Comparative Biochemistry and Physiology, Part B 171 (2014) 12–17

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Molecular characterization of the glucose-regulated protein 78 (GRP78) gene in planarian *Dugesia japonica*

Ke-Xue Ma, Guang-Wen Chen *, Chang-Ying Shi, Fang-Fang Cheng, He Dou, Cheng-Cheng Feng, De-Zeng Liu

College of Life Sciences, Henan Normal University, Xinxiang 453007, PR China

ARTICLE INFO

Article history:
Received 29 November 2013
Received in revised form 2 March 2014
Accepted 4 March 2014
Available online 12 March 2014

Keywords:
Planarian
cDNA cloning
GRP78
Stress
Gene expression

ABSTRACT

GRP78 (78 kDa glucose-regulated protein) has ubiquitously existed in nearly all organisms from yeast to humans, reflecting the central roles it plays in cell survival. In this report, we isolated and sequenced the full-length cDNA of GRP78 (designated DjGRP78) from the planarian *Dugesia japonica*. The cDNA is 2121 bp, including an open reading frame (ORF) of 1983 bp encoding a polypeptide of 660 amino acids with three HSP70 family signatures. DjGRP78 contains signal peptides at the N-terminus and a KTEL peptide motif at the C-terminus, which suggests that it localizes in the endoplasmic reticulum (ER). Fluorescent real time RT-PCR was employed to detect the expression pattern of *Djgrp78* in response to different stressors. Our results show that heat shock and heavy metals (Hg^{2+} and Pb^{2+}) induce *Djgrp78* expression, but starvation does not. Interestingly, we found that *Djgrp78* was up-regulated in planarians with septic tissues, and also verified that it was up-regulated in response to bacterial challenge. Our data indicate that *Djgrp78* may be a multifunctional gene, and play important roles in physiological and pathological stress in planarians.

1. Introduction

The endoplasmic reticulum (ER) is a membranous organelle found in all eukaryotic cells that is crucial for normal cell function and development (Schröder, 2008; Giorgi et al., 2009). It plays a fundamental role in synthesis, folding, sorting, and delivery of proteins to the appropriate cellular destinations. ER function is affected by various intracellular and extracellular stressors, and prolonged ER stress leads to cell death. Cells from all organisms increase the expression of a class of ER stress proteins in response to ER stress, which serve as molecular chaperones and are involved in protein translocation, protein folding and assembly, and the regulation of protein secretion (Kaufman, 1999). Glucose-regulated protein (GRP) 78 (also known as HSPA5 or BiP), is a member of the heat shock protein 70 (HSP70) family of proteins, which are involved in ER stress. GRP78 expression is induced during oxidative stress (Kitamura and Hiramatsu, 2010), chemical toxicity (Falahatpisheh et al., 2007; Stacchiotti et al., 2009), treatment with Ca^{2+} ionophores and inhibitors of glycosylation (Kaufman, 1999). This induction, which is part of the unfolded protein response (UPR), is required to alleviate ER stress, maintain ER function, facilitate protein folding and thus protect cells from the aforementioned toxic insults (Kaufman, 1999; Sherman and Goldberg, 2001). Other reports suggest that GRP78 may protect the host cell against cell death by suppressing oxyradical accumulation and stabilizing mitochondrial function (Yu et al., 1999). GRP78 also has an anti-apoptotic function to prevent ER stress-induced cell death and is used as a biomarker for the onset of the UPR (Lee, 2001). Recent works suggest that GRP78 may play a role in immune defense against foreign pathogens (Luan et al., 2009; Morito and Nagata, 2012).

In spite of numerous investigations into the role of GRP78 in mammals, there has been very limited investigation into it's role in freshwater planarians. Freshwater planarians are unique animals, showing powerful regenerating ability and strong tolerance to prolonged starvation (Bowen et al., 1976; Newmark and Sánchez Alvarado, 2002). As aquatic animals, planarians are easily threatened by water pollution and are often used as test organisms in water environmental toxicology (Pra et al., 2005). We observed that some planarians can survive for several weeks with septic tissues (induced by lysing parts of their bodies). Septic tissues can be easily attacked by a wide array of microbes present in water. Therefore, we are very interested in whether GRP78 is involved in the adaptive stress and innate immune responses in planarians. In the present paper, we cloned the full length cDNA of GRP78 from the planarian *Dugesia japonica* (*Djgrp78*) for the first time, and studied the expression profiles of *Djgrp78* in response to different stressors and bacterial challenge. The results of this study will allow greater understanding of the physiological and immunological roles of GRP78 in planarians.

* Corresponding author at: College of Life Sciences, Henan Normal University, No. 46, Jianshe Road, Xinxiang 453007, PR China. Tel.: +86 373 3326190; fax: +86 373 3326162.
E-mail address: Chengw0183@sina.com (G.-W. Chen).

http://dx.doi.org/10.1016/j.cbpb.2014.03.001
1096-4959/

2. Materials and methods

2.1. Animals and treatments

The planarians *D. japonica* used in this study were collected from Tagang Reservoir, Xinxiang City, China. They were cultured in autoclaved tap water in the dark at 18 °C and fed once a week with fresh fish spleen. Animals of similar body size were used for experiments after 7–10 day starvation. For heavy metal stress, planarians (20 animals/group) were exposed to Hg^{2+} ($HgCl_2$, 1, 10, 50, 200 μg/L) and Pb^{2+} [$Pb(NO_3)$, 10, 100, 250, 500 μg/L] respectively, for 48 h. For thermal stress, animals (20 animals/group) were cultured at 25 °C for 2 days, followed by 30 °C for 2 days. A group of control organisms was maintained in parallel for all experiments but not subjected to metal or thermal stress.

Planarians with septic tissues (designated as unhealthy animals) were selected for pathological stress experiments (see Fig. 6B,C). For the bacterial challenge experiments, we cultured the intact and regenerating animals (animals were cut before and after the pharynx) in sterile water containing *Escherichia coli* DH5α (1×10^7 cuf/mL). Samples were collected at 4, 8, 12, 16 and 24 h post-challenge for RNA extraction. Normal regeneration was conducted in sterile water at 18 °C for control experiments.

2.2. RNA extraction and Djgrp78 cDNA cloning

Total RNA was extracted using Trizol reagent (Invitrogen, USA) and 2 μg RNA was used for reverse transcription. A 388 bp EST fragment for *Djgrp78* gene was previously obtained by differential-display RT-PCR in our laboratory. Based on the known EST of *Djgrp78* cDNA, *Djgrp78* 3′-RACE specific primer (5′-ATG ATG CCC AAA GAC AAG CTA CTA GTG-3′) and *Djgrp78* 5′-RACE specific primer (5′-CTC TTT GAT CGA AAT CTT CAC CTC C-3′) were designed for the amplification of cDNA ends (RACE). Both 5′-RACE and 3′-RACE were carried out using a TaKaRa RACE cDNA amplification kit according to the manufacturer's instructions. The PCR products were gel-purified, ligated into the pUCm-T vector, and submitted for sequencing. The resulting sequences were verified and subjected to cluster analysis.

2.3. Amplification of Djgrp78 ORF sequences from genomic DNA

Genomic DNA was extracted using the Takara genomic DNA extraction kit Ver.3.0 according to the manufacturer's instructions. Based on the *Djgrp78* cDNA sequences, we designed a pair of specific primers (forward: 5′-AAC TGT TGT TGA ATC CAT GAA ATC G-3′; reverse: 5′-GGT TTA CAA TTC AGT CTT AGC ATC ATC-3′) to amplify *Djgrp78* ORF sequences from genomic DNA. The PCR program was carried out at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 3 min and a final extension step at 72 °C for 10 min. The PCR products were sequenced.

2.4. Homology analysis

The homology analysis of nucleotide and protein sequences was carried out using blastn and blastp at the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequence and protein motif features were analyzed with the Expert Protein Analysis System (http://www.expasy.org). Multiple alignments of the GRP78 protein sequences were performed using the software Dnaman 6.0. A phylogenic tree was constructed using the programs Clustal X 1.83 and Mega 3.1 based on the amino acid sequences of DjGRP78 and other known GRP78 sequences. Bootstrap analysis was used with 1000 replicates to test the relative support for the branches produced by neighbor-joining analysis.

2.5. Expression pattern of Djgrp78 in response to different stressors

To assess gene expression of *Djgrp78* in different stress conditions, the transcript levels in stressed animals were compared to those of unstressed animals. Fluorescent real time RT-PCR was performed at least three times with independent RNA samples. Specific sense and antisense primers (forward: 5′-GAG TTA TCG GTG GAG TTG AAG AA-3′; reverse: 5′-CTT GAA TTG TGA CGG TAG GTT GA-3′) were designed to amplify a *Djgrp78* cDNA fragment of 186 bp. SYBR Green chemistry-based RT-PCR was carried out with ABI PRISM 7500 Sequence Detection System (Applied Biosystems). Planarian *elongation factor 2* (*Djef2*) (forward: 5′-TTA ATG ATG GGA AGA TAT GTT G-3′; reverse: 5′-GTA CCA TAG GAT CTG ATT TTG C-3′) was used as the reference gene in all experiments (Pineda et al., 2002). The expression ratios were determined using the method $2^{-\Delta\Delta CT}$, described by Livak and Schmittgen (2001). The data obtained from qRT-PCR analysis for the expression of *Djgrp78* were subjected to one-way analysis of variance (one-way ANOVA). Differences were considered significant at $P < 0.05$.

2.6. Hyperlinking to databases

Nucleotide sequence and protein sequence data are available in the GenBank database under the accession numbers: JN968463 and AET10307, respectively.

3. Results

3.1. Molecular characteristics of the GRP78 gene in the planarian Dugesia japonica

The full-length cDNA of DjGRP78 is 2121 bp, including a 5′-terminal untranslated region (UTR) of 44 bp, a 3′-terminal UTR of 94 bp with a canonical polyadenylation signal sequence AATAAA and a poly (A) tail (Fig. 1), and an ORF of 1983 bp encoding a polypeptide of 660 amino acids with a predicted molecular mass of 72.66 kDa and theoretical isoelectric point of 5.38. The Signal P software analysis indicates that DjGRP78 contains a signal peptide of 18 amino acids (**MKSITVLLISLLVCGIYA**). SMART analysis shows that DjGRP78 displays three conserved heat shock protein 70 (HSP70) family signature motifs: **IDLGTTYS** at the position of 38–45, **VFDLGGGTFDVSLL** at 226–239, and **IVLVGGSTRIPKVQQ** at 363–377. The C terminal of DjGRP78 contains the **KTEL** peptide motif (residues 657–660) (Fig. 1). This motif is essential for retention in the ER lumen (Monro and Pelham, 1987; Persson et al., 2005), which suggests that DjGRP78 localizes in the ER. In addition, we sequenced the ORF sequences from genomic DNA, and found only one intron (44 bp) existed in the *Djgrp78* gene structure. Interestingly, the intron disrupts the codon TG^T at the position of 46 that encodes for cysteine (C). The 5′-putative and 3′-putative splice junctions follow the typical canonical consensus “GT-AG” rule (Fig. 1).

3.2. Homology analysis of DjGRP78

The deduced amino acid sequence of DjGRP78 is highly similar to GRP78s in invertebrates and vertebrates (more than 75% similarity in all matches). There is high similarity to GRP78s of *Hydra magnipapillata* (75.15%), *Caenorhabditis elegans* (76.44%), *Danio rerio* (76.55%), and *Homo sapiens* (76.4%). Based on the nucleotide sequence of DjGRP78, we blasted the *Schmidtea mediterranea* genome database (Robb et al., 2008), and obtained a genome sequence of SmedGRP78 (v31.002536). The genome sequence of SmedGRP78 contains a 46 bp intron, and an ORF of 1983 bp encoding a polypeptide of 660 amino acids with a predicted molecular mass of 72.87 kDa and theoretical isoelectric point of 5.5. SmedGRP78 is highly similar to DjGRP78 (94.07% amino acid identity), especially in HSP70 family signatures and the ER retrieval signal **KTEL** (data not shown).