C-TAD) of HIF-1 α is also hydroxylated under normoxic conditions blocking its interaction with transcriptional coactivators such as p300, thereby inhibiting transcription of downstream HIF target genes (Bruick, 2003; Lando et al., 2002a,b).

Most of our knowledge of molecular responses to hypoxia comes from in vitro studies of terrestrial mammalian systems. Relatively little is known about molecular responses of aquatic organisms to hypoxia. The oxygen content in aquatic environment varies markedly daily, seasonally, and spatially. Due to the low oxygen content of water, increased respiration by benthic (micro)organisms stimulated by excess organic material, a condition known as eutrophication, can cause hypoxia or anoxia (Rabalais et al., 2002). It is thus not surprising that environmental oxygen levels play a significant role in the evolution of aquatic animals. They have developed

various physiological and biochemical adaptations to enable survival in hypoxic and anoxic environments, including air breathing organs, specialized metabolic pathways enabling long-term anoxic survival, and modifications of hemoglobin molecules to optimize oxygen transport. At the same time, they present a unique opportunity to study the evolution, function, and regulation of oxygen-dependent genes and their role in the environmental adaptation (Soitamo et al., 2001). Since widely divergent organisms have the ability to adapt to variable oxygen concentrations, mechanisms of hypoxic sensing and response may have been established early in evolutionary history.

The first full-length cDNA (3605 bp) of HIF-1 α in fish was cloned from rainbow trout *Oncorhynchus mykiss*, which encodes a protein sequence of 766 amino acids that shows a 61% similarity to human and mouse HIF-1 α (Soitamo et al., 2001). To date ~20 additional HIF-1/2/3/4 α cDNAs have been reported in several fish species (Law, 2002; Law et al., 2006; Strausberg et al., 2002; Powell and Hahn, 2002), but HIF-1 (-2, -3 or -4) α has not yet been characterized in crustacea and the mechanisms and conditions by which HIF regulation occurs in hypoxic shrimp have not been elucidated. Here, we present the sequence of HIF cDNA from the grass shrimp *Palaemonetes pugio* and its response to moderate and severe hypoxia. This marsh-resident species is commonly found in habitats that are often hypoxic, and can adapt to very low oxygen levels found in their immediate environment (Finley et al., 1998; Lee et al., 1998). In general, grass shrimp has been shown to be uniquely physiologically adapted to stressful tidal marsh habitats (Welsh, 1975). The use of a non-model, yet commonly occurring resident species in these studies allows laboratory results to be more easily related and applied to field measurements.

2. Materials and methods

2.1. Extraction of total RNA and cloning of full-length cDNA

Total RNA was isolated from grass shrimp (*P. pugio* Holthuis, 1949) hepatopancreas using Stat-60 (Tel-Test, Friendswood, TX, USA) according to the manufacturer's protocol. After precipitation, RNA was stored in RNA Secure (Ambion). Single-stranded cDNA was generated from mRNA by reverse transcription (RT) with Superscript II RNase H⁻ Reverse Transcriptase (Invitrogen) and oligo dT primers (5'-TTTTTTTTTTTTTTVN-3').

Block Maker (Henikoff et al., 1995) was used to find several highly conserved blocks in a set of HIF protein sequences from different species (*Danio rerio* AAH46875, *Mus musculus* AAC53455, *Rattus norvegicus* AAD24413, *Xenopus laevis* CAB96628, *Homo sapiens* AAC50152, *Drosophila melanogaster* AAC47303, *Caenorhabditis elegans* AAK62778, *O. mykiss* AAK30364, and *Gallus gallus* BAA34234), where a block is an aligned array of amino acid sequence segments without gaps that represents a highly conserved region of homologous proteins. The resulting blocks in the Blocks Database format were imported into the CODEHOP program (Rose et al., 1998) to design the forward and reverse primers for PCR amplification, HIFF1, HIFF2, HIFR1, and HIFR2 (Table 1).

Table 1
Primers used for grass shrimp RT-PCT and RACE

Primer name	Nucleotide sequences
HIFF1	5'-GGG CGG AAG GAG AAG TCC MGN GAY GCN GC-3'
HIFF2	5'-ACA CAA CGT CAC CAC CCA CYT NGA YAA RGC-3'
HIFR1	5'-GGT ACT GGC CGG TCG TCM CYT GNC CYT T-3'
HIFR2	5'-GGT CCG CAG CTG CAA ATC RTC RTC NAT-3'
900F1	5'-CAG AGG AAG GTC AAG CAG GGT CAC A-3'
900F2	5'-GCT GCC ACT TCA AGA GTT CGG GAT ATA GAG-3'
900R1	5'-CCA TCG TCA TCC CGG ACA TCG TA-3'
900R2	5'-CGC GGT CTC ATG TTG CCT CCT TTA-3'
700F1	5'-GCT TGT GAA GGG CGA GGA CGA GT-3'
700F2	5'-GCC CTG GAC TCG GAA CTC ATC AAA G-3'
700R1	5'-AAT GAT GTC GCC TTC GGT AGA GAG CAC-3'
700R2	5'-GTT AGA CAA GCA TGG CAG AGG GC-3'
F1	5'-CCA GGA AGT AGC CCA GAA TAT GAC G-3'
F2	5'-ACG CAC ACC AGA GCC ACC TAA AGC-3'
F3	5'-GCG GTA AAG ATG GAG ATG ATG GAG-3'
F4	5'-GTA GCT CAC CTC TCC AAG ATC ACC A-3'
T1	5'-GAC TAC ACA CCA GAT GAA CTG CAA GG-3'
T2	5'-GTC CCT CTA CCC CTT GTG TCA CG-3'

PCR conditions for the primary PCR were 94 °C for 2 min for 1 cycle, 35 cycles of 94 °C for 30 s, 45 °C for 30 s, and 68 °C for 2 min, followed by 68 °C for 7 min. Secondary PCR cycle conditions were 94 °C for 2 min for 1 cycle, 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 68 °C for 1 min, followed by 68 °C for 7 min. The PCR reactions using AccuPrime™ SuperMix I (Invitrogen) gave two amplification products, ~700 (hif700) and ~900 bp (hif900). Bands were visualized by ethidium bromide on a 1% agarose gel, purified with QIAquick Gel Extraction Kit Protocol (Qiagen), and T/A cloned using pGEM®-T Easy Vector System (Promega), and sequenced. The ~900 bp and ~700 bp PCR products were identified as fragments of Trachealess and HIF-1 α (see Results).

RACE was performed using the SMART-RACE kit (BD Bioscience) to generate the 3' and 5' ends of the Trachealess and HIF cDNA. Gene-specific primers were designed based on the initial cDNA sequences of hif700 and hif900. Primary and secondary primers for 3' and 5' ends of hif900 were 900F1 and 900F2, and 900R1 and 900R2, respectively. For hif700, the primary and secondary primers for 3' and 5' ends were 700F1 and 700F2, and 700R1 and 700R2, respectively (Table 1). Touchdown PCR conditions for the primary PCR were 5 cycles of 94 °C for 30 s and 72 °C for 3 min, 5 cycles of 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min, 35 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 3 min, followed by 72 °C for 5 min. Secondary PCR cycle conditions were 40 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min, followed by 72 °C for 5 min. The PCR products were purified and cloned and sequenced as described above.

Because the sequencing reactions gave ambiguous results for the 3' and 5' ends, additional sets of primary and nested primers were designed for trachealess and HIF, to get the full-length cDNA sequences. For trachealess the primary and secondary primers were T1 and T2. For HIF the first set of primary and secondary primers was F1 and F2, and the second set of primers was F3 and F4 (Table 1). The PCR cycling parameters for the primary and secondary PCR were the same as above. The product bands were isolated from the gel, purified and sequenced.

All primers were synthesized by Invitrogen (Carlsbad, CA, USA). DNA sequences were determined by the University of Maine Sequencing Center (Orono, ME, USA) and in house using a Beckman CEQ 8000 Genetic Analysis System. Sequence analyses and homology searches were performed using the online BLAST suite of programs (NCBI). Conserved domains in the sequences were identified using the NCBI Conserved Domains database and by alignment of grass shrimp HIF with HIF-1 α from organisms with known HIF domain structures.

2.2. Hypoxia exposures

Grass shrimp were collected in the vicinity of Ocean Springs, Mississippi, in Davis Bayou using dip nets. Adult females and males were segregated by sex and maintained in the laboratory at 15 psu and 27 \pm 1 °C for 7 to 30 days prior to experimentation. During acclimation and experimentation periods, grass shrimp were fed brine shrimp nauplii once daily and commercial flake food once daily. During all acclimation and experimentation periods, shrimp were held in artificial seawater (Fritz Super Salt, Fritz Industries, Mesquite TX) diluted to 15 psu.

Exposures were conducted in an intermittent flow-through system described by Manning et al. (1999). Normoxic (7.5 ppm DO) and hypoxic (2.5 ppm and 1.5 ppm DO) conditions within the treatment aquaria were established and maintained as described before (Brouwer et al., 2004, 2005; Brown-Peterson et al., 2005; Brouwer et al., 2007). The flow-through test system provided 1 l every 20 min (resulting in 3 complete volume additions/day) to each of the 35-l test aquaria using a separate water delivery partitioner for each of the normoxic and hypoxic treatments. Oxygen levels were controlled by bubbling nitrogen into a holding tank which gravity fed to the partitioner used to deliver flow-through hypoxic seawater. A 24-h timer was used to activate a solenoid valve which controlled nitrogen introduction into the holding tank at intervals that maintained oxygen in the holding tank at a level which resulted in the desired oxygen concentration when introduced into the test aquaria. An additional partitioner provided flow-through normoxic seawater, and normoxic conditions were maintained by gently bubbling oxygen into the cells of the water partitioner prior to delivery of water to the individual aquaria. Female grass shrimp were housed individually in retention chambers constructed from 10-cm Petri dish bottoms with a 15-cm high collar of 500- μ m nylon mesh placed into 35-l flow-through glass aquaria in a water bath held at 27 \pm 1 °C. In all experiments, oxygen was monitored continuously in one hypoxic flow-through aquaria, and DO, temperature and salinity were measured in all flow-through aquaria once or twice daily using a YSI Model 600XLM data sonde. After 3, 7 and 14 days of exposure, 10 shrimp per treatment were sacrificed and weighed (40.2–41.7 mg). The thorax/hepatopancreas of the shrimp was stored in 1 ml RNAlater at –20 °C for nucleic acid extraction.

2.3. Phylogenetic analysis

A multiple alignment of 21 HIF-1/2/3/4 α amino acid sequences was performed with ClustalX 1.83 (Thompson et al.,

1994, 1997; Jeanmougin et al., 1998). The aligned sequences were then used to calculate distances between pairs of protein sequences. The neighbor-joining method (Saitou and Mei, 1987) was applied to the distance matrix to calculate a tree using 1000 bootstrap trials to derive confidence values for the groupings in the tree. Corrections for multiple substitutions were made as described by Kimura (1983). Alignment positions where any of the sequences had gaps were excluded from the analysis. An additional tree was constructed using the maximum parsimony method implemented by Felsenstein's PHYLIP package v3.6 (Felsenstein, 1985). The aligned sequences in PHYLIP format were imported into the SEQBOOT algorithm to generate 500 data sets by bootstrap resampling (Felsenstein, 1989). The multiple data sets were used to calculate most parsimonious trees with PROTPARS. The resulting tree output file was used as input in the program CONSENSE that calculates a majority rule consensus tree with confidence intervals. *C. elegans* HIF-1 was used as outgroup.

2.4. HIF expression analysis

HIF mRNA levels were determined using custom cDNA macroarrays printed with cDNA from 78 clones from a hypoxia-responsive suppression subtractive hybridization (SSH) cDNA library (Brouwer et al., 2005, 2007), and with cDNA from the ~700 bp gsHIF clone. gsHIF PCR products with a final concentration of 100 ng/μl were robotically spotted in duplicate onto neutral nylon membranes using 100 nl pins as described by Larkin et al. (2003).

Total RNA was extracted from normoxic (7.5 ppm DO) and hypoxic (2.5 ppm and 1.5 ppm DO) grass shrimp as described above. Five to eight individual shrimp, out of ten sampled, in each treatment group gave sufficient amounts of RNA for reverse transcription and labeling. Radiolabeled probes were generated by random primer labeling of DNase-treated (DNA-free, Ambion) total RNA with [α -³³P]dATP (2'-deoxyadenosine 5'-triphosphate). Hybridization and wash steps were performed as previously described (Larkin et al., 2003).

The membranes were exposed to a phosphor screen (Molecular Dynamics) at room temperature for 48 h. The blots were quantitatively evaluated using a Typhoon 8600 imaging system (Molecular Dynamics). For each cDNA clone the general background of each membrane was subtracted from the average value of the duplicate spots on the membrane. Intensity values for

all genes were transformed to the log base 2 and normalized to the median array intensity. HIF expression data were analyzed by conducting a one-way ANOVA across time for both DO regimens. Data were tested for homogeneity of variance and normality of distribution using SigmaStat 3.11 (SYSTAT Software, Inc. San Jose, CA, USA). Where normality test failed a Kruskal–Wallis one-way ANOVA on ranks was performed.

3. Results

3.1. Cloning and sequencing of tracheless from grass shrimp

Using total RNA extracted from the hepatopancreas of grass shrimp and degenerate primers designed by CODEHOP, two initial PCR products of ~900 bp and ~700 bp were obtained. Sequencing showed the ~900 bp product to be 870 bp long, with an open reading frame of 290 amino acids without start and stop codons, with a 78% sequence identity to tracheless protein from *Tribolium castaneum* (GenBank Accession no: XP_967112). 3' and 5'RACE were performed in an attempt to obtain the complete tracheless coding sequence. 3'RACE products formed one contiguous sequence with the 870 bp product with a 327 bp overlap. All 3'RACE products had a TGA stop codon in the same position. All 5'RACE products had an identical 252 bp sequence at the 3' end which formed one contiguous sequence with the 870 bp product with a 120 bp overlap. The 5' end sequences of the 5'RACE clones were ambiguous and there was no clear translation start. Hence we show only the 84 amino acids, corresponding to the conceptual translation of the 252 bp that are identical in all 5'RACE products (Fig. 1). The 501 amino acid sequence shown in this figure represents therefore a fragment of tracheless protein, with part of the N-terminal sequence (~45 amino acids) missing. It should be noted that tracheless proteins that show greatest homology with grass shrimp tracheless, *T. castaneum* (67%), and *Bombyx mori* (65%; GenBank Accession no. BAA22946) tracheless proteins (Matsunami et al., 1999) are 834 and 849 amino acids long, respectively.

3.2. Cloning and sequencing of HIF from grass shrimp

Sequencing showed the ~700 bp PCR product to be 673 bp long. BLASTX search revealed 49% identity with HIF-1 protein from *T. castaneum* (GenBank Accession No. XP_967427). The

```

1 XXXFAPEQOW RQLPDGSILE LRKEKSRDAA RSRRGKENYE FYELAKMLPL PPAITSQLDK
61 ASIIRLTISY LKLRDFTLHG DPPWPRDHS GTKNLKGNM RPRTMSGTM DIFETHQGTG
121 ILQSLDGFAP TLAADGRFLY ISETVSIYLG LSOVEMTGSS VFDYIHQODH QELADQLGLT
181 LATGQPLPSP SSLGSEEGQA GSQGTMPDV ATCMAVTSTS QHKGYDRAFC IRMKSTLTR
241 GCHFSSSGYR VVLILGHLRP QYVFSHSRKS APTLMGLVAL AIALPPPSVH EVRLESDMFV
301 TRITFDRIA HCEPKVADLL DYTPDELQGR SLYPLCHGQD VDKLRKTHVD LIEKGQVMSF
361 YFRLLNKTGG YTWMQTCATV VINNKGDEQ NIICVNYIIS RTQYDTLVMD QTQLDPALAN
421 MKRDDLDYTN PATPEPEGS CGVVGEESS SPRSTANTTG GGGSIGTRS PPTGGPPEIQI
481 STPLRGTPSV PLDGAQIKTE VITL

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XXX indicate N-terminal sequence upstream from FAPE is ambiguous. No distinct translation start is found.

20-72: bHLH Domain; 116-177 : PAS Domain; 296-395: PAS Domain.

Fig. 1. Amino acid sequence of grass shrimp Tracheless.

```

1  MPHKKKGVKA  TGKSPAKEKR  RNSEKRKEKS  RDAARCRRGK  ESEIFTELAN  ALPLPARTIS
61  MLDKASVMRL  TIAFLKTRAL  CHACLTKKSD  MEGGKKLDLE  MDSLFMKALD  GFLLVSTEG
121 DIIYSSENIA  TFLGLPQVDV  MGQCLYEYTH  PCDHEEARAL  VSAKGPPQEP  RHAFLRLKCT
181 LTAKGRSVNL  KSASYKVV RV  SGBLVKGEDE  SWLVALGTPV  PPHSNIEFPL  DKQTFVSKHS
241 LDMKFTYVDD  NVSEFCGHAS  SELLRSLYE  MHHALDSELI  KDAYKTLRIK  GQVETGRYRF
301 LAREGGYVWV  VTQATLIHGP  KDHKPQYVVC  LNYVVSGVES  PGEVLSEQQF  LNCCKNNNNK
361 STKSLDSAVP  VALSVPTSAT  QKVFQPKPEV  QPKVVNGVPA  SSPVSRVIPA  PAPPLQPPTP
421 VAATSKIFTP  RTEEMKGYL  IFPEDQPYGV  ELKDEPDDL  HLAPSGGDT  C  VPLEVP  IFKP
481 ELDDVFTQIP  ISYTDGALFT  SPTAIPENIL  EPGSSPEYDE  YEVTD  RGKAC  NRLSGGKITV
541 NNNGSCSPSS  ICSSPGSACG  LRTPEPPKAL  ISQA  AVFQSS  PGSKPSHRRV  VESNRPISAT
601 ESLFTQLNET  AHESYVNI  EL  KVENQNMDLD  EFD  MRAPFT  C  LSNELMLNQ  DDL  MWGAQPD
661 SLP  MGKRNSK  YTSLLNGDED  SSLAQLLRDR  DPPIAGSGPE  KNLES  RNPSD  PHCSQYQSK
721 FLDGGGSFVD  PNQVLP  GHFG  GKDGD  DGGGD  LVEDDPPQVM  MHETVE  PPPP  LINVESHQNE
781 LTSAKRQHSP  NSSPLLGHK  K  LCSLIYEHRQ  RSSPLQDHQ  Q  PSPQRSPEGS  QHQP  QMQSH
841 HSGIRELTTP  YAP  TMQQLLI  SKEPITVRGG  R  PGGVGGGG  GGGGLSAPLS  LHK  GDSVLR
901 NLLNLNGEIE  DASREGEVQ  V  FAAP  IRLTQD  RMTAML  LADD  GSSHLSYPKL  RILT  GSGGSF
961 MQAGQHSLKI  SSGFTKGGT  R  GGD  DNGSNS  AGGGK  SFPG  ESFIQR  RRQ  DPLL  LVDPDL
1021 TIP  SLSELSQ  LDF  EVNAPAN  I  GNLLQ  GADL  LMALDQA
    
```

24-76: bHLH Domain; 100-161: PAS Domain; 234-331: PAS Domain; 459-464: proline hydroxylation motif in N-terminal oxygen-dependent degradation domain (N-ODD); 634-640: proline hydroxylation motif in C-terminal C-ODD; 1025-1046: C-terminal transactivation domain (C-TAD); 1034-1038: asparagine hydroxylation motif

Fig. 2. Amino acid sequence of grass shrimp HIF.

complete HIF sequence was obtained using SMART 5' and 3' RACE. However, the first 3'RACE product did not give the full-length product. Use of new primer sets (F1/F2 and F3/F4, Table 1) designed from the sequence of the previously found 3'RACE product resulted in the full-length 3' sequence. The complete gsHIF sequence (GenBank Accession no. AY655698) was 3288 bp long, with an open reading frame

encoding a 1057 amino acid protein with the initiation methionine at position 135 bp and stop codon TAG at 3308 bp and a molecular weight of 114.67 kDa (Fig. 2). There is an overall 46% homology with *T. castaneum* HIF-1 protein. The amino acid sequence of grass shrimp HIF is the second longest in size compared to HIF-1 α of most vertebrates (~800 aa) and *Drosophila* (1507 aa).

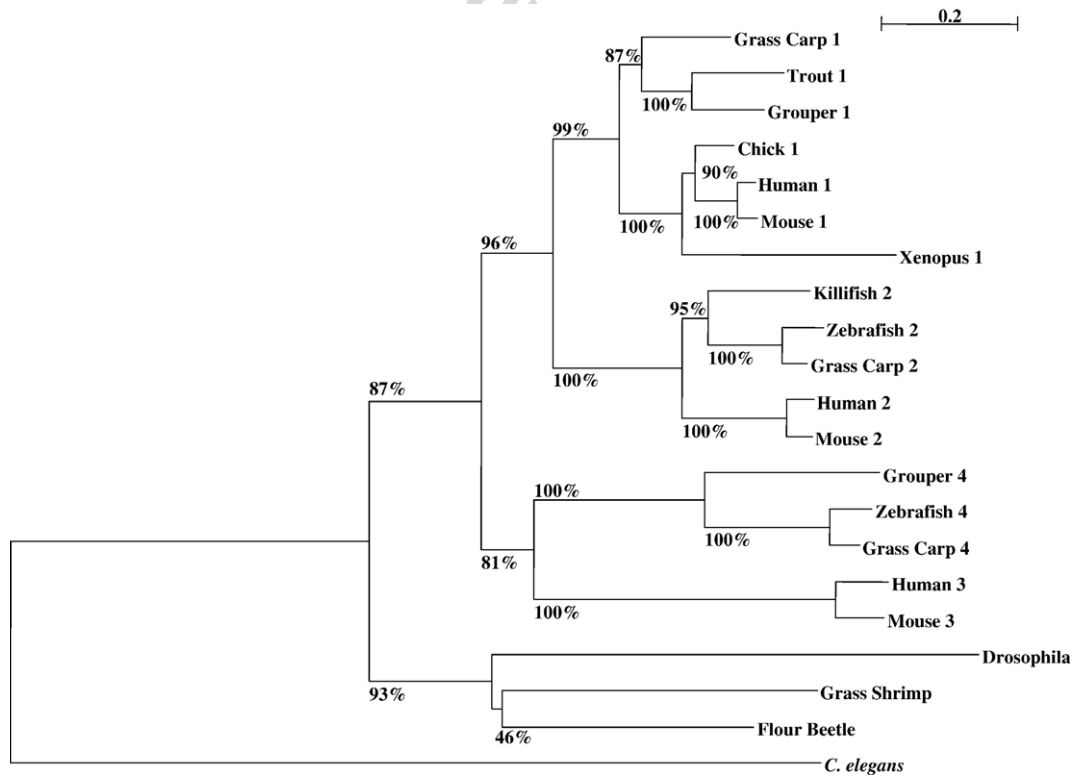


Fig. 3. Neighbor-joining tree derived from 21 HIF amino acid sequences. Bootstrap values on each branch indicate the percentage of trees (1000 replicates) in which that branch is present. 1, 2, 3 and 4 represent HIF-1 α , -2 α , -3 α and -4 α , respectively. GenBank accession numbers for each species from top (Grass Carp1) to bottom (*C. elegans*) are: AAR95697; AAK30364; AAW29027; BAA34234; AAF20149; NP_034561; CAB96628; AAL95711; ABD33838; AAT76668; AAC51212; NP_034267; AAW29028; AAQ94179; AAR95698; AAD22668; AAC72734; AAC47303; AAT72404; XP_967427; CAA19521.

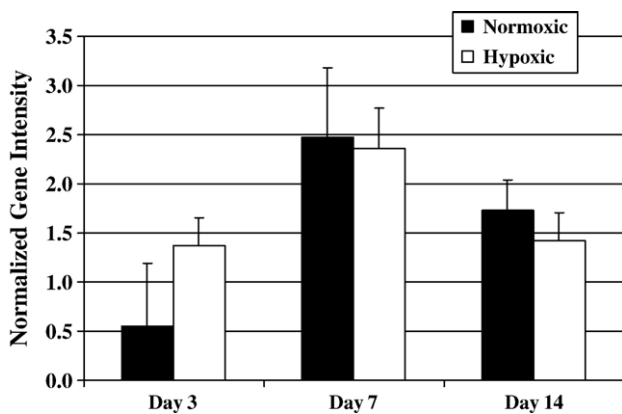


Fig. 4. Changes in grass shrimp HIF expression in response to chronic hypoxia exposure (2.5 ppm) at days 3, 7, and 14.

3.3. Molecular phylogenetic analyses

Neighbor-joining and maximum parsimony analysis produced trees of the same topology with similar high bootstrap scores. Both methods revealed 2 well-supported clades. (1) Invertebrate (arthropod) HIFs (*Palaemonetes*, *Drosophila* and *Tribolium*) with 93% bootstrap support in the neighbor-joining tree (Fig. 3) and 87% in the maximum parsimony tree (data not shown), and (2) vertebrate HIF-1/2/3/4 α with 87 (neighbor joining) and 82% (maximum parsimony) bootstrap support. Two subclades comprised of HIF-1/2 α (96% bootstrap support) and HIF-3/4 α (81% bootstrap support) were present in the vertebrate HIF protein family (Fig. 3).

3.4. In vivo expression and response pattern of HIF to severe and moderate hypoxia

To study the expression of gsHIF under normoxic and hypoxic conditions, grass shrimp were exposed to normoxic, moderate hypoxic (2.5 ppm), and severe hypoxic (1.5 ppm) conditions and shrimp were sampled on days 3, 7, and 14. The measured DO values were 7.71 ± 1.61 ppm and 2.47 ± 0.50 ppm for the moderate hypoxia exposure, and 7.50 ± 0.53 ppm and 1.55 ± 0.23 ppm for the severe hypoxia exposure. The normalized normoxic and hypoxic expression levels of gsHIF, in response to 2.5 ppm DO are shown in Fig. 4. One-way ANOVA across time showed no statistical difference between groups ($p=0.065$). Similarly, using Kruskal–Wallis ANOVA on ranks, no difference between groups was observed in the 1.5 ppm DO exposure ($p=0.587$).

4. Discussion

Like HIF-1 α , the tracheless protein (TRH) is a member of the bHLH-PAS family of transcription factors, which shows high identity with HIF-1 α in the bHLH domain (Isaac and Andrew, 1996). TRH is required for tube formation and is a key regulator of salivary gland and tracheal development in *Drosophila*. Growth of tracheal tubes, which comprise the oxygen delivery system in insects, is stimulated by hypoxia and involves the nitric oxide/cyclic GMP (NO/cGMP) signal

transduction pathway (Wingrove and O'Farrell, 1999). NO, hypoxia and HIF-1 α are functionally linked. The gene of the inducible form of nitric oxide synthase (iNOS) is upregulated by HIF and NO impairs normoxic degradation of HIF-1 α by inhibition of prolyl hydroxylases (Melillo et al., 1995; Metzen et al., 2003). *Drosophila* larvae exhibit rapid NO/cGMP-mediated responses to hypoxia including behavioral changes allowing larvae to escape local hypoxia (Wingrove and O'Farrell, 1999). Grass shrimp show similar types of behavior by climbing out of water during periods of oxygen deficiency (Anderson, 1985). It will be interesting to determine whether hypoxia-induced behavioral responses in grass shrimp share a regulatory mechanism with the HIF and NO/cGMP system as found in *Drosophila*.

The fragment of grass shrimp tracheless protein obtained in this study shows a high level of conservation with other tracheless proteins in the bHLH/PAS regions. Grass shrimp tracheless bHLH domain (20–72) shows 98% identity with bHLH from *D. melanogaster* (GenBank Accession no. AAA96754) and *B. mori* (BAA22946) tracheless. The PAS-A (116–177) domain is most similar to that of *Drosophila* (88%) and the PAS-B (296–395) domain is most similar to that of the red flour beetle *T. castaneum* (GenBank Accession no: XP_967112) (78%). The transcriptional activation domain in the C-terminal portion of the *Drosophila/Bombyx/Tribolium* molecule (Isaac and Andrew, 1996) is not found in the 501 amino acid sequence of the grass shrimp tracheless protein.

HIF protein of the grass shrimp shows a high level of conservation with other HIF-1 α proteins in the bHLH/PAS regions, with amino acid sequences 23–76, 109–179, and 234–321 corresponding to bHLH, PAS-A, and PAS-B domains respectively. In general, the bHLH domain is involved in DNA binding and dimerization, and the PAS domain in target gene specificity, transactivation, and dimerization. In addition to the bHLH/PAS domains, HIF-1 α proteins contain domains and sequence motifs that are involved in nuclear translocation, transactivation and posttranslational modifications, which control HIF protein stability and transcriptional activity.

HIF-1 α is rapidly degraded under normoxic conditions, mediated by post-translational hydroxylation of conserved proline residues within a polypeptide segment known as the oxygen-dependent degradation domain (ODD), which comprises residues 401–603 in human HIF-1 α (Huang et al., 1998; Masson et al., 2001) and 692–863 in *Drosophila* Sima (HIF-1) (Lavista-Llanos et al., 2002; Nambu et al., 1996). One of the proline residues subjected to hydroxylation in vertebrate HIF-1 α resides at the N-terminal end of the ODD within a LXXLAP sequence motif (residues 397–402 in human HIF-1 α), which is conserved in grass shrimp (residues 459–464, LTHLAP). A second Pro residue (Pro-564) resides in the C-terminal ODD (residues 561–567, MLAPYIP in human HIF-1 α), with corresponding sequences in *Drosophila* present in residues 847–853 (MRAPYIP) and in grass shrimp in residues 634–640 (MRAPFIP). The hydroxylated proline residues in ODD are recognized by pVHL, the product of the von Hippel–Lindau tumor suppressor gene, which functions as an E3 ubiquitin ligase and targets HIF-1 α for polyubiquitination and proteasome-dependent degradation (Semenza, 1998). The

prolyl hydroxylase enzymes that catalyze the hydroxylation of these critical proline residues use oxygen as a substrate. Because oxygen is rate limiting for their activity, these enzymes appear to function as oxygen sensors and provide a direct link between oxygen concentration and the HIF-mediated hypoxic response pathway (Bruick and McKnight, 2004; Bruick, 2003). Accordingly, HIF-1 α protein increases exponentially in human HeLa cells exposed to decreasing oxygen concentrations, with a half-maximal response between 1.5 and 2% O₂ and a maximal response at 0.5% (Jiang et al., 1996). In rainbow trout and chinook salmon cells, maximum accumulation of HIF-1 α occurs at much higher oxygen levels (5% O₂), a typical oxygen tension of venous blood in normoxic animals, suggesting a role for oxygen-dependent gene regulation not only during environmental hypoxia, but also in the normal physiology of these fish (Soitamo et al., 2001). Whether this difference in HIF-1 α stabilization applies to terrestrial and aquatic organisms in general remains to be determined.

Under hypoxic conditions proline residues are unmodified and HIF-1 α accumulates and translocates to the nucleus, a step which is mediated by a bipartite nuclear localization signal (NLS) in the C-terminus of the human protein (Luo and Shibuya, 2001). Grass shrimp HIF does not have this signal sequence, but has two potential NLSs between aa 4–20 and 25–41, which have the characteristic bipartite NLS structure consisting of two adjacent basic domains separated by a 10 amino acid spacer sequence. Interestingly, human HIF-1 α contains a similar bipartite NLS between aa 17 and 33, which mediates nuclear import of a GFP-HIF-1 α /1–74 chimeric protein (Kallio et al., 1988).

Modulation of transactivation domain function is a second major mechanism by which HIF-1 α activity is controlled. Vertebrate HIF-1 α contains two transactivation domains (TADs) responsible for recruitment of transcriptional coactivators essential for gene expression: N-TAD, the amino-terminal transactivation domain, comprised of amino acid residues ~540–580 in mammals, and C-TAD, the carboxyl-terminal transactivation domain, comprised of amino acid residues 786–826 in mammals (Jiang et al., 1997; Pugh et al., 1997; Bruick and McKnight, 2004). N-TAD is highly conserved in vertebrate HIF-1 α and contains the second proline hydroxylation motif in the C-ODD. Regulation of its activity is likely to be a by-product of protein stability (Pugh et al., 1997; Bruick and McKnight, 2004). Grass shrimp HIF appears to lack N-TAD. However, as discussed above, the conserved proline hydroxylation motif still exists.

C-TAD operates independently of the ODD and is able to recruit coactivator complexes such as CBP/p300 only under hypoxic conditions (Kallio et al., 1998; Kung et al., 2000; Bruick, 2003). The molecular event that controls C-TAD activity involves the hydroxylation under normoxic conditions of a conserved Asn residue in aa 801–805 (EVNAP) of human HIF-1 α . This Asn hydroxylation motif is conserved in grass shrimp HIF in aa 1034–1038. The hydroxylation of Asn blocks the interaction of C-TAD with the p300/CBP transcriptional coactivators. Abrogation of Asn hydroxylation under hypoxic conditions allows for the interaction of C-TAD with CBP/p300 (Lando et al., 2002a,b). The asparaginyl hydroxylase enzyme that catalyzes the reaction belongs to the same family of 2-oxoglutarate/Fe(II)-dependent oxygenases as the prolyl hydro-

xylases (Masson and Ratcliffe, 2003; Lando et al., 2002a). Both prolyl and asparaginyl hydroxylases serve as direct oxygen sensors and must be turned on to fully induce HIF in mammals (Lando et al., 2002a,b). HIF activity is thus subjected to multiple independent levels of regulation responsible for graded responses to subtle changes in oxygen concentration.

Grass shrimp HIF mRNA levels are not noticeably affected by hypoxia. This observation, combined with the conservation of the ODD, suggests that gsHIF protein levels may be controlled at the (post)translational level as found for vertebrate HIF-1 α and -2 α . Recently 2 HIF isoforms have been identified whose induction appears to occur at the transcriptional level: HIF-3 α in mammals (Heidbreder et al., 2003) and HIF-4 α in fish (Law et al., 2006). Molecular phylogeny analysis, using neighbor-joining and maximum parsimony methods, shows that grass shrimp HIF clusters firmly with HIFs from other invertebrates, which are distinct from the vertebrate HIF-1/2/3/4 α family. The transcriptionally controlled mammal HIF-3 α and fish HIF-4 α proteins form a distinct subclade within the vertebrate HIF (1/2/3/4 α) clade. These results suggest that the gene duplication giving rise to the invertebrate and vertebrate HIFs preceded the duplication resulting in the posttranslationally and transcriptionally controlled forms of vertebrate HIF. Whether invertebrates also have HIFs that are under transcriptional control is unknown at present.

5. Conclusion

As a first step towards understanding the molecular mechanisms that underlie the adaptation of hypoxia-tolerant crustacea to low dissolved oxygen concentrations, we have cloned an HIF-1 α homolog from the grass shrimp *P. pugio*. The encoded amino acid sequence shows high level of conservation with vertebrate HIF-1 α in the bHLH, PAS-A, PAS-B, ODD (with the two proline hydroxylation motifs) and C-TAD (with the asparagine hydroxylation motif) domains. Conservation of important structural motifs suggests that the function, stability and transactivation of grass shrimp HIF are controlled by similar molecular mechanisms as the vertebrate HIF-1 α proteins. However, grass shrimp HIF contains a large polypeptide sequence (aa 790–1020) which has no matching sequences in GenBank. Whether this region conveys unique functional properties to grass shrimp HIF remains to be determined. Similar to what is found for vertebrate HIF-1 α , grass shrimp HIF is constitutively expressed and not induced to an appreciable extent by hypoxia.

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