

# Role of Hypoxia-Inducible Factor $\alpha$ in Response to Hypoxia and Heat Shock in the Pacific Oyster *Crassostrea gigas*

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**Abstract** The Pacific oyster *Crassostrea gigas* inhabits the intertidal zone and shows tolerance to stress conditions such as hypoxia and heat shock. Although some information is available about the genes expressed in response to hypoxia, little is known about the molecular mechanism of the regulation of their expression in mollusks, including the Pacific oyster. Hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) is a master regulator of hypoxia-responsive transcription. In this study, we cloned HIF- $\alpha$  from the oyster and investigated its response to unique stress conditions, including air exposure, for the first time in mollusks. The cDNA of oyster *Hif- $\alpha$*  is 3,182 bp long, of which 2,094 bp encodes a protein of 698 amino acid residues. Northern and Western blot analysis showed that expression of oyster HIF- $\alpha$  mRNA and protein were induced by air exposure, and that expression was induced periodically during air exposure. In addition, induction of *Hif- $\alpha$*  mRNA increased by a maximum 8.0-fold by heat shock. Under heat shock at 35°C (lethal temperature for the oyster), however, it was induced later than at 30°C. After recovery from hypoxia and/or heat shock, *Hif- $\alpha$*  mRNA also upregulated. These data suggest that the oyster has a strategy to induce *Hif- $\alpha$*  mRNA in order to survive hypoxia and heat shock, and that HIF signaling is necessary for recovery from stress.

**Keywords** Air exposure · *Crassostrea gigas* · Heat shock · Hypoxia · Hypoxia-inducible factor

## Introduction

Oxygen is important for the survival of all metazoan organisms. Their lives depend on oxygen to produce energy by aerobic respiration. They synthesize ATP through the mitochondrial tricarboxylic acid cycle in the process of obtaining and utilizing oxygen from air and/or water. In environments where there is a low concentration of available oxygen (hypoxia), however, they have to depend on the glycolysis pathway for survival (Ballantyne 2004; Brahimi-Horn and Pouyssegur 2007; Semenza 2007). It is important for aerobic organisms to respond to hypoxia promptly and to regulate the expression of several genes that are related to glycolysis and cellular homeostasis (Hochachka and Lutz 2001). The hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) acts as the master regulator of the expression of these genes in response to hypoxia (Wang and Semenza 1995; Wang et al. 1995; Hochachka and Lutz 2001; Lee et al. 2004; Pouyssegur et al. 2006). The half-life of mammalian HIF-1 $\alpha$  is very short (<5 min) under normoxic conditions (Wang et al. 1995; Jewell et al. 2001). Under hypoxic conditions, however, HIF-1 $\alpha$  accumulates and forms a heterodimeric DNA-binding complex with HIF-1 $\beta$  [also known as the aryl hydrocarbon receptor nuclear translocator (Arnt)] (Wang et al. 1995), and binds to the hypoxia response element (HRE), 5'-RCGTG-3', on the promoter region of target genes (Semenza and Wang 1992; Wang and Semenza 1993; Semenza et al. 1994). HIF-1 $\alpha$  belongs to the large gene family of basic-helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) proteins (Wang et al. 1995). Its stability and transcriptional activity is regulated by oxygen

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concentration via prolyl hydroxylase domain proteins (PHDs) (Jaakkola et al. 2001) and factor inhibiting HIF-1 (FIH) (Mahon et al. 2001). Under normoxic conditions, PHDs hydroxylate two conserved proline residues (Pro-402 and Pro-564 in human HIF-1 $\alpha$ ) of the LXXLAP motif in the oxygen-dependent degradation domain (ODDD) and N-terminal transactivation domain (N-TAD) (Epstein et al. 2001; Masson et al. 2001; Huang et al. 2002; Appelhoff et al. 2004; Semenza 2004; Tuckerman et al. 2004; Berra et al. 2006). These two 4-hydroxyproline residues are recognized by an E3 ubiquitin ligase, von Hippel–Lindau tumor-suppressor protein (VHL); subsequently, ubiquitinated HIF-1 $\alpha$  is degraded by the ubiquitin–proteasome system (Huang et al. 1998; Yu et al. 2001; Hon et al. 2002). FIH hydroxylates an asparagine residue (Asn-803 in human HIF-1 $\alpha$ ) in the C-terminal transactivation domain (C-TAD) under normoxic conditions (Hewitson et al. 2002; Linke et al. 2004; Stolze et al. 2004). Because this hydroxylation disturbs its interaction with the transcriptional co-activator p300 and CREB-binding protein (CBP), the transcriptional activity of HIF-1 $\alpha$  is inactivated under this condition (Kallio et al. 1998; Lando et al. 2002).

HIF-1 $\alpha$  is a widely conserved protein, and it has been identified in nematodes (Jiang et al. 2001), insects (Nambu et al. 1996), crustaceans (Li and Brouwer 2007), fishes (Soitamo et al. 2001), and mammals (Wang et al. 1995). In mammals, two HIF-1 $\alpha$  isoforms have been reported: HIF-2 $\alpha$  and HIF-3 $\alpha$  (Tian et al. 1997; Gu et al. 1998; Kietzmann et al. 2001). In rats, expression levels of *Hif-1 $\alpha$*  and *Hif-2 $\alpha$*  mRNAs were not affected by hypoxia; however, those of *Hif-3 $\alpha$*  were upregulated after 2 h of hypoxia (Heidbreder et al. 2003). Generally, it seems that *Hif-1 $\alpha$*  mRNA is constitutively synthesized and not affected by hypoxia, but there are some data suggesting that it is inducible, albeit transiently, by hypoxia (Wang et al. 1995; BelAiba et al. 2007). In fishes, a novel HIF-1 $\alpha$  isoform, HIF-4 $\alpha$ , has been reported, but HIF-3 $\alpha$  has not been identified (Law et al. 2006). Similar to its mammalian counterpart, fish *Hif-1 $\alpha$*  mRNA was not affected by hypoxia (Soitamo et al. 2001; Law et al. 2006), but fish *Hif-4 $\alpha$*  mRNA was upregulated after 4 and 96 h of hypoxia (Law et al. 2006). In the Atlantic croaker *Micropogonias undulatus*, a hypoxia-tolerant marine teleost, *Hif-1 $\alpha$*  and *Hif-2 $\alpha$*  mRNAs were upregulated under hypoxic conditions (Rahman and Thomas 2007). In the crucian carp *Carassius carassius*, a poikilothermic vertebrate, HIF-1 $\alpha$  protein was detectable, and the amount found in cold-acclimated specimens increased without degradation despite normoxia (Rissanen et al. 2006). In the nematode *Caenorhabditis elegans*, HIF was required not only for heat acclimation but also for cross-tolerance against CdCl<sub>2</sub> (Treinin et al. 2003). These data suggest that species living in different environments show a variety of stress response mechanisms against hypoxia that

enable them to adapt to their own environment, and that HIF-1 $\alpha$  does not always regulate transcription of the genes involved in response to hypoxia.

Marine mollusks, especially shellfishes inhabiting the intertidal zone, have a particular ability to survive harsh environments (Ballantyne 2004). They experience not only heat shock but also severe hypoxia with the tidal cycle. Therefore, their hypoxic tolerance is an interesting feature of environmental adaptation. The Pacific oyster *Crassostrea gigas*, a sessile bivalve inhabiting the intertidal zone, also has the ability to adapt to hypoxia (David et al. 2005). To determine ability to tolerate stress conditions or as potential biomarkers, the stress genes including heat shock proteins (HSPs) and the energy metabolism genes have been investigated in marine shellfish (Buckley et al. 2001; Piano et al. 2002, 2005; Boutet et al. 2003; Lannig et al. 2006; Cherkasov et al. 2007). Although there were several investigations about hypoxic adaptation (Ballantyne 2004; Schulte 2004; David et al. 2005; Michaelidis et al. 2005), they did not provide a clear overview of adaptation to hypoxia in mollusks. Under experimental long-term hypoxic conditions, stress proteins comprising HSP and several genes involved in energy metabolism were upregulated in the oyster (David et al. 2005). Our previous studies showed that endoplasmic reticulum chaperones in oyster were induced during air exposure (Kawabe and Yokoyama 2009, 2010). However, it is unclear what molecules regulate the expression of these genes. Because HIF-1 $\alpha$  is a master transcription factor under hypoxia, HIF-1 $\alpha$  might regulate gene expression in marine mollusks under hypoxic conditions. In marine invertebrates, however, the full-length cDNA for this transcription factors has been reported only in crustaceans, such as the grass shrimp, *Palaemonetes pugio* (Li and Brouwer 2007). In spite of the remarkable adaptability of intertidal mollusks to hypoxia, little information is available about HIF-1 $\alpha$  in these animals. Here, to extend our knowledge of the response to air exposure in the oyster, we focused on HIF-1 $\alpha$ . This is the first report on the cloning of the full-length cDNA of *Hif- $\alpha$*  in a mollusk (*C. gigas*). We prepared a polyclonal anti-oyster HIF- $\alpha$  antibody and quantified the expression levels of oyster HIF- $\alpha$  protein using Western blot analysis during air exposure. We also quantified in detail the expression levels of oyster *Hif- $\alpha$*  mRNA using Northern blot analysis during several stress conditions.

## Materials and Methods

### Materials

Pacific oysters (*C. gigas*) were purchased from an oyster farm in Obama Bay, Fukui Prefecture, Japan. Specimens for

hypoxic and air exposure treatments were acclimated to seawater for more than 3 days in Obama Bay or in a 60-l tank at  $13\pm 1^\circ\text{C}$  in December 2004. Specimens for heat shock treatment were acclimated to seawater for more than 3 days in Obama Bay and then in a 60-l tank at  $20\pm 1^\circ\text{C}$  in August 2005.

#### RNA Preparation

Total RNA was extracted from oyster tissues using Sepasol-RNA I super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. Samples were stored at  $-80^\circ\text{C}$  until assay.

#### Reverse Transcription–Polymerase Chain Reaction (RT–PCR)

The RACE-Ready cDNA used as a template PCR was synthesized from 300 ng mRNA, which was purified from 100  $\mu\text{g}$  total RNA prepared from the gill using the Oligotex<sup>TM</sup>-dT30 Super mRNA Purification Kit (Takara, Shiga, Japan) and the SMART<sup>TM</sup> RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. The degenerate oligonucleotide primers for PCR were as follows: the first sense and antisense primers were 5'-CAYCCNTGYGAYCAYGARGA-3' and 5'-GCYTGNGTNCNACCCANAC-3' and the nested sense and antisense primers were 5'-MGNATGAARTGYACNYTNAC-3' and 5'-CNACCCANACRWANCCNCC-3', respectively: M=A+C, N=A+C+G+T, R=A+G, Y=C+T, and W=A+T (Table 1). These primers correspond to the amino acid sequences HPCDHEE, VWVE(V)TQA, RM(L)KCTL, and GGY(F) VWVE(V), respectively, which are identical to those of *Danio rerio* HIF-1 $\alpha$  (AY326951), *Gallus gallus* HIF-1 $\alpha$  (AB013746), *Homo sapiens* HIF-1 $\alpha$  (U22431), and *P. pugio* HIF- $\alpha$  (AY655698). The PCR was performed in a 10  $\mu\text{l}$  reaction mixture comprising Blend Taq –Plus– (Toyobo, Osaka, Japan), buffer for Blend Taq (Toyobo),

0.2 mM dNTPs, and 0.6  $\mu\text{M}$  of each primer with the following parameters: one cycle at  $94^\circ\text{C}$  for 2 min followed by 40 cycles at  $94^\circ\text{C}$  for 1 min,  $52^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 1 min, and one cycle at  $72^\circ\text{C}$  for 10 min. The second PCR was performed with the following parameters: one cycle at  $94^\circ\text{C}$  for 2 min followed by 40 cycles at  $94^\circ\text{C}$  for 1 min,  $48^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 1 min, and one cycle at  $72^\circ\text{C}$  for 10 min. The inserts, approximately 430 bp, were subcloned into the pDrive Cloning Vector using the Qiagen PCR Cloning Kit (Qiagen, Hilden, Germany) and sequenced using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

#### Rapid Amplification of cDNA Ends (RACE)

The cDNA library for 5' rapid amplification of cDNA ends (5'-RACE) was synthesized from 2  $\mu\text{g}$  of total RNA from the gill using the 5'/3' RACE Kit, 2nd Generation (Roche, Penzberg, Germany) and the gene-specific oligonucleotide primer (GSP) 5'-GAGGGGTGAGGAATGGGTTC-3', according to the manufacturer's protocols for the synthesis of dA-tailed first-strand cDNA (dA-tailed cDNA). 3'-RACE-Ready cDNA was synthesized from 300 ng mRNA, which was purified from 100  $\mu\text{g}$  of total RNA prepared from the gill using the Oligotex<sup>TM</sup>-dT30 Super mRNA Purification Kit (Takara) with the SMART<sup>TM</sup> RACE cDNA Amplification Kit (Clontech) according to the manufacturer's protocol.

5'-RACE was performed in a 20  $\mu\text{l}$  reaction mixture comprising Blend Taq –Plus– (Toyobo), buffer for Blend Taq (Toyobo), 0.2 mM dNTPs, 0.75  $\mu\text{M}$  Oligo dT-anchor primer, 0.25  $\mu\text{M}$  GSP, and dA-tailed cDNA with the following parameters: one cycle at  $94^\circ\text{C}$  for 2 min followed by 40 cycles at  $94^\circ\text{C}$  for 1 min,  $55^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 2 min, and one cycle at  $72^\circ\text{C}$  for 10 min. The second PCR was performed with the following parameters: one cycle at  $94^\circ\text{C}$  for 2 min followed by 40 cycles at  $94^\circ\text{C}$  for 1 min,  $60^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 2 min, and one cycle at  $72^\circ\text{C}$  for 10 min. The PCR for 3'-RACE was performed

**Table 1** Nucleotide sequences of degenerate and gene-specific primers used for RT–PCR and RACE

Usage	Direction	Sequence
RT–PCR	Sense	5'-CAYCCNTGYGAYCAYGARGA-3'
RT–PCR	Antisense	5'-GCYTGNGTNCNACCCANAC-3'
2nd RT–PCR	Sense	5'-MGNATGAARTGYACNYTNAC-3'
2nd RT–PCR	Antisense	5'-CNACCCANACRWANCCNCC-3'
cDNA synthesis for 5'-RACE	Antisense	5'-GAGGGGTGAGGAATGGGTTC-3'
5'-RACE PCR	Antisense	5'-CCCCACACTCCTTCTTTATC-3'
5'-RACE 2nd PCR	Antisense	5'-GTTGCTGACTTGAGGTTGAC-3'
3'-RACE PCR	Sense	5'-GTGAGGTCATAGACCAGGCC-3'
3'-RACE 2nd PCR	Sense	5'-GGGACAAGTGATGACAAGGC-3'

M, A+C; N, A+C+G+T; R, A+G; Y, C+T; W, A+T

in a 20- $\mu$ l reaction mixture comprising Advantage 2 Polymerase Mix (Clontech), Advantage 2 PCR Buffer (Clontech), 0.2 mM dNTPs, 0.2  $\mu$ M each primer, and 3'-RACE-Ready cDNA, with the following parameters: one cycle at 94°C for 1 min followed by 35 cycles at 94°C for 15 s, 68°C for 30 s, and 72°C for 3 min, and one cycle at 72°C for 10 min. The GSPs for RACE were as follows: the first and the nested primers for 5'-RACE were 5'-CCCCA CACTCCTTCTTTATC-3' and 5'-GTTGCTGACTT GAGGTTGAC-3', and for 3'-RACE were 5'-GTGAGGT CATAGACCAGGCC-3' and 5'-GGGACAAGTGATGA CAAGGC-3', respectively (Table 1). The cDNA fragments obtained from 5'-RACE and 3'-RACE were cloned and sequenced as described above.

#### Accession Numbers of Nucleotide Sequences

The nucleotide sequence of oyster *Hif- $\alpha$*  has been registered in the DDBJ/EMBL/GenBank databases under accession number AB289857.

#### Hypoxic Treatment

The oysters ( $N=52$ ) were acclimated to aerated seawater ( $pO_2=199$  mmHg) at  $13\pm 1^\circ\text{C}$ . The hypoxic seawater was prepared by bubbling with nitrogen gas until  $pO_2 < 40$  mmHg. A total of 37 acclimated oysters were transferred to hypoxic seawater at  $13\pm 1^\circ\text{C}$ . After hypoxic treatment for 48 or 72 h, the oysters were allowed to recover by retransfer to aerated seawater at  $13\pm 1^\circ\text{C}$  for 1 h.

#### Air Exposure Treatment

The oysters were exposed to air under experimental conditions as described previously (Kawabe and Yokoyama 2009). The environmental temperature ranged from a minimum of 4°C to a maximum of 20°C during the studies. The oysters ( $N=169$ ) were acclimated to seawater ( $pO_2=199$  mmHg) at  $13\pm 1^\circ\text{C}$ . A total of 89 acclimated oysters were exposed to air at  $4\pm 1^\circ\text{C}$  for 15 days in a cold room. Another 68 acclimated oysters were exposed to air at  $20\pm 1^\circ\text{C}$  for 7 days in a multi-thermo incubator (MTI-202; Tokyo Rikakikai, Tokyo, Japan).

#### Heat Shock Treatment

Oysters ( $N=110$ ) were acclimated to aerated seawater at  $20\pm 1^\circ\text{C}$ . A total of 50 acclimated oysters were transferred to aerated seawater ( $pO_2=199$  mmHg) at  $30\pm 1^\circ\text{C}$  for 96 h. Another 50 acclimated oysters were transferred to aerated seawater at  $35\pm 1^\circ\text{C}$  for 48 h. After heat shock treatment for 10 h, the oysters were retransferred to aerated seawater at  $20\pm 1^\circ\text{C}$  for 48 h.

#### Preparation of Polyclonal Anti-oyster HIF- $\alpha$ Antibody

The synthetic peptide of HIF- $\alpha$ , CQNPDLLAALELFLPD-SELM to which cysteine was added for the coupling, was coupled to the carrier protein keyhole limpet hemocyanin (KLH; Sigma, St. Louis, MO, USA) with *m*-maleimidobenzoyl-*n*-hydroxysuccinimide ester (MBS; Sigma) as described previously (Liu et al. 1979). This antigen solution (synthetic peptide conjugated to KLH-MB) was stored at  $-20^\circ\text{C}$  until use. For initial immunization, 200  $\mu$ l antigen solution comprising 100  $\mu$ g of synthetic peptide with 1.5 ml complete Freund's adjuvant (Difco, Detroit, MI, USA) was administered to a rabbit by subcutaneous injection. Three weeks after the initial immunization, booster immunization with 1.5 ml Incomplete Freund's Adjuvant (Difco) comprising 50  $\mu$ g of synthetic peptide was given four times at 10-day intervals. At week 11, antiserum was collected from an ear vein and stored at  $-80^\circ\text{C}$  until use.

#### Western Blot Analysis

After collection, oyster tissues were immediately ground in liquid nitrogen and stored at  $-80^\circ\text{C}$  until use. Ground tissue (50 mg) was homogenized with 250  $\mu$ l cold phosphate-buffered saline (PBS) (pH 7.4) comprising 1% (v/v) Triton X-100 and  $1\times$  Protease Inhibitor Cocktail (Sigma). The homogenates were centrifuged at  $17,000\times g$  for 15 min. Total soluble protein (20  $\mu$ g) was electrophoresed on 7.5% sodium dodecyl sulfate–polyacrylamide gel according to Laemmli (1970). Proteins were electrically transferred to nitrocellulose membranes (Advantec, Dublin, CA, USA) using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA). As an internal control, another total soluble protein (20  $\mu$ g) was electrophoresed on another gel and stained with Coomassie Brilliant Blue R-250 (CBB R-250).

The membranes were washed with PBS containing 0.1% Tween 20 (PBS-T). After washing, the membranes were blocked at room temperature for 2 h with PBS-T containing 5% skim milk (Yukijirushi, Tokyo, Japan) and then incubated at 37°C for 1 h with a polyclonal anti-oyster HIF- $\alpha$  antibody diluted 1:200 in PBS-T. After washing with PBS-T, the membranes were incubated at 37°C for 1 h with a monoclonal anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted at a ratio of 1:100,000 in PBS-T. The membranes were exposed to medical X-ray film (RX-U; Fujifilm) after incubation with CDP-star detection reagent (Amersham Biosciences, Piscataway, NJ, USA). Film images were scanned using an ImageScanner (Amersham Biosciences) and image analysis was performed using ImageMaster 2D Platinum version 5.0 (Amersham Biosciences). Spot volume (%) is calculated as a ratio of the total signal volume detected from the entire membrane and normalized to the total spot volume per membrane.

## Northern Blot Analysis

Oysters processed immediately after harvesting were used as controls. Total RNA was extracted from the control oysters (acclimated to 13°C) and air-exposed oysters (4°C for 1, 3, 5, 7, 10, and 15 days, and 20°C for 1, 3, 5, and 7 days). Northern blot analysis of oyster *Hif-α* was performed with 10 μg total RNA as described previously (Kawabe and Yokoyama 2009). The digoxigenin-labeled probe of oyster *Hif-α* (889 bp, 861–1749 bases in Fig. 1a) was prepared by PCR using DIG-11-dUTP (Roche). Film images were scanned as described above.

## Statistical Analysis

Data were analyzed by one-way ANOVA followed by Fisher's PLSD post hoc test (StatView 5.0; SAS, Cary, NC, USA). Statistical significance was accepted when  $P < 0.05$ .

## Results

### Full-Length cDNA of Oyster *Hif-α*

In this study, the full-length cDNA of mollusk *Hif-α* was cloned for the first time from the oyster *C. gigas*. The cDNA of oyster *Hif-α* was 3,182 bp long of which 2,094 bp encoded a protein of 698 amino acid residues (Fig. 1a). The theoretical molecular weight and isoelectric point of oyster HIF-α, found using ProtParam (<http://kr.expasy.org/tools/protparam.html>), are approximately 79 kDa and 6.22, respectively. The PSORT II server (<http://psort.ims.u-tokyo.ac.jp/form2.html>) revealed that oyster HIF-α has three putative nuclear localization signals (amino acids 12–28, 149–153, and 599–606). In addition, oyster HIF-α contained a bHLH DNA binding domain (amino acids 12–66), two PAS domains (amino acids 88–149 and 218–288), and a PAS-associated C-terminal motif (PAC) domain (amino acids 292–335), which were identified by an InterProScan sequence search (<http://www.ebi.ac.uk/Tools/InterProScan/>). The deduced amino acid sequence of oyster HIF-α was 33%, 29%, 34%, 34%, 37%, 30%, and 20% homologous to HIF-1α of *H. sapiens*, *G. gallus*, *Xenopus laevis*, *Ctenopharyngodon idella*, *P. pugio*, *Drosophila melanogaster*, and *C. elegans*, respectively (Table 2). Although homology based on all amino acid sequences was low, each of the four conserved regions (bHLH, PAS-A, PAS-B, and PAC) of oyster HIF-α showed high homology to the corresponding regions of vertebrates, crustaceans, and insects. It is known that the bHLH domain is required for DNA binding and the PAS domain is required for heterodimerization with HIF-1β (Lee et al. 2004). Therefore, it is thought that oyster HIF-α is a potential transcription factor forming a heterodimer with

HIF-1β, as in other HIF-1αs. Oyster HIF-α conserved an LXXLAP motif (amino acids 399–404) containing the target proline residue of PHD (Fig. 1b). In N-TAD, however, oyster HIF-α had an incomplete LXXLAP motif (amino acids 500–505); leucine residues were replaced with methionine and arginine residues. Interestingly, oyster HIF-α has no asparagine residue, which is the target of FIH-1; that is, there is no C-TAD in the oyster HIF-α. Therefore, the activity of oyster HIF-α might not be controlled predominantly by the regulatory mechanism of transcriptional activity via formation of a complex between HIF-α and p300/CBP coactivator. Figure 2 shows the phylogenetic tree of HIF-α constructed by CLUSTALW with the neighbor-joining method using *C. elegans* HIF-α as an outgroup. The phylogenetic tree showed that invertebrate HIF-α forms a cluster different from that of vertebrate HIF-α. In the invertebrate cluster, oyster HIF-α formed a cluster different from that of arthropod HIF-α.

### Stabilization of Oyster HIF-α Protein During Air Exposure

Because exposure to air, like exposure to hypoxic seawater, is a hypoxic condition for oysters (Kawabe et al. 2010), we investigated the stabilization of oyster HIF-α protein during long-term air exposure. In advance of Western blot analysis, the specificity of polyclonal anti-oyster HIF-α antibody was confirmed by the enzyme-linked immunosorbent assay with synthetic HIF-α peptides (data not shown). Figure 3a shows the result of Western blot analysis using the polyclonal anti-oyster HIF-α antibody. No band was detected in the adductor muscle, mantle, and gill under normoxic conditions. After exposure to air for 3 days, a polyclonal anti-oyster HIF-α antibody detected a single protein band of 120 kDa in these three tissues. This molecular weight detected by the Western blot analysis was higher than the theoretical molecular weight deduced from oyster *Hif-α* cDNA, which is approximately 79 kDa. There is nothing surprising in the result of Western blot analysis because posttranslational modifications (such as phosphorylation) of HIF-1α protein are observed in several species (Wang et al. 1995; Richard et al. 1999; Rissanen et al. 2006). In mammals, the apparent molecular weight of HIF-1α protein was 120 kDa which is higher than the theoretical molecular weight of 93 kDa (Wang et al. 1995). In fish, the apparent molecular weight of HIF-1α protein was 91 and 94 kDa which are higher than the theoretical molecular weight of 85 kDa (Rissanen et al. 2006). We concluded that the detected protein band shows oyster HIF-α from the facts that HIF-1α protein receives the posttranslational modification (Richard et al. 1999) and that a polyclonal anti-oyster HIF-α antibody detected a single protein band which is detectable under hypoxic conditions. The finding of a 120-kDa protein band suggests



**Table 2** Deduced amino acid sequence comparison of oyster HIF- $\alpha$  with other HIF-1 $\alpha$  (%)

	bHLH	PAS-A	PAS-B	PAC	ORF
<i>H. sapiens</i> HIF-1 $\alpha$	58	46	52	68	33
<i>G. gallus</i> HIF-1 $\alpha$	61	48	50	70	29
<i>X. laevis</i> HIF-1 $\alpha$	60	45	54	68	34
<i>C. idella</i> HIF-1 $\alpha$	61	46	57	65	34
<i>P. pugio</i> HIF- $\alpha$	61	50	57	52	37
<i>D. melanogaster</i> Sima	61	41	53	47	30
<i>C. elegans</i> HIF- $\alpha$	34	33	25	38	20

ORF open reading frame

the existence of posttranslational modifications of oyster HIF- $\alpha$ .

To characterize the behavior of oyster HIF- $\alpha$  under air exposure in detail, we investigated the expression of oyster HIF- $\alpha$  at the protein level in the gill, a respiratory organ (Fig. 3b). On the first day of air exposure at 4°C, the expression of HIF- $\alpha$  in these oysters was significantly higher than that in the control (day 0) ( $P=0.0216$ ). The

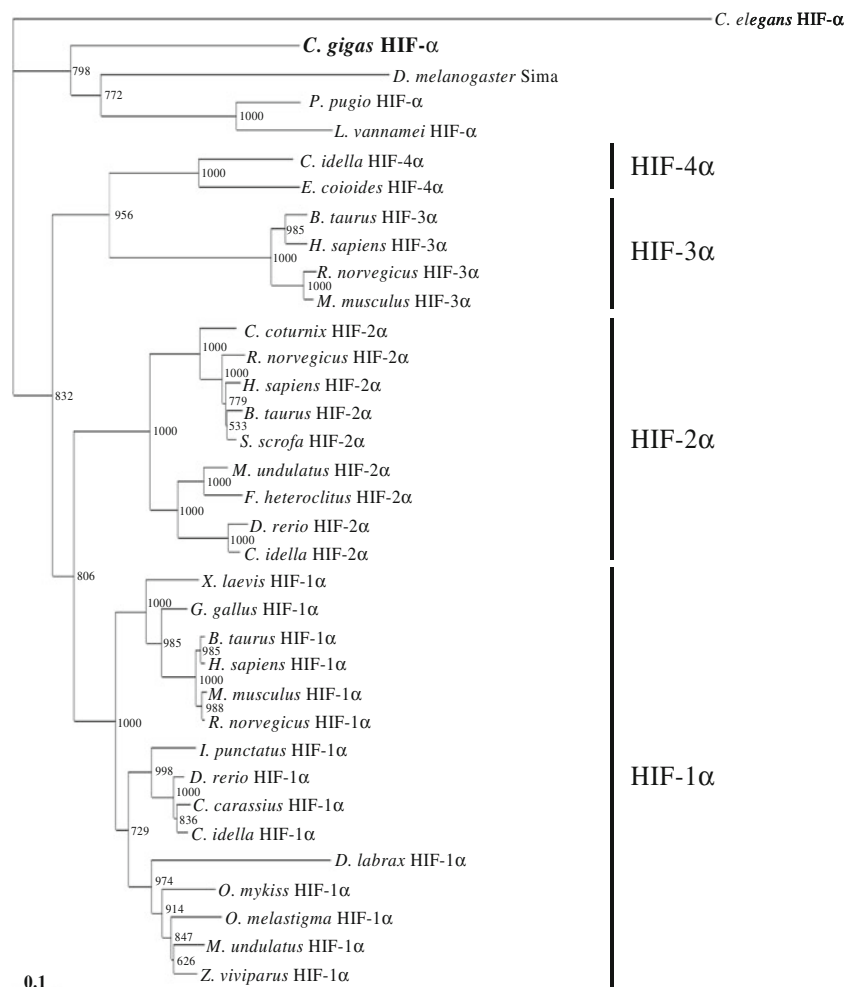
accumulation of oyster HIF- $\alpha$  protein was maintained until day 2; however, it decreased toward the basal level by day 5, reaching a value that was significantly lower than the value observed at day 1 ( $P=0.0251$ ). Interestingly, at day 10 after air exposure at 4°C, HIF- $\alpha$  expression in these oysters was again significantly higher than that in the controls and the day 5 value ( $P<0.05$ ). This accumulation of HIF- $\alpha$  protein was maintained until day 14.

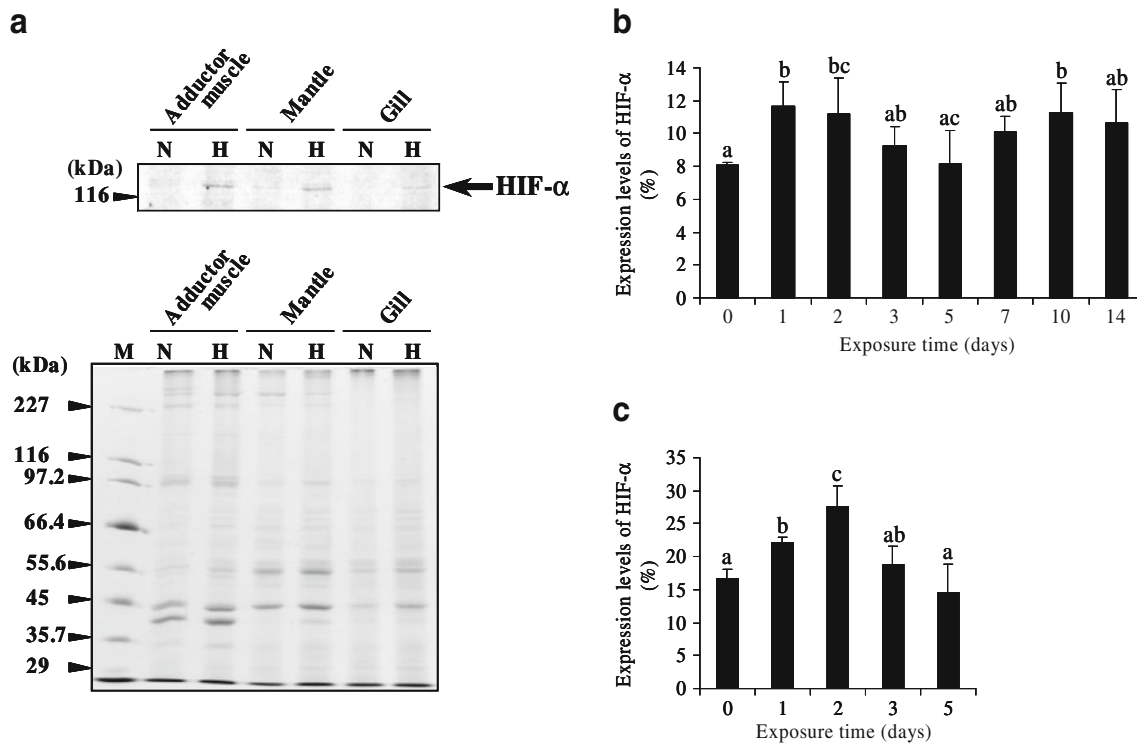
The expression patterns of oyster HIF- $\alpha$  protein were similar on air exposure at 20°C and 4°C (Fig. 3c). On the first day of air exposure at 20°C, the expression of HIF- $\alpha$  in these oysters was significantly higher than that in controls (day 0) ( $P=0.0287$ ). On day 2 of air exposure, the expression level again increased significantly and was maximal in comparison with controls ( $P=0.0002$ ). However, the expression level decreased significantly to the basal level by day 5 ( $P<0.0001$  compared with day 2).

#### Expression of Oyster *Hif*- $\alpha$ mRNA in Tissues

Figure 4a shows the results of Northern blot analysis of oyster *Hif*- $\alpha$  mRNA. The expression of *Hif*- $\alpha$  mRNA

**Fig. 2** Phylogenetic analysis of HIF- $\alpha$  family from various species. GenBank accession numbers of predicted amino acid sequences for each species from top (*C. elegans* HIF- $\alpha$ ) to bottom (*Z. viviparus* HIF-1 $\alpha$ ) are AF364604, AB289857, DMU43090, AY655698, FJ807918, AY450270, AY735011, BC153226, AB054067, AJ277827, BC120587, AF212989, AJ277828, U81984, AB018399, EF375723, DQ363932, AF402782, DQ375242, AY577524, BC043769, AB013746, AB018398, U22431, AF003695, AF057308, DQ020167, AY326951, DQ306727, AY450269, DQ171936, AF304864, DQ317443, DQ363931, and DQ089695. The neighbor-joining method was used to generate this phylogenetic tree, with *C. elegans* HIF- $\alpha$  as an outgroup. Scale bar indicates the number of 0.1 residue substitutions per site. Numbers at the nodes indicate bootstrap values





**Fig. 3** Western blot analysis of oyster HIF- $\alpha$ . **a** Accumulation of oyster HIF- $\alpha$  protein in adductor muscle, mantle, and gill under hypoxic conditions (*top panel*). Total soluble proteins (20  $\mu$ g) were prepared from oyster tissues acclimated to normoxic seawater at 13°C (N) and exposed to air at 4°C for 3 days (H). The specific signals of oyster HIF- $\alpha$  protein were visualized by Western blot analysis with polyclonal anti-oyster HIF- $\alpha$  antibody. As an internal control, another

total soluble protein (20  $\mu$ g) was electrophoresed and stained with CBB R-250 (*bottom panel*). M molecular markers. **b** Histogram showing changes in expression levels of oyster HIF- $\alpha$  protein in the gill during air exposure at 4°C (N=3). **c** Histogram showing changes in expression levels of oyster HIF- $\alpha$  protein in the gill during air exposure at 20°C (N=4). Columns and bars show mean $\pm$ SD. Different letters indicate a significant difference ( $P<0.05$ )

differed significantly among control oyster tissues. The expression level in the gill was higher than that in the adductor muscle ( $P=0.0483$ ) and mantle ( $P=0.0038$ ) (Fig. 4b). In subsequent expression analyses of *Hif- $\alpha$*  mRNA, we focused on the gill in which the expression of *Hif- $\alpha$*  mRNA was the highest.

#### Expression of Oyster *Hif- $\alpha$* mRNA During Air Exposure

In general, the expression levels of *Hif-1 $\alpha$*  mRNA are not affected by hypoxia in mammals, fish, and shrimps (Soitamo et al. 2001; Stroka et al. 2001; Pagé et al. 2002; Lee et al. 2004; Li and Brouwer 2007). Interestingly, however, that of oyster *Hif- $\alpha$*  mRNA was noticeably affected by air exposure (Fig. 5). On the first day of air exposure at 4°C, the expression of *Hif- $\alpha$*  in oysters was 1.6-fold higher than that in controls (day 0) (Fig. 5a). At day 3 of air exposure, the expression level was 3.5-fold ( $P=0.0471$ ); however, it decreased to 1.5-fold the control value at day 5. Unexpectedly, the level of *Hif- $\alpha$*  mRNA expression was observed to fluctuate during air exposure at 4°C. At day 7 of air exposure at this temperature, expression increased significantly to 3.6-fold the control value ( $P=0.0417$ ), but the level decreased

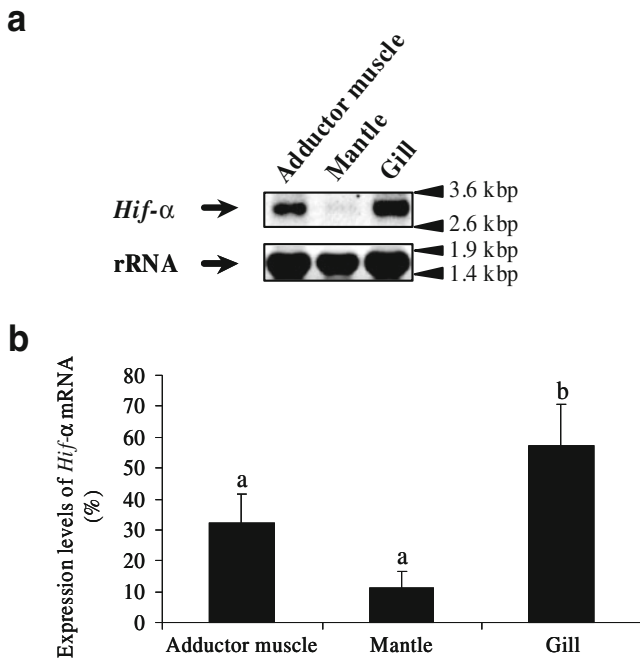
again by day 10. It increased again to 3.5-fold the control value at day 15 ( $P=0.0531$ ). Interestingly, the fold changes showed a constant value of approximately 3.5 (at days 3, 7, and 15) in comparison with controls.

During air exposure at 20°C, the expression patterns of *Hif- $\alpha$*  mRNA (Fig. 5b) were similar to those of proteins. On the first day of air exposure at 20°C, the expression of *Hif- $\alpha$*  was significantly higher (3.6-fold) in these oysters than the control value ( $P=0.0439$ ). At day 3 of air exposure, it reached a maximum of 5.6-fold the control value ( $P=0.0021$ ). Similar to the changes seen on exposure to air at 4°C, the expression level was significantly lower at day 5 in comparison with day 3 ( $P=0.0419$ ). The fold changes at days 5 and 7 were 3.0 and 3.3, respectively. Interestingly, these fold changes were higher than those observed on air exposure at 4°C until day 5.

#### Influence of Hypoxia on Expression of Oyster *Hif- $\alpha$* mRNA

Oyster *Hif- $\alpha$*  mRNAs were apparently induced during air exposure. In bivalves, air exposure not only constitutes a hypoxic stress (Widdows et al. 1979; Jokumsen and Fyhn 1982) but also leads to fluctuation of hemolymph pH





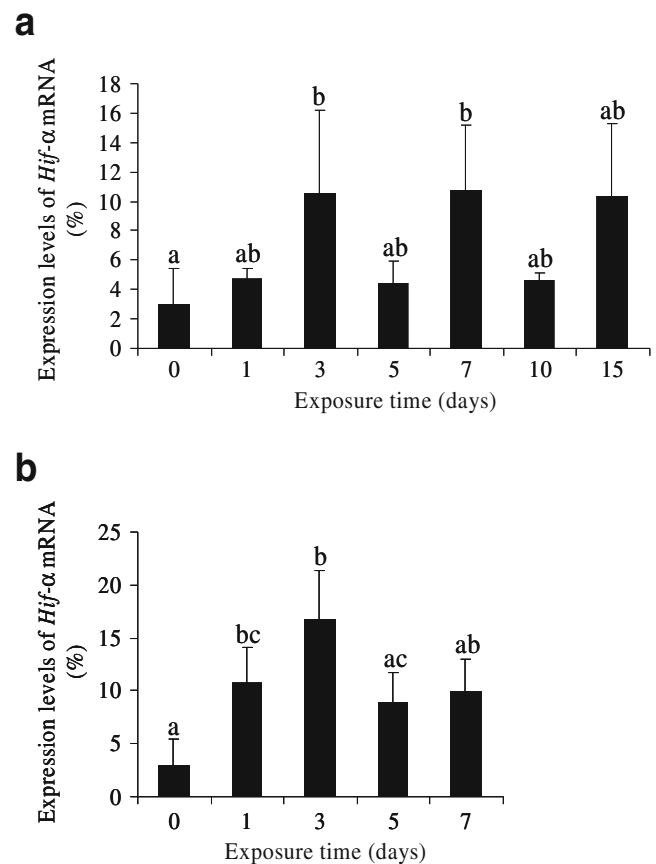
**Fig. 4** Different expression levels of oyster *Hif-α* mRNA in the three types of tissues. **a** Northern blot analysis of oyster *Hif-α* in control oysters (acclimated to 13°C). Total RNA (10 μg) was extracted from the adductor muscle, mantle, and gill. Specific signals of oyster *Hif-α* mRNA were visualized by Northern blot analysis using a digoxigenin-labeled oyster *Hif-α* probe. rRNA stained with methylene blue is shown as the loading control. **b** Histogram showing expression levels of oyster *Hif-α* mRNA in three tissues of the control oyster (acclimated to 13°C). Columns and bars show mean±SD ( $N=3$ ). Different letters indicate a significant difference ( $P<0.05$ )

(Jokumsen and Fyhn 1982; Michaelidis et al. 2005). To identify the factor that induces oyster *Hif-α* mRNA, we examined its expression level during hypoxia (Fig. 6). The expression of *Hif-α* mRNA was not noticeably affected until after 18 h of hypoxia (Fig. 6a). After 36 h of hypoxia, it was 6.5-fold the control value ( $P=0.0536$ ). After 48 h of hypoxia it reached a maximum value that was 7.9-fold the control value ( $P=0.0166$ ). However, expression was significantly lower after 72 h of hypoxia in comparison with 48 h ( $P=0.0447$ ). To investigate how the expression level of *Hif-α* mRNA responded to recovery from oxygen deficiency, we performed a recovery test by retransferring oysters to aerated seawater for 1 h (Fig. 6b). Interestingly, *Hif-α* mRNA increased significantly to 12.4- and 9.5-fold the control value on recovery from 48 and 72 h of hypoxia, respectively ( $P=0.0004$  and  $P=0.0042$ ). On recovery from 72 h of hypoxia, it increased significantly in comparison with 72 h of hypoxia ( $P=0.0120$ ).

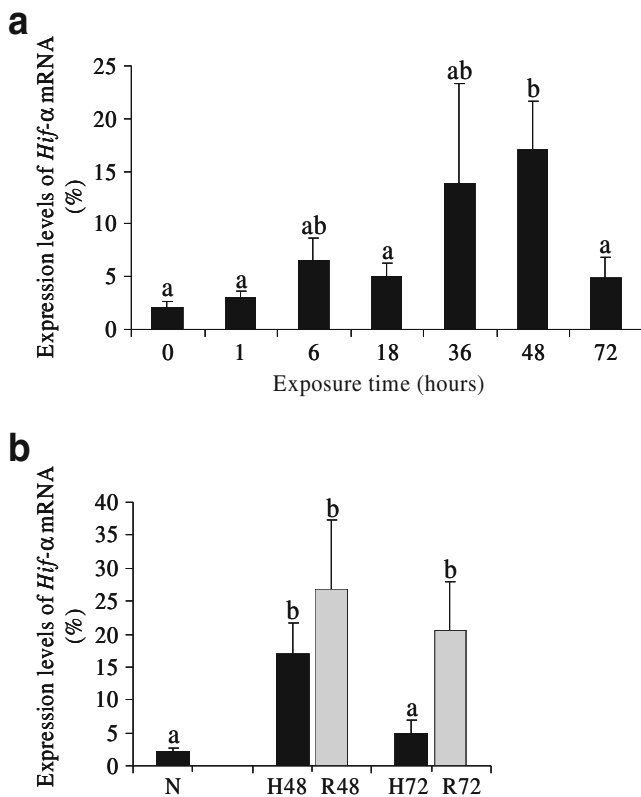
#### Influence of Heat Shock on Expression of Oyster *Hif-α* mRNA

On exposure to air, oyster *Hif-α* mRNA was strongly induced at 20°C (Fig. 5). We therefore examined the

expression level of oyster *Hif-α* mRNA during heat shock (Fig. 7). The survival rate of the oysters was 100% throughout heat shock at 30°C. After 0, 4, 10, and 48 h of heat shock at 35°C, survival rates were 100%, 100%, 97%, and 75%, respectively. This result indicates that heat shock at 35°C was lethal for oysters in the present study. Although oyster *Hif-α* mRNA was induced by heat shock, the expression pattern differed between heat shock at 30°C and 35°C. Under heat shock at 30°C, the expression of *Hif-α* mRNA in oysters was not noticeably affected until after 10 h; however, it increased significantly to 8.0-fold the control value after 24 h ( $P<0.0001$ ) (Fig. 7a). After 31 h of heat shock, expression decreased significantly to 3.4-fold the control value in comparison with 24 h ( $P<0.0001$ ). After 48 h of heat shock, expression increased again to reach 5.4-fold the control value, and was significantly higher than the value at 31 h ( $P=0.0353$ ). This significant accumulation of *Hif-α* mRNA in comparison with controls



**Fig. 5** Different expression levels of oyster *Hif-α* mRNA in the gill during air exposure. **a** Histogram showing changes in the expression level of oyster *Hif-α* mRNA during air exposure at 4°C. **b** Histogram showing changes in the expression level of oyster *Hif-α* mRNA during air exposure at 20°C. Total RNA (10 μg) was extracted from the gills of control oysters (0 day) and air-exposed ones (4°C for 1, 3, 5, 7, 10, and 15 days, and 20°C for 1, 3, 5, and 7 days). Columns and bars show mean±SD ( $N=3$ ). Different letters indicate a significant difference ( $P<0.05$ )



**Fig. 6** Different expression levels of oyster *Hif-α* mRNA in the gill during hypoxia and recovery. **a** Changes in the expression levels of *Hif-α* mRNA during hypoxia at 13°C. Total RNA (10 μg) was extracted from gills of control oysters (0 h) and ones exposed to hypoxic seawater (seawater at 15% oxygen saturation for 1, 6, 18, 36, 48, and 72 h) by bubbling with nitrogen gas. **b** Effect of recovery from hypoxia on expression levels of oyster *Hif-α* mRNA. Total RNA (10 μg) was extracted from gills of oysters acclimated to normoxic seawater (N), exposed to hypoxic seawater for 48 and 72 h (H48 and H72), and recovered for 1 h from H48 and H72 (R48 and R72). Columns and bars are mean±SD (N=3). Different letters indicate a significant difference ( $P<0.05$ )

was maintained until 96 h of heat shock ( $P=0.0002$ ). Under heat shock at 35°C, the expression of *Hif-α* mRNA was not noticeably affected until after 24 h; then, it increased significantly to reach 2.5-fold the control value after 31 h ( $P=0.0422$ ) (Fig. 7b). After 48 h of heat shock, expression increased again to reach 8.0-fold the control value, and was significantly higher than the value at 31 h ( $P<0.0001$ ).

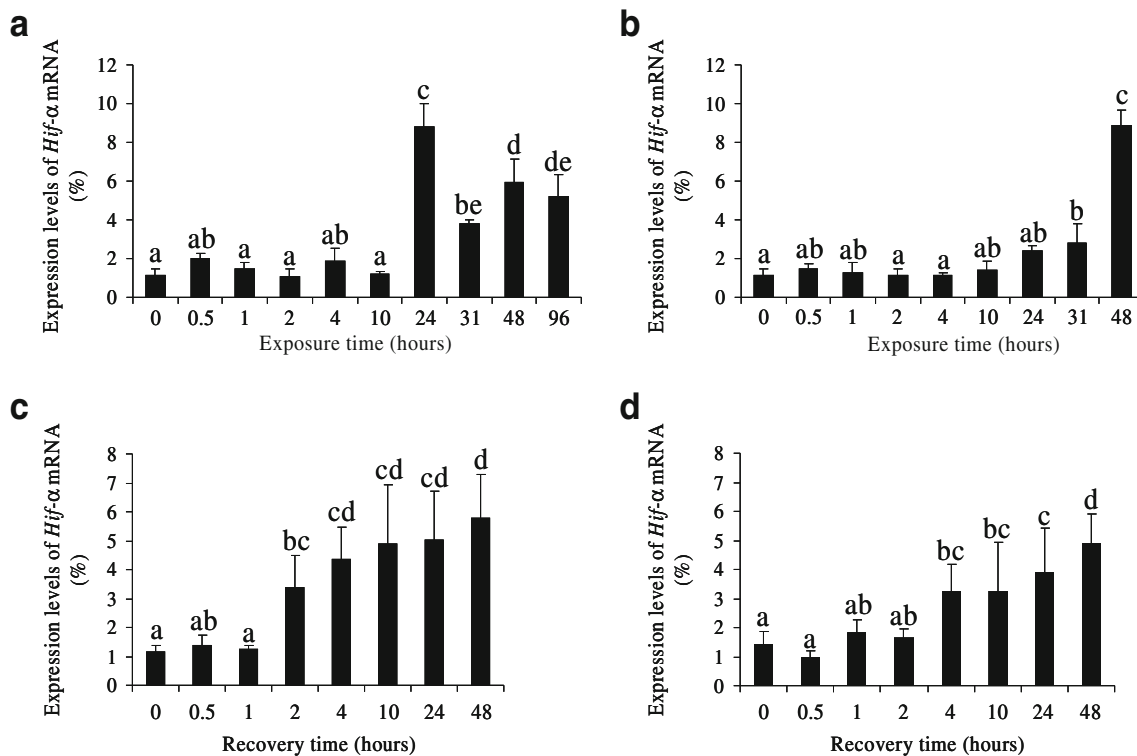
To investigate how the expression level of oyster *Hif-α* mRNA recovered from heat shock, we retransferred oysters to aerated seawater at 20°C for 48 h after 10 h of heat shock (Fig. 7c, d). Interestingly, during recovery from heat shock at 30°C, *Hif-α* mRNA was induced earlier than during recovery from a 35°C shock. During recovery from 30°C, the expression of *Hif-α* mRNA was not noticeably affected until after 1 h of recovery, but it increased significantly to 2.9-fold the value for no-recovery (recovery time 0) after 2 h of recovery ( $P=0.0354$ ) (Fig. 7c). This significant

accumulation of *Hif-α* mRNA was maintained until 48 h of recovery ( $P<0.005$ ). After 48 h of recovery, expression increased significantly to 4.9-fold of no-recovery level in comparison with 2 h of recovery ( $P=0.0219$ ). During recovery from 35°C, expression was not noticeably affected until after 2 h of recovery, but it increased significantly to 2.3-fold of the no-recovery level after 4 h of recovery ( $P=0.0280$ ) (Fig. 7d). This significant accumulation of *Hif-α* mRNA was maintained until 48 h of recovery ( $P<0.05$ ). After 48 h of recovery, *Hif-α* mRNA increased significantly to reach 3.5-fold of the no-recovery level in comparison with 4 h of recovery ( $P=0.0450$ ).

## Discussion

This is the first report of cloning of *Hif-α* and of its responses to several stress conditions in a mollusk. Under hypoxic conditions, bivalves show good tolerance to hypoxia by producing anaerobic energy (Ballantyne 2004). Investigations on mollusk hypoxic responses revealed that gene expression levels and activities of several glycolytic enzymes were upregulated under hypoxia (Bacchiocchi and Principato 2000; David et al. 2005; Moullac et al. 2007). In bivalves, anaerobic metabolites differ from those of vertebrates, which are succinate, propionate, and opines (Widdows, et al. 1979; Ballantyne 2004; Michaelidis et al. 2005). In fact, during hypoxia in oysters, the pH of the hemolymph decreases as a result of accumulation of these acidic end-products by glycolysis (Michaelidis et al. 2005; Kawabe et al. 2010). The expression of *Hsp* mRNAs is also induced in response to hypoxic conditions in oysters (David et al. 2005; Kawabe and Yokoyama 2009, 2010). However, it is not known whether the transcriptional regulator and its target genes are different between hypoxia-tolerant and other organisms.

In this study, we cloned the full-length cDNA of HIF-α for the first time from the oyster *C. gigas*. We found that oyster HIF-α conserved four main functional domains (bHLH, PAS-A/B, PAC) (Table 2). It is suggested that oyster HIF-α has a role as a transcriptional factor by heterodimerizing with HIF-β. However, there are some differences in the amino acid sequence between oyster HIF-α and other HIF-1αs. It is noteworthy that Leu500 and Leu503 in the LXXLAP motif in the N-TAD, which is related to the stabilization of HIF-1α protein, are replaced with methionine and arginine residues (Fig. 1b). *C. elegans* HIF-α is known to conserve an LXXLAP motif at only one site (Epstein et al. 2001), and *P. pugio* HIF-α conserves a complete and an incomplete motif (Li and Brouwer 2007). Huang et al. (2002) reported that mutations of two leucine residues in the LXXLAP motif are unimportant for proline hydroxylation by PHDs. These substitutions of leucine residues occur in arthropod HIF-α (Nambu et al. 1996; Li



**Fig. 7** Expression levels of oyster *Hif-α* mRNA in the gill during heat shock and recovery. Histogram showing changes in the expression levels of *Hif-α* mRNA in the gill during heat shock at 30°C (a) and 35°C (b). Total RNA (10 μg) was extracted from oysters acclimated to 20°C seawater and exposed to heat shock (30°C for 0.5, 1, 2, 4, 10, 24, 31, 48, and 96 h, and 35°C for 0.5, 1, 2, 4, 10, 24, 31, and 48 h). Effect of recovery from heat shock at 30°C (c) and 35°C (d) on the

expression level of oyster *Hif-α* mRNA. After heat shock treatment for 10 h (recovery time 0 h), oysters were allowed to recover in 20°C seawater. Total RNA (10 μg) was extracted from oysters exposed to heat shock for 10 h and allowed to recover for 0.5, 1, 2, 4, 10, 24, and 48 h. Columns and bars are mean±SD ( $N=3$ ). Different letters indicate a significant difference ( $P<0.05$ )

and Brouwer 2007). Oyster HIF- $\alpha$  conserves Ala504 in the LXXLAP motif. Therefore, Pro505 in this motif might be hydroxylated by PHDs under normoxic conditions. The FIH-1 target residue, an asparagine residue, was deleted in oyster HIF- $\alpha$  (Fig. 1a). Putative asparagine residue targeting of FIH-1 is conserved in arthropod HIF- $\alpha$ , but not in *C. elegans* HIF- $\alpha$ . Because the affinity for oxygen and 2-oxoglutarate differs between FIH-1 and PHDs, a model was proposed in which gene regulation of HIF-1 $\alpha$  activated by FIH-1 occurs in severely hypoxic conditions (Pouyssegur et al. 2006). Two grades of regulation of HIF-1 $\alpha$  activity may have been evolutionarily acquired following the mollusk. Primary sequence analysis indicates that oyster HIF- $\alpha$  is novel with an incomplete LXXLAP motif on N-TAD and deletion of C-TAD.

Northern blot analysis revealed that the expression level of oyster *Hif-α* mRNA was highest in the gill. In the oyster, the gill functions in gas exchange. Therefore, the gill might be sensitive to hypoxia and protect against hypoxic conditions accompanying the tidal cycle in such a manner as to accumulate *Hif-α* mRNA. Under hypoxic conditions in the present study, oyster HIF- $\alpha$  protein accumulated to a similar degree as in other organisms, but unexpectedly

decreased after accumulation (Fig. 3). Transcription levels of *Phds* are induced by hypoxia and this induction is HIF-1 dependent (Berra et al. 2006). Although the mechanism of re-activation of PHDs under hypoxia is unknown (Berra et al. 2006), degradation of oyster HIF- $\alpha$  might be due to re-activation of PHDs. In mammals, transcription of *Hif-1 $\alpha$*  is only transiently induced and/or not affected by hypoxia (Wang et al. 1995; BelAiba et al. 2007). In the Atlantic croaker, a hypoxia-tolerant teleost fish, accumulation of *Hif-1 $\alpha$*  mRNA was observed after 3 days, 1 week, and 3 weeks of hypoxia (Rahman and Thomas 2007). However, mRNA was not observed to decrease during hypoxia in the croaker. The present study provides the first detailed description of the expression profile of *Hif-α* mRNA during hypoxic conditions. Under exposure to air, the expression levels of oyster *Hif-α* mRNA increased first and then decreased, and the changes in the expression level were in synchrony with the changes in protein levels. These fluctuations were observed during exposure to hypoxic seawater. Our results suggest that there is a positive feedback loop for the induction of *Hif-α* in the oyster. Under hypoxic/anoxic conditions, the global translation rates decrease to 50% (Larade and Storey 2002; Spriggs et

al. 2010). During hypoxia, the translation of mammalian HIF-1 $\alpha$  is maintained by 5' cap/eukaryotic initiation factor 4E-independent mechanism which is recruited for the translation initiation complex by internal ribosome entry site (IRES) in the large size of 5' untranslated region (5' UTR) of mRNA (Lang et al. 2002; Spriggs et al. 2010). Because the 5'UTR of oyster *Hif- $\alpha$*  mRNA is long (409 bp) (Fig. 1a), this region may contain IRES. As a result, the translation of oyster HIF- $\alpha$  may be maintained during long-term hypoxic conditions. Under hypoxic conditions, oyster *Hif- $\alpha$*  mRNA was induced after 18 h (Fig. 6a). This induction of oyster *Hif- $\alpha$*  mRNA may depend on activated HIF and/or another factor induced by HIF. However, it is not known whether there are HREs on the promoter regions of *Hif- $\alpha$*  not only in oyster but also in other species. To reveal the mechanism of transcriptional control for oyster *Hif- $\alpha$*  under hypoxic conditions, it is necessary to determine the promoter region and to investigate this promoter activity in detail.

Interestingly, the expression levels of oyster *Hif- $\alpha$*  mRNA were affected by heat shock. In addition, the response time of *Hif- $\alpha$*  induction at 30°C was shorter than that at 35°C. Unexpectedly, oyster *Hif- $\alpha$*  was also upregulated during recovery from short-term heat shock. To our knowledge, this is the first report of a heat shock response of *Hif- $\alpha$*  at the mRNA level. Treinin et al. (2003) reported that a strain of *C. elegans* with an *Hif* loss-of-function mutation could not acclimate to heat shock and the survival rate was increased by HIF up-regulation. These data suggest that HIF is essential for heat acclimation in *C. elegans*. Our present data suggest that acute heat shock delayed heat adaptation mediated by HIF- $\alpha$  in the oyster. Recovery from hypoxia and/or heat shock upregulated the expression levels of *Hif- $\alpha$*  mRNA in the oyster. HIF target genes were reported to be induced in normoxia after exposure to a mild decrease in oxygen, but expression levels of *Hif-1 $\alpha$*  mRNA were not affected (Kamat et al. 2007). This phenomenon is termed the “memory” of HIF signaling. Oysters inhabit the intertidal zone, where the environmental temperature and oxygen concentration change during the tidal cycle. In order to survive in this severe environment, oysters might gain the “memory” of HIF signaling through induction of *Hif- $\alpha$*  mRNA. Recently, Baird et al. (2006) reported a new HIF-1 pathway in *D. melanogaster* an HIF-1-dependent heat shock pathway acting through the heat shock transcription factor (HSF) during hypoxia. There are two HREs in the second intron of the *Hsf* gene in *Drosophila*. Because HSF induces transcription of several genes related to stress proteins, *Drosophila* survives hypoxia by activation of the HIF–HSF pathway. The pathway whereby oyster *Hif- $\alpha$*  mRNA is induced during heat shock and recovery is unknown. Further analysis to characterize transcriptional factors and

their target genes may reveal whether this pathway is peculiar to marine bivalves inhabiting the intertidal zone.

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