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Molecular Cloning and Expression of the Mitochondrial 60-kDa Heat Shock Protein from an Arctic Copepod *Calanus glacialis*

Il-Chan Kim, Chang-Eun Lee, Hyun Hee Cho, Soon Gyu Hong, Jin Sung Lee, Hyoungseok Lee and Hong Kum Lee*

Polar BioCenter, Korea Polar Research Institute (KOPRI), Songdo Techno Park, Incheon 406-103, Korea

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ABSTRACT

Heat shock proteins (Hsps) play an essential role in cell defense against cell damages by several environmental and physiological stresses. We cloned and sequenced full-length heat shock protein 60-kDa (Hsp60) from the Arctic copepod *Calanus glacialis* cDNA. The complete nucleotide sequence of *Calanus glacialis* Hsp60 (*Cg* Hsp60) gene contained 2,092 bp in length. The open reading frame (ORF) was 1,743 bp flanked by a 5'-UTR of 90 bp and a long 3'-UTR of 259 bp, and encoded a putative protein of 581 amino acids with a calculated molecular weight of 62 kDa. It showed high similarity to those of *Culicoides variipennis* (73%), *Aedes aegypti* (72%), and *Tribolium castaneum* (71%) at the amino acid sequence level. In phylogenetic analysis, it clustered with other arthropods sequences. We also confirmed that the recombinant protein of *C. glacialis* Hsp60 by the SDS-PAGE and immunoblotting analysis after expression of recombinant gene construct in *Escherichia coli*.

Key words: *Calanus glacialis*, heat shock protein 60-kDa, Arctic, cloning, mitochondrial.

INTRODUCTION

Heat shock proteins (Hsps) are a multigene family induced by a wide variety of other factors, including thermal, physical, chemical, and biological stress. Hsps play an essential role in cell survival in prokaryotic or eukaryotic cells subjected to several stresses, and display potential as biomarkers of environmental pollution (Stürzenbaum et al., 2005). In case of *E. coli*, for example, the Hsp60 reveals 1 to 2% of the total protein content under normal conditions, but its concentration is increased four- to fivefold after heat shock (Shinnick, 1991).

Hsps act as molecular chaperones for proper folding and assembly of polypeptides during the protein biogenesis (Zügel and Kaufmann, 1999). Chaperones perform essential functions to reverse polypeptide unfolding and to prevent protein aggregation under stress conditions (Becker and Craig, 1994; Craig et al., 1994; Hartl, 1996). Chaperonins form oligomeric complexes and are composed of two different types of subunits: 60 kD and 10 kD proteins, known as cpn60 and cpn10 kD proteins, respectively. Many molecular chaperones described so far are members of the Hsp60 and Hsp70 families (Zügel and Kaufmann, 1999). In nonstressed cells, Hsps are present in low concentration, while in stressed cells they accumulate

*To whom correspondence should be addressed.
Email: hklee@kopri.re.kr.

at high levels.

Hsp60, also known as phage growth λ E large (GroEL) in bacteria, is ubiquitous in both prokaryotes and eukaryotes, and encode highly conserved housekeeping protein that assist in proper protein folding for the survival of these cells (Kwok et al., 1999). GroEL family members exist especially in mitochondria of eukaryotic cells in association with a cofactor of the Hsp10 (GroES) family (Kim et al., 2007) and is absolutely essential for the proper functioning of cells under normal and stress conditions (Choresh, 2004). To participate in the process of folding and assembly of proteins, the chaperonin GroEL and co-chaperonin GroES form the GroE chaperone machine (Hartl and Hayer-Hartl, 2002). GroES are required for folding of the polypeptide intermediates and release from the chaperonin (Stürzenbaum et al., 2005). By collaboration with GroES, GroEL prevents misfolding and aggregation of partially denatured proteins through an ATP-dependent process (Thies et al., 1999).

In a number of bacteria, Hsp60 also has been recognized as common antigens in the immune response to bacterial infection and in autoimmune diseases. Retzlaff et al. (1994) reported that bacterial Hsp60 together with Hsp70 (DnaK) regulates immunity by directly inducing cytokine mRNA production in macrophages.

Also, Hsp60 has function as protecting regeneration cells from stress and apoptosis during regeneration. Through study results using the *nbl* mutant, which displayed a defect in blastema formation and maintenance from zebrafish, Shinji et al. (2005) found that hsp60 expression is increased in blastema cells during zebrafish fin regeneration, and that hsp60 function is required for blastema formation and viability. From these findings, Shinji et al. (2005) suggested that hsp60 may play an important role in blastema formation and maintenance during fin regeneration.

Hsps as molecular chaperones have function as facilitating the synthesis, folding, assembly and intracellular transport of proteins, and reducing protein denaturation and aggregation (Dorota and Barbara, 2003).

Copepod species of the genus *Calanus* (Copepoda; Calanoida) form a predominant proportion of the zooplankton biomass in the Arctic Ocean (Conover, 1988; Conover and Huntley, 1991; Mumm et al., 1998; Thibault et al., 1999; Kosobokova and Hirche, 2000). Calanoid copepods play an essential role as consumers of primary production (Runge, 1988), and are used as prey species for organisms at the higher trophic levels such as carnivorous zooplankton (Auel et al., 2002; Falk-Petersen et al., 2002), polar cod (*Boreogadus saida*) and seabirds (Bradstreet and Cross, 1982; Hop et al., 2002; Karnovsky et al., 2003) in marine food chains (Tobias et al., 2006). *Calanus* species are distributed unevenly according to depth, and the distribution pattern is changing seasonally (Richter, 1994, 1995; Auel and Hagen, 2002). High abundances of *Calanus* spp. have been found in the upper water column (0-200m) south and west of Svalbard in winter, and are known to descend to depths and enter a resting stage (diapause) to survive the long unproductive winter (Hagen, 1999 ; Sato et al., 2002). The genus *Calanus* currently consists of 14 species (Hulsemann, 1991), and one of these species, *Calanus glacialis*, is classified as the same group together with *C. finmarchicus* and *C. marshallae* that are markedly similar in morphology (Frost, 1974). Because of the morphological similarity and the overlapping geographical ranges, however, the morphological identification among *Calanus* species has raised persistent problems (Bucklin et al., 1995). *C. glacialis* growth and reproduction happen during the spring and summer months, and the spawning time coincides with the period of maximum phytoplankton bloom during the spring (Tande, 1982).

In recent years, copepods have been an interesting object of toxicological, genetic and molecular biological studies as a result of information obtainedfrom study of their gene profiling (Lee et al., 2005, 2006). They have been proposed to be useful model organisms for marine invertebrates (Marcial et al., 2003; Seo et al., 2006a, b). *C. glacialis* also is thought potentially a suitable species to study response for marine pollution and molecular biology for cold adaptation mechanism in the Arctic region. However, in spite of its utilization possibility as a model organism as well as its ecological importance as a prey species, there is little available information on molecular genetics for *Calanus* species.

In this paper, we identify *C. glacialis* by sequence analysis based on the LSU rDNA gene, and show the

cDNA sequence of *Calanus glacialis* Hsp60 and discuss its characteristics. This is the first report on hsp60 gene of the Arctic copepod *C. glacialis*.

MATERIALS AND METHODS

Sample collection and identification

Copepod species KIC10701 were collected from seawater around the Korea Arctic Research Station Dasan at Ny-Ålesund, Svalbard, Norway (79°N, 12°E) in July 2005. Collected samples were homogenized in Trizol reagent for RNA extraction or stored in 100% absolute ethanol for DNA preparation. To identify collected copepod, the partial LSU rDNA were amplified using the primers LR0R (Rehner and Samuels, 1994) and LR5 (**Table 1**) (Vilgalys and Hester, 1990). LSU rDNA was used for phylogenetic identification as the gene contains high phylogenetic information and sequence database for *Calanus* species was well established. PCR was performed using Quick PCR Premix (Bioneer, Korea). Amplifications were performed in a T-gradient thermocycler (Biometra, Germany) with the following cycling parameters: 5 min initial denaturation at 94℃, 35 cycles of 1 min denaturation at 94℃, 1 min annealing at 52℃, and 1 min 30 sec extension at 72℃ and 10 min final extension at 72℃. The PCR product sizes were determined by comparison to the 1 kb DNA ladder (SolGent, Korea), and PCR products were purified with PCR purification kit (Bioneer, Korea). Sequences of the LSU rDNA regions were determined by overlapping sequencing reactions on complementary DNA strands with primers, LR0R and LR5 using ABI 3730XL automated sequencer (Applied biosystems company. USA).

Total RNA extraction and cDNA synthesis

Whole copepods were first ground with a pestle in liquid nitrogen and then homogenized in five volumes of TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) using a tissue grinder. The total RNA was extracted according to manufacturer's protocol, and isolated RNA was kept at -80℃ till further use. To synthesis complementary DNA (cDNA), 2 mg of total RNA was used, the cDNA was synthesized by AMV RT (Avian Myeloblastosis Virus Reverse Transcriptase) - PCR kit (GeneChoice, Inc.) following the user's manual.

Phylogenetic analysis

The sequence generated in this study and twelve LSU rDNA sequences retrieved from the GenBank database were proofread, edited and aligned using the jPHYDIT program (Jeon et al., 2005). *Echtrogaleus coleoptratus* (DQ180344) was used as an outgroup for rooting the trees. Phylogenetic trees were inferred from the data sets by methods of neighbor-joining method (NJ), maximum parsimony (MP) and maximum likelihood (ML) using PAUP 4.0b10 (Swofford, 2002). Parsimony analysis was conducted with tree bisection reconnection (TBR) branch swapping and MAXTREES set to auto-increase. All gaps were treated as missing data. Maximum likelihood methods with heuristic option were performed with MAXTREES set to auto-increase with TBR branch swapping. NJ tree was reconstructed under the Kimura's 2-parameter model (Kimura, 1980). Supports for internal branches in three analyses were tested by the bootstrap analyses of 1,000 replications.

Table 1. Primer sequences used in polymerase chain reaction.

Primer names	Primer sequences	Remarks
LR0R	5'-GTA CCC GCT GAA CTT AAG C-3'	Identification
LR5	5'-ATC CTG AGG GAA ACT TC-3'	
5'GSP3-1	5'-GGT GAG ATG TAT CCA CGG TC-3'	*Cg*Hsp60 5'-RACE
5'GSP3-2	5'-GCA GAG ATT GTG GCA ACT TG-3'	
3'raceF1	5'-CGA ATG CAA GAC ATG GCT GTT-3'	*Cg*Hsp60 3'-RACE
3'raceF2	5'-CGA CTG ACG CTC TTA ATG CCA-3'	
full-HSP-F	5'-ATG TTG AGC CTA GCA CGT GTA-3'	*Cg*Hsp60 ORF amplification
full-HSP-R	5'-CAT CAT GCC CCC CAT TCC TCC-3'	

5' and 3' RACE PCR

To obtain full-length cDNA sequence of *Calanus glacialis* Hsp60 from the partial sequence that derived from Gene Sequencer 20 sequencing, we conducted the 5' and 3' rapid amplification of cDNA ends (RACE) PCR techniques using GeneRacer kit (Invitrogen) according to the manufacturer's instructions. Briefly, for 3' ends RACE PCR, first-strand cDNA was synthesized using poly (A) mRNA with adapter oligo (dT) primer (Invitrogen). The 3'-RACE product of *C. glacialis* Hsp60 was amplified by PCR using 3'raceF1, 3'raceF2 primers (**Table 1**) and 3'-RACE adaptor primer (AUAP) in following reaction: 0.2 ㎍ cDNA template, 10 ㎛M 3'raceF1 primer and 3'-Adaptor primer, 10 ㎛M of each dNTP, and LA *Taq* DNA polymerase (5 U/L) and 50 ㎕ of reaction volume.

For 5' end RACE PCR, two primers (5'GSP3-1 and 5'GSP3-2, **Table 1**) were designed. The first cDNA was synthesized with gene specific primer 5'GSP3-1, and then was performed column purification and TdT tailing of cDNA. PCR amplification was sequentially carried out using specific primer 5'GSP3-2 together with 5'-abridged anchor primer (AAP).

Both 5' and 3' end RACE PCR amplifications were performed with the following conditions: 94℃/5 min; 35 cycles of 98℃/30 sec, 55 ~ 58℃/1 min according to primers; 72℃/1 min; and 72℃/10 min.

After the amplified PCR products were visualized on 1% agarose gels, products cloned into pGEM T-easy vector (Promega, WI, USA) and sequenced. All the oligonucleotide primer synthesis and sequence analysis work was performed by SolGent (Korea).

Construction and expression of recombinant *Calanus glacialis* Hsp60 from *Escherichia coli*

For the expression of *Cg* Hsp60 gene in *E. coli*, the sense primer (full-HSP1) and anti-sense primer (full-HSP2, **Table 1**) were synthesized for PCR cloning. cDNA amplificated by RNA from *C. glacialis* was used as a template. The PCR condition was 35 cycles of 98℃ for 30 sec, 52℃ for 1 min, 72℃ for 1 min with a final extension at 72℃ for 10 min. The amplified 1.5 kb fragment was directly inserted into the pBAD Topo TA vector (Invitrogen). *Escherichia coli* Top10 cell was transformed with ligated DNA. The transformants were spread onto LB agar plates containing 100 ㎍/㎖ ampicillin and incubated at 37℃. Recombinant protein was induced by the addition of L-arabinose (Sigma) to a final concentration of 0.02%. Cells were harvested by centrifugation at 13,000 rpm for 5 min after 3 hrs of induction, and lysed using sonication.

Proteins were directly analyzed by 10% SDS polyacrylamide gel. The samples were heat-treated for 5 min at 95℃ before being loaded and ran at 80 V and 120 V for 150 min using a Bio-Rad Mini-Protein III Gel Kit. After the electrophoresis, the protein gels were stained with Bio-SafeTM Coomassie (Bio-rad).

Immunoblotting

For immunoblot experiments, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to manufacturer's instructions (Bio-Rad). Proteins were transferred electronically to PVDF membrane (Millipore). Following transfer, the membrane was blocked in 5% of skim milk, incubated for 1 hr at room temperature with Anti-His_6 (1:500, Roche). After extensive washing, Peroxidase-conjugated AffiniPure $F(ab')_2$ Fragment Donkey Anti-Mouse IgG (H+L) (1:20,000, Jackson ImmunoResearch Laboratories) was incubated for further 1 hr at room temperature. The PVDF membrane was then washed again, and SUPEX Western blot detection kit (Neuronex) was used to visualize signals and image were detected with luminescent image analyzer LAS-3000 (Fujifilm)

RESULTS AND DISCUSSION

Molecular identification of the arctic copepod

The sequence length of partial LSU rDNAs of the arctic copepod was approximately 950 bp (primer pair LR0R-LR5), which showed 100% identity (675/675) with the Antarctic *Calanus glacialis* (DQ250166).

The aligned dataset of 107 sequences was composed of 737 nucleotide sites after excluding ambiguously aligned sites. Among them, 445 positions were constant, 150 positions were variable but non-informative, and 142 positions were parsimony-informative. From the heuristic search based on parsimony criterion, one most parsimonious tree (TL = 544 steps, CI = 0.752, RI = 0.560) was retrieved (**Fig. 1**). Lineages that were supported by the high bootstrap supports in the parsimony analysis were recovered in the neighbor-joining and maximum likelihood analyses. Branches

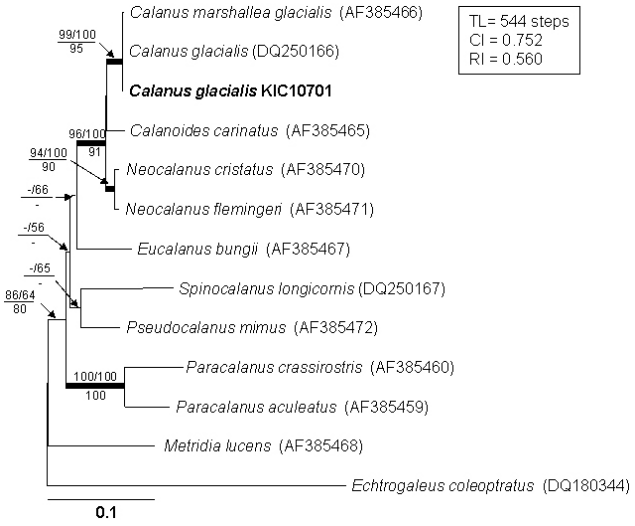

Figure 1. The most parsimonious tree of the copepod species derived from nuclear large subunit ribosomal RNA gene sequences. Branches maintained in three different analyses (MP, NJ and ML) were presented by bold lines. Numbers above branches that are before the slash are MP bootstrap proportions and those that are after the slash are NJ bootstrap proportions. Values below branches are NJ bootstrap proportions. A plant species, *Echtrogaleus coleoptratus* (DQ180344) was used as an outgroup.

supported in three different analyses were presented by bold lines in the tree (**Fig. 1**). In this tree, the arctic species and other two species of *Calanus glacialis* composed a distinct monophyletic clade at 99% (MP), 100% (NJ) and 95% (ML) confidence level.

Because of the morphological similarity of the *Calanus*s pecies, genetic characters may be provide accurate indicators of taxonomy of the genus (Bucklin et al., 1995). The arctic calanoid copepod species was analyzed using the sequences of nuclear large subunit rDNAs (LSU rDNAs) for identification and investigation of the phylogenetic relationships among related copepod species. As a result of morphological examination and molecular analysis of the collection, the copepod species was determined as *Calanus glacialis*. In a phylogenetic study based on mitochondrial large subunit ribosomal RNA gene (Bucklin et al., 1995), *C. glacialis* belonged to 'finmarchicus group' with *C. marshallae* and *C. finmarchicus*.

Cloning and sequencing of *Calanus glacialis* Hsp60 gene

To obtain the full-length cDNA sequence of *Calanus glacialis* Hsp60, we designed 5'- and 3'-RACE PCR using partial sequences of *Cg*Hsp60 derived from Gene Sequencer 20 analysis. The 5'- and 3'-ends of *C. glacialis* Hsp60 obtained by 5'- and 3'-RACE were products of 750 bp and 1 kb, respectively. The complete nucleotide sequence of *Calanus glacialis* Hsp60 gene was 2,092 bp in length. The open reading

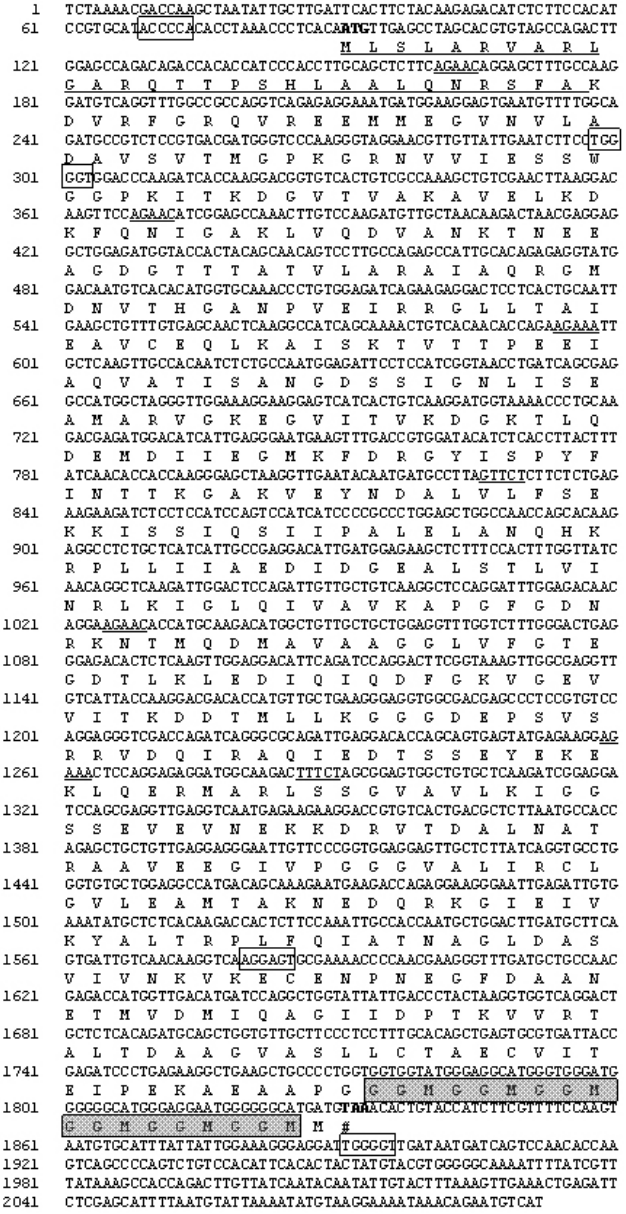

Figure 2. Nucleotide and deduced amino acid sequences of the 60 kDa heat shock protein cDNA from *Calanus glacialis*. The start codon and the stop codon is marked bold. Predicated cleavage site is shown from the first to 29th residue, and putative heat shock factor (HSF) motifs (AGAAN) and alcohol dehydrogenase gene regulator 1 (ADR1) motifs (NGGRGK) are underlined and boxed, respectively. The GGM repeats are shown in a gray box.

frame (ORF) was 1,743 bp flanked by a 5'-untranslated region of 90 bp and a long 3'-untranslated region of 259 bp (**Fig. 2**). By searching motifs from the MOTIF Search database (http://motif.genome.jp/), seven putative heat shock factor (HSF) motifs (AGAAN) and four alcohol dehydrogenase generegulator 1 (ADR1) motifs (NGGRGK) were found using an *E*-value of 1.0 as a cut-off score (**Fig. 2**). HSF is a transcription factor that regulates the expression of heat shock proteins under cellular stress conditions (F-Kierzkowska et al., 2003), and ADR1 regulates the alcohol dehydrogenase (ADH) catalysis of the oxidation of a wide variety of xenobiotic or endogenous alcohols (Eklund et al., 1990; Duester, 1991). Using the signal peptide online tool (SignalP 3.0 Server, http://www.cbs.dtu.dk/services/SignalP/), the predicated cleavage site was confirmed between the 29th and 30th amino acids from the start codon (**Fig. 2**). The *Cg*-Hsp60 sequence was registered in GenBank databases under the accession number EU263637. The *Cg*-Hsp60 cDNA encoded a putative protein of 581 amino acids with a theoretical isoelectric point (p*I*) value of 5.18 and a predicted molecular weight of 62 kDa. The nucleotide sequence and deduced amino acid sequence of DNA encoding the Hsp60 are presented in **Figure 2**.

Sequence alignment and phylogenetic analysis

To investigate the amino acids similarity and phylogenetic relationship of the *C. glacialis* Hsp60 gene, the putative amino acid sequence of *Cg*-Hsp60 was compared with those from 14 other species containing arthropods, fishes, mammalian, and amphibian through GeneDoc program (http://www.psc.edu/biomed/genedoc/) and BLAST NCBI search (http://www.ncbi.nlm.nih.gov/BLAST/). A plant species, *Echtrogaleus coleoptratus* (DQ180344), was used as an outgroup. *C. glacialis* Hsp60 displays 73% identity to *Culicoides variipennis* Hsp60 (GenBank no. AAB94640), 72% identity to *Aedes aegypti* Hsp60 (EAT36327), 71% identity to *Tribolium castaneum* Hsp60 (XP_971630), *Lucilia cuprina* Hsp60 (ABO09590) and *Liriomyza huidobrens* Hsp60 (AAW30392), 70% identity to *Drosophila melanogaster* Hsp60 (O02649), 69% identity to *Myzus persicae* (CAB58441), 68% identity to *Paralichthys olivaceus* Hsp60 (ABB76381), *Homo sapiens* Hsp60 (ABB01006) and *Caenorhabditis elegans* Hsp60 (AAK84594), 67% identity to *Xenopus laevis* Hsp60 (NP_001083970), *Carassius auratus* Hsp60 (ABI26641), *Paracentrotus lividus* Hsp60 (CAB56199) and *Danio rerio* Hsp60 (AAH68415), and 55% identity to *Arabidopsis thaliana* (BAD43178).

From analysis of protein domains by PROSITE program (http://www.expasy.org), a chaperonins cpn60 signature domain that is highly conversed region in Hsp60 genes and a attachment site for phosphopantetheine that is the prosthetic group of acry carrier proteins were confirmed (**Fig. 3**). Two characteristics, the GGM repeats and the mitochondrial presequence, showing in mitochondrial Hsp60s (mt-Hsp60s) also display in amino acid sequences of *Cg*-Hsp60. The first is that *Cg*-Hsp60 has a conserved GGM repeats at its C-terminal end. The GGM repeats are known as typical signature in mt-Hsp60s (**Fig. 2**), and the structure and function of this region are not known (Gupta, 1995; Sanchez et al., 1999). The second is that the 1,745 bp (581 amino acids) sequence contains a presequence of 29 amino acids at the N-terminus that is required for import into the mitochondria (**Fig. 2**). The mitochondrial presequence is less conserved among the various species (Choresh et al., 2004). These results suggest that *Cg*-Hsp60 gene in this study belongs to the eukaryotic mt-Hsp60 family.

To clarify the molecular evolutionary relationships among *Cg*-Hsp60 and those of other species, the sequence alignments of the Hsp60 genes were used to produce the phylogenetic tree of the other species included in our analysis (**Fig. 4**). Mammalian, fishes, amphibian and echinoderm Hsp60s, including *Danio rerio, Carassius auratus, Paralichthys olivaceus, Home sapiens, Xenopus laevis, Xenopus laevis, and Paracentrotus lividus*, were clustered as the same evolution group; while arthropods Hsp60s, including *Culicoides variipennis, Aedes aegypti, Drosophila melanogaster, Lucilia cuprina, Liriomyza huidobrensis, Tribolium castaneum, Myzus persicae*, and *Calunus glacialis*, were clustered as another group. As depicted in **Figure 4**, the *Cg*-Hsp60 sequence of *C. glacialis* was more closely related to that of *Myzus persicae* of arthropods Hsp60 than to those of fishes, amphibian, or echinoderm. As expected, the phylogenetic tree showed that the Hsp60 from *C. glacialis* is highly phylogenetically and evolutionarily closer to the other Hsp60 sequences of arthropods with high similarities

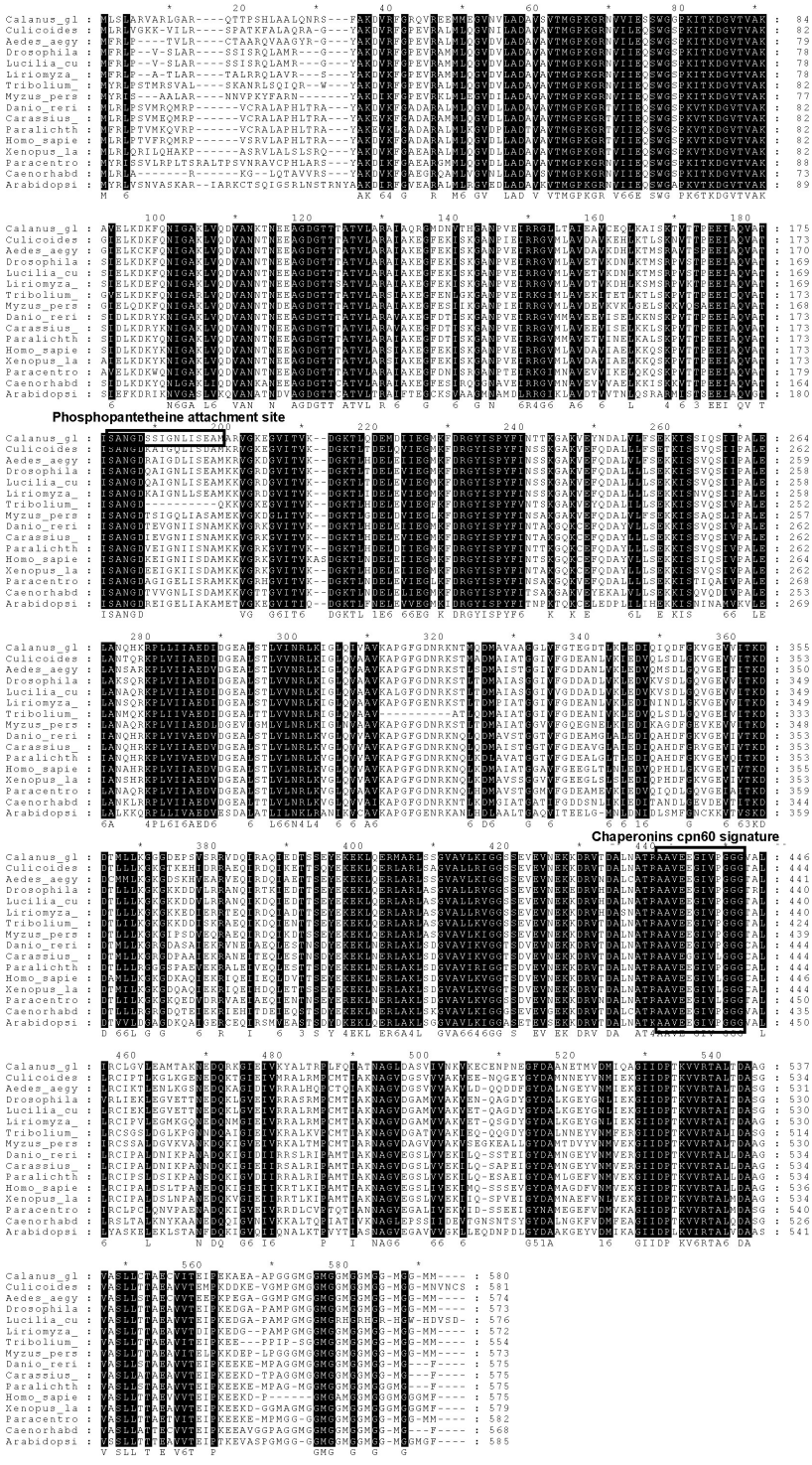

Figure 3. Multiple alignment of the deduced amino acid sequence of the *C. glacialis* Hsp60 with that of 15 other organisms. The sequence information was retrieved from the NCBI Genbank. Sequence alignment was performed using GeneDoc program (edu/biomed/ genedoc/). Black highlights show *C. glacialis* Hsp60 identical sequences. Gray highlights show *C. glacialis* Hsp60 similar sequences. Chaperonins cpn60 signature domain that is highly conversed region in Hsp60 genes and phosphopantetheine attachment site are indicated. *Culicoides variipennis* (AAB94640), *Aedes aegypti* (EAT36327), *Tribolium castaneum* (XP_971630), *Lucilia cuprina* (ABO09590), *Liriomyza huidobrens* (AAW30392), *Drosophila melanogaster* (O02649), *Myzus persicae* (CAB58441), *Paralichthys olivaceus* (ABB76381), *Homo sapiens* (ABB01006), *Caenorhabditis elegans* (AAK84594), *Xenopus laevis* (NP_001083970), *Carassius auratus* (ABI26641), *Paracentrotus lividus* (CAB56199) and *Danio rerio* (AAH68415), and *Arabidopsis thaliana* (BAD43178).

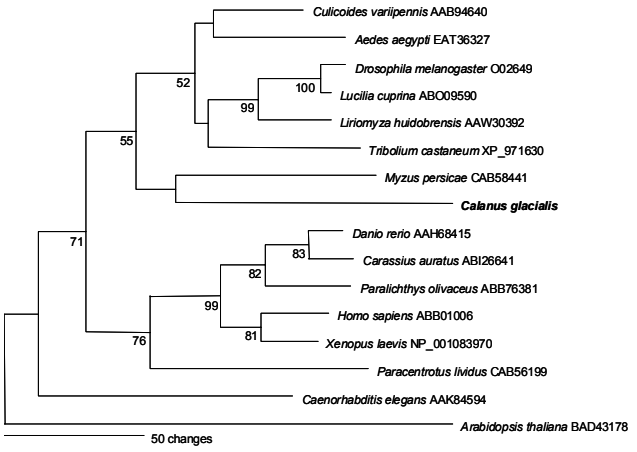

Figure 4. Phylogenetic relationship of Hsp60 reconstructed by parsimony analysis based on amino acid sequences to other Hsp70 family members from a variety of species. The numbers given under branches are the frequencies (> 50%) with which a given branch appeared in 1,000 bootstrap replications. The strains and the GenBank accession numbers are the same as in **Figure 3**.

of 69% – 73%. Moreover, the chaperonins cpn60 signature domain was well conversed in *Cg* Hsp60 amino acid with those of other species.

Polyacrylamide gel electrophoresis (PAGE) and immunoblotting

To express recombinant *Cg* Hsp60 in *E. coli*, target gene was directly inserted into the pBAD Topo TA vector. Hsp60 from *Calanus glacialis* was over-expressed in *E. coli* Top10 cells. As shown in **Figure 5A**, Sodium dodecyl sulphate (SDS) - PAGE analysis and Coomassie blue staining revealed distinct protein bands in samples induced by L-arabinose, but not in the samples without L-arabinose or the host strain Top10. For solubility analysis of recombinant *Cg* Hsp60, the transformed cells were disrupted by sonication and centrifuged at 13,000 rpm for 20 min. The supernatants (soluble form) and the precipitated pellets (insoluble form) were loaded onto 10% SDS polyacrylamide gel. *Vibrio* sp. recombinant chitinase is used as the positive control. As expected, the molecular weight of major protein band is the same as predicted size of approximately 62 kDa. The protein size difference shown in **Figure 5** results from the approximately 4 kDa structure of the constructed expression vector with co-expressed parts, including the 6 × His-tag and its own conformation structure.

Attempts to overexpress the complete *Cg* Hsp60 gene in *E. coli* revealed the protein expression at relatively low level. It can be caused by genetic codon usage difference of *C. glacialis* from *E. coli*. If there are a number of codons for Arg (AGA, AGG), Ile (AUA), Gly (GGA), Leu (CUA) and Pro (CCC) that are rarely used in *E. coli* of the *Cg* Hsp60 gene sequences, the protein expression has known to be restricted within *E. coli* system. We found several repeated sequences, AGA or AGG, for encoding Arginine in *Cg* Hsp60 gene. We guess that the weak expression of recombinant *C. glacialis* Hsp60 is induced by the difference of genetic codon usage between the both species, *E. coli* strain Top10 and *C. glacialis*.

To further verify the expression of His-tagged *Cg* Hsp60 fusion protein, Western blot analysis was performed using a His-tag monoclonal antibody (Invitrogen, CA). As shown by Western blot analysis (**Fig. 5B**), this His-tag antibody was cross-reacted to a recombinant *Calanus* Hsp60 protein that reflects same size to the expressed protein seen on SDS-PAGE.

In conclusion, although Hsp60 gene has been previously identified in many species, this paper is the first report on cloning, sequencing, and expression of Hsp60 in the Arctic copepod *Calanus glacialis*. Based on *Cg* Hsp60 amino acid sequence data, the *C. glacialis* Hsp60 has 73%, 72%, 71%, 71%, and 69% identity with Hsp60 of *Culicoides variipennis*, *Aedes aegypti*, *Tribolium castaneum*, *Lucilia cuprina*, and *Myzus persicae*, respectively. Hsp60 gene from *C.*

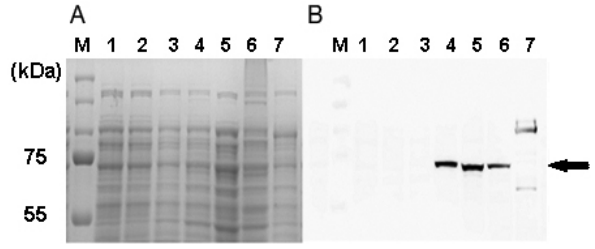

Figure 5. Localization of the recombinant *Cg* Hsp60 protein. (A) SDS–PAGE of purified recombinant *Cg* Hsp60 (staining, coomassie solution). (B) Immunoblot analysis of the recombinant protein. M. protein molecular weight marker, 1. uninduced vector without recombinant protein, 2. uninduced recombinant *Cg* Hsp60, 3. vector control, 4. total crude extract, 5. soluble form, 6. insoluble form, 7. positive control, *Vibrio* sp. recombinant chitinase. The arrows indicate the Hsp60 recombinant protein.

glacialis is highly phylogenetic and evolutionarily closer to the other Hsp60 sequences in arthropods, and has a predicted cleavage site, seven putative heat shock factor (HSF) motifs, four alcohol dehydrogenase gene regulator 1 (ADR1) motifs, a phosphopantetheine attachment site, and a chaperonins cpn60 signature domain that is conserved in the Hsp60 family. *Cg* Hsp60 also has the GGM repeats of its C-terminal and the mitochondrial presequence of its N-terminal that are known as typical characteristics in mt-Hsp60 family. From these results, we suggest that *C. glacialis* Hsp60 gene is purified by this study belongs to the eukaryotic mt-Hsp60 family. We also confirmed that the recombinant protein of *Calanus glacialis* HSP-60 made approximately 62 kDa after expression of recombinant gene construct in *E. coli* system.

We are currently investigating the thermotolerance effect and the enzymatic activities of a recombinant *Cg*-Hsp60. The present results in this work and our future works with *C. glacialis* Hsp60 will may provide valuable information to understand its function in adaptation mechanism and responses to the environment changes of a copepod *Calanus glacialis* Hsp60 in extreme situations such as the Antarctic and the Arctic regions.

ACKNOWLEDGEMENTS

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