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cDNA cloning and expression of *grp94* in the Pacific oyster *Crassostrea gigas*

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ABSTRACT

The 94-kDa glucose-regulated protein (GRP94) is an endoplasmic reticulum (ER) chaperone. We cloned the first mollusk *grp94* from a cDNA library of the Pacific oyster *Crassostrea gigas*. Analysis of *C. gigas grp94* (c*ggrp94*) clone containing 3212 bp DNA revealed that the cDNA contains a 2391 bp open reading frame that encodes a 797 amino acid protein of 91.6 kDa. The deduced amino acid sequence of cgGRP94 is 67%, 68%, and 67% homologous to the GRP94 of *Homo sapiens*, GP96 of *Strongylocentrotus purpuratus*, and GP96 of *Xenopus laevis*, respectively. CgGRP94 contains an N-terminal 22 amino acid sequence, which is characteristic of a signal sequence. It also contains a HATPase_c domain. In addition, it contains the KDEL (-Lys-Asp-Glu-Leu) peptide motif at the C-terminus, which suggests that cgGRP94 localizes in the ER. Northern blot analysis showed that c*ggrp94* mRNA is expressed at high levels in the gill which c*ggrp94* mRNA is induced during air exposure condition. Expression patterns of c*ggrp94* mRNA differed between gill and mantle, and c*ggrp94* mRNA was induced at high temperature during air exposure condition. These indicate that c*ggrp94* mRNA is induced by hypoxia and heat shock stress, and there are different strategies for air exposure condition between gill and mantle.

1. Introduction

For all organisms, appropriate folding of newly synthesized (unfolded) or misfolded proteins is necessary for life (Fink, 1999), especially under conditions of stress such as heat shock (Bukau et al., 2006; Grover, 2002). Like most molecular chaperones, heat shock proteins (HSP) play a role in protein folding and are induced by abnormal temperatures (Grover, 2002). Molecular chaperones localize to a variety of cellular compartments, such as mitochondria, endoplasmic reticulum (ER), and cytosol (Argon and Simen, 1999; Buchner, 1999; Fink, 1999; Hendrick and Hartl, 1995; Netzer and Hartl, 1998).

The 94-kDa glucose-regulated protein (GRP94), which belongs to the HSP90 family, is an ER chaperone (Argon and Simen, 1999). It was first described as a protein influenced by glucose starvation (Shiu et al., 1977). This protein is also called Endoplasmin (Koch et al., 1986), 96-kDa glycoprotein (GP96) (Srivastava et al., 1986, 1987; Maki et al., 1990) or HSP108 (Kulomaa et al., 1986; Sargan et al., 1986). GRP94 mainly localizes in the ER, and it is thought that it is required for the folding and maturation of secretory and membrane proteins (Argon and Simen, 1999). It is believed that GRP94 has a role in ER quality control (QC), along with other ER chaperones because its expression is up regulated by ER stress, such as disruption of ER calcium concentration and glucose starvation (Argon and Simen, 1999; Yang and Li, 2005). Expression of GRP94 is also induced by low extracellular pH, expression of mutated proteins, and viral infection (Argon and Simen, 1999). GRP94, which is a highly conserved protein (Argon and Simen, 1999; Larreta et al., 2000), has also been found in humans (*Homo sapiens*, Maki et al., 1990), bovine (*Bos taurus*, Watanabe et al., 2001), African clawed frog (*Xenopus laevis*, Robert et al., 2001), zebrafish (*Danio rerio*, Sumanas et al., 2003), sea urchin (*Strongylocentrotus purpuratus*, Smith et al., 1996a,b), plant (*Xerophyta viscose*, Walford et al., unpublished), and protozoa (*Leishmania infantum*, Larreta et al., 2000). Currently, there is no information available on mollusk GRP94.

The Pacific oyster *Crassostrea gigas* is a sessile bivalve mollusk. The oyster inhabits the intertidal zone in which the environmental temperature and oxygen concentration are subject to change during the tidal cycle. In addition, similar to other bivalves (Widdows et al., 1979; Buckley et al., 2001) the oyster shows good tolerance to various stress conditions such as hypoxia and heat shock (Clegg et al., 1998; David et al., 2005). Clegg et al. (1998) observed that HSPs of approximately 70-kDa (HSP70s) were induced in the oyster *C. gigas* by heat shock. An increase in HSP70 concentration was also observed in the oyster *Ostrea edulis* by heat shock (Piano et al., 2002). Under long-term hypoxia (30% O_2-saturated seawater by nitrogen flow), David et al. (2005) observed that genes associated to stress proteins (e.g., *hsp70*) and energy metabolism were up or down regulated in oyster tissues. It is thought that air exposure condition results in low concentration of available oxygen, hypoxia, for bivalves (Widdows et al., 1979). Widdows et al. (1979) observed that oxygen partial pressure (pO_2) of water in the mantle cavity declined rapidly and anaerobic end-products accumulated in *Mytilus* spp. during air exposure. Michaelidis et al. (2005) also observed that anaerobic end-products accumulated in oyster during air

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exposure. In this condition, stress protein genes may be up or down regulated in oysters. Although there is some information about cytosolic HSPs and their responses to heat shock, there is little knowledge of the ER chaperones and their responses to air exposure in oysters.

In this study, we cloned the first full-length cDNA of the mollusk *C. gigas grp94* (c*ggrp94*). We also quantified the expression of c*ggrp94* mRNA in oyster tissues using Northern blot analysis during air exposure.

2. Materials and methods

2.1. Materials

The Pacific oyster *C. gigas* was purchased from an oyster farm in Obama Bay, Fukui Prefecture, Japan. Specimens for air exposure were acclimatized to seawater for more than 3 days within Obama Bay at 13 °C in December 2004.

2.2. Preparation of RNA

Total RNA was extracted from oyster tissues using the Sepazol-RNA I super (Nacalai Tesque) according to the manufacturer's protocols. Samples were stored at −80 °C.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

One μg of total RNA from the oyster mantle was reverse transcribed with 200 U of M-MLV RT (Gibco-BRL) in a 20 μL reaction mixture comprising 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 1 mM dNTPs, 5 μM random hexamers, and 20 U of RNase inhibitor (Toyobo) according to the manufacturer's protocols for the synthesis of the first strand cDNA. Synthesized cDNA was used as a template for polymerase chain reaction (PCR) in a 20 μL reaction mixture comprising HotStarTaq Master Mix (Qiagen) and 1 μM of each primer. The degenerate oligonucleotide primers for PCR were as follows: sense primer, 5′-CARTTYGGNGTNGGNTTCTA-3′ and antisense primer, 5′-TCYTTCATNCKNTCNACGTA-3′: K = G + T, N = A + C + G + T, R = A + G, and Y = C + T (Table 1). These primers correspond to the amino acid sequences GQFGVGFY and YVERMKE, respectively, which are identical to those of *H. sapiens* GRP94 (hsGRP94) (P14625), *S. purpuratus* GP96 (spGP96) (AY187547), and *X. laevis* GP96 (xlGP96) (AY187545). The PCR was performed with the following parameters: 1 cycle at 95 °C for 15 min followed by 30 cycles at 94 °C for 1 min, 52 °C for 1 min and 72 °C for 90 s, and 1 cycle at 72 °C for 10 min. The inserts, approximately 1500 bp, were subcloned into the pDrive Cloning Vector (Qiagen) using the QIAGEN PCR Cloning Kit and sequenced using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

2.4. Semiquantitative RT-PCR

One μg of total RNA from oyster tissues, namely the adductor muscle, mantle, and gill, was reverse transcribed with 10 U of ReverTra Ace-α-™ (Toyobo) in a 20 μL reaction mixture comprising 5 mM $MgCl_2$, 1 mM dNTPs, 1.25 μM random hexamers and 10 U of RNase inhibitor (Toyobo) according to the manufacturer's protocols for the synthesis of the first strand cDNA. Synthesized cDNA was used as a template for PCR in a 20 μL reaction mixture comprising *Taq* DNA Polymerase (Promega), 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dNTPs, and 1 μM each primer. The gene-specific oligonucleotide primers (GSPs) for RT-PCR were as follows: the sense primer and the antisense primer were 5'AGACCGTGTGGGACTGGGAACTGAT-3′ and 5′-TTTCTCAACCTCGCTCCTGCTGGTT-3′, respectively (Table 1). The PCR was performed with the following parameters: 1 cycle at 94 °C for 2 min followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, and 1 cycle at 72 °C for 10 min. The PCR products were separated by electrophoresis on a 1% agarose gel followed by staining with ethidium bromide.

Table 1
Sequences of degenerate and gene-specific primers used for RT-PCR, semiquantitative RT-PCR, and RACE.

Usage	Direction	Sequence
RT-PCR	Sense	5′-CARTTYGGNGTNGGNTTCTA-3′
RT-PCR	Antisense	5′-TCYTTCATNCKNTCNACGTA-3′
Semiquantitative RT-PCR	Sense	5′-AGACCGTGTGGGACTGGGAACTGAT-3′
Semiquantitative RT-PCR	Antisense	5′-TTTCTCAACCTCGCTCCTGCTGGTT-3′
5′-RACE PCR	Antisense	5′-TTTCTCAACCTCGCTCCTGCTGGTT-3′
5′-RACE 2nd PCR	Antisense	5′-GACCTCTCCTTCTGCGGTGAAATGG-3′
3′-RACE PCR	Sense	5′-CCCTGGTAGCTAGCACCTATGGC-3′

K, G + T; N, A + C + G + T; R, A + G; Y, C + T.

2.5. Rapid amplification of cDNA ends

The cDNA library for 5′-rapid amplification of cDNA ends (5′-RACE) was synthesized from 1 μg of mRNA, which was purified from the 250 μg of total RNA prepared from the gill using the Oligotex™-dT30 Super kit (Roche), using the Marathon™ cDNA Amplification Kit (Clontech) according to the manufacturer's protocols for the synthesis of double-stranded (ds) cDNA (ds cDNA). The 3′-RACE-Ready cDNA was synthesized from 300 ng of mRNA, which was purified from the 100 μg of total RNA prepared from the gill using the Oligotex™-dT30 Super mRNA Purification Kit (Takara), using the SMART™ RACE cDNA Amplification Kit (Clontech) according to the manufacturer's protocols.

The 1st PCR of 5′-RACE was performed in a 10 μL reaction mixture comprising HotStarTaq Master Mix (Qiagen), 1 μM each primer, and ds cDNA for 5′-RACE with the following parameters: 1 cycle at 95 °C for 15 min followed by 35 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min, and 1 cycle at 72 °C for 10 min. The 2nd PCR of 5′-RACE was performed in a 20 μL reaction mixture comprising 1 U Expand High Fidelity^{PLUS} (Roche), Expand High Fidelity^{PLUS} Reaction Buffer (Roche), 0.2 mM dNTPs, 1 μM each primer, and the 1st PCR products, with the following parameters: 1 cycle at 94 °C for 2 min followed by 30 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 4 min, and 1 cycle at 72 °C for 10 min. The PCR of 3′-RACE was performed in a 20 μL reaction mixture comprising Advantage 2 Polymerase Mix (Clontech), Advantage 2 PCR Buffer (Clontech), 0.2 mM dNTPs, 0.2 μM each primer, and 3′-RACE-Ready cDNA, with the following parameters: 1 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 1 cycle at 72 °C for 10 min. The GSPs for RACE were as follows: the first and the nested primers for 5′-RACE and the primer for 3′-RACE were 5′-TTTCTCAACCTCGCTCCTGCTGGTT-3′ and 5′-GACCTCTCCTTCTGCGGTGAAATGG-3′ and 5′-CCCTGGTAGCTAGCACCTATGGC-3′, respectively (Table 1). The cDNA fragments obtained from the 5′-RACE and 3′-RACE were cloned and sequenced as described above.

2.6. Accession numbers of nucleotide sequences

The nucleotide sequence of c*ggrp94* has been registered with the DDBJ/EMBL/GenBank databases under accession number AB262084.

2.7. Nucleotide and amino acid sequence analysis

The nucleotide and amino acid sequences were searched by BLAST (http://blast.genome.jp/) and CLUSTALW (http://www.ddbj.nig.ac.jp/search/clustalw-j.html) programs. Phylogenetic analysis using the CLUSTALW program with the neighbor-joining method was performed from all amino acid sequences of *C. gigas* GRP94 and GRP94 homologues. The *L. infantum* GRP94 was used as an out-group for the phylogenetic trees.