Fish & Shellfish Immunology 34 (2013) 617–622

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

GRP78 from grass carp (*Ctenopharyngodon idella*) provides cytoplasm protection against thermal and Pb^{2+} stress

Yujiao Zhu [a], Qidi Fan [a], Huiling Mao [a,*], Yong Liu [a], Chengyu Hu [a,b,**]

[a] Department of Bioscience, College of Life Science and Food Engineering, Nanchang University, Nanchang 330031, China
[b] Institute of Life Science, Nanchang University, Nanchang 330031, China

ARTICLE INFO

Article history:
Received 9 October 2012
Received in revised form
4 December 2012
Accepted 4 December 2012
Available online 27 December 2012

Keywords:
Glucose regulated protein 78
Stress protein
Cytoplasm protection
Chaperone
Grass carp

ABSTRACT

Glucose regulated protein (GRP) located in endoplasmic reticulum (ER) was a member of heat shock protein (Hsp) family. The protective mechanism adapted to ER stimuli was closely related to GRP. GRP78, known as BiP, was one of central regulator responded to stress in ER. Grass carp (*Ctenopharyngodon idella*) *GRP78* (*CiGRP78*) was up-regulated in almost tissues, especially in liver, under heat shock (34 °C), cold stress (4 °C) or lead nitrate (0.25 mmol/L) stress. In order to understand the function of *Ci*GRP78 in cellular protection, *CiGRP78* ORF cDNA was inserted into the plasmid of pET-32a(+) or pEGFP-C1 respectively, then the recombinant plasmids were transformed or transfected into *Escherichia coli* cells, mouse myeloma cells (SP2/0) or grass carp kidney cells (CIK). In the cells, *CiGRP78* was over-expressed following thermal, cold or Pb^{2+} stress. Results showed that *Ci*GRP78 not only contributed to protecting prokaryotic cells against thermal or cold extremes, but also played the same role in SP2/0 and CIK cells. After treatment with heat stress at 42 °C for 1 h, although the viability of the cells declined a lot, CIK cells with pEGFP-C1/*Ci*GRP78 exhibited a higher survival rate (28%) than wild-type cells (7%) or cells with only pEGFP-C1 (5.1%). When the time lag extended to 2.5 h, the survival rates were 19%, 5.7%, 4.8% respectively. In addition, *Ci*GRP78 would also provide a transient cytoplasm protection against Pb^{2+} stress in a dose- and time-dependent manner. After treatment with lead nitrate at concentration of 10 μmol/L for 12 h, 24 h or 36 h, the survival rates of cells with pEGFP-C1 or wild-type cells were 46.7% or 46.7% (12 h), 25% or 22% (24 h), 10% or 11% (36 h) respectively. When the cells were treated with lead nitrate at the concentration of 25 μmol/L, the survival rates of cells with pEGFP-C1 or wild-type cells were 45.5% or 30% (12 h), 16.7% or 25% (24 h), 6.5% or 8% (36 h), respectively. *Ci*GRP78 provided a distinct protection in CIK cells at the low concentration for 24 h. The survival rates of CIK cells with pEGFP-C1/*Ci*GRP78 treated with lead nitrate at concentration of 10 μmol/L or 25 μmol/L were 65.9% or 58.8% respectively. When the cells were treated with lead nitrate at concentration of 50 μmol/L for 24 h, the survival rate of the CIK cells was only about 30%. If the process-time was extended to 36 h, *Ci*GRP78 could not provide any cytoplasm protection for CIK cells.

1. Introduction

The glucose regulated protein 78 (GRP78), also known as BiP, was first discovered in chicken embryo fibroblasts cultured in glucose free medium [1]. As one member of heat shock protein 70 (Hsp70) subfamily, GRP78 may be a central regulator of endoplasmic reticulum (ER) homeostasis due to its multiple functions in protein folding, assembly and trafficking [2–4]. In particular, GRP78 could respond to stimuli in ER [5], so it belongs to stress proteins.

GRP78 was necessary for protein folding, Ca^{2+} stability and adjusting ER transmembrane signal sensors activity [6], and it was required to protect cells against intracellular adverse environment, such as oxidative stress and Ca^{2+} depletion [7,8]. GRP78 could defend cells from cytotoxicity and apoptosis [9] and play an essential role in the aging process and age-related diseases [10]. In addition, GRP78 was closely related to the tumorigenesis and development of tumor. For instance, GRP78 was expressed at high levels in human lung cancer tissues and hepatic tumor cells [11,12]. GRP78 was known to bind procaspase-7 (C-7) and block its activation [13], so it would increase the viability of cells against various

* Corresponding author. Tel.: +86 791 8396 9531; fax: +86 791 8396 9530.
** Corresponding author. Department of Bioscience, College of Life Science and Food Engineering, Nanchang University, Nanchang 330031, China. Tel.: +86 791 8878 5566; fax: +86 791 8396 9530.
E-mail addresses: 13507085896@163.com (H. Mao), hucy2008@163.com, hucy2008@21cn.com (C. Hu).

1050-4648/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fsi.2012.12.005

extracellular stimuli. Moreover, GRP78 could respond to heavy metal stress, indicating that it would play a potential role in protection against heavy metal toxicity [14]. GRP78, as a major ER chaperone, was able to transfer mis-folded proteins from ER and inhibit the formation of abnormal protein, thus alleviate Ca^{2+} imbalance caused by heavy metal in ER [15,16]. GRP78 served as a Ca^{2+} buffering chaperone to keep Ca^{2+} homoeostasis following ER stress, so it could compensate the deprivation of Ca^{2+} and the interruption of phosphorylation signaling pathways due to the influence of Pb^{2+} on Ca^{2+} homeostasis [16–18].

As a kind of poikilotherm, fish was more sensitive to extracellular stimuli (e.g. water temperature, heavy metal, oxygen, osmotic pressure and disease). These stimuli would affect the distribution and physical condition of fish. The studies on fish stress proteins were very important and necessary. In recent years, *GRP78* had been reported and identified in many fishes, including rainbow trout (*Oncorhynchus mykiss*) (AB196459) [19], Japanese flounder (*Paralichthys olivaceus*) (DQ662232) and grass carp (*Ctenopharyngodon idella*) (FJ436356) [20]. However, the expression pattern of *GRP78* was often covered in these papers, but its function had little been involved in fish.

Grass carp, one of the most important freshwater aquaculture species in China, was susceptible to stimuli throughout the breeding process, resulting in a high mortality rate and an enormous economic loss. In order to understand the function of *Ci*GRP78, we carried out the cytoplasm protection of *Ci*GRP78 against thermal (at 42 °C), cold (at 4 °C) and Pb^{2+} stress. The results suggested that *Ci*GRP78 would protect cells against thermal and Pb^{2+} stress.

2. Materials and methods

2.1. Fish, thermal and cold stress, Pb^{2+} stress and sample collection

Grass carp (mean body weight 30 g) was purchased from Jiangxi Provincial Fisheries Research Institute and acclimatized for 2 weeks in a quarantine area. For temperature stress experiments, 10 fish were put into 25 L aerated aquaria at 34 ± 1 °C or 4 °C for 2 h, respectively. On the other hand, 6.667 mg $Pb(NO_3)_2$ were put into 20 L aerated aquaria and made up into the Pb^{2+} concentration of 0.25 mmol/L. After treated with 0.25 mmol/L Pb^{2+} for 24 h at 25 °C, the fishes were immediately returned to acclimation tanks in normal condition for 20 min. Then, tissues including liver, spleen, kidney, brain and intestine were sampled and frozen in liquid nitrogen. Non-stress fish were used as a control group.

2.2. Tissue expression analysis of *Ci*GRP78

Total RNA was extracted according to the manufacturer's instruction of RNA Extraction Kit (Sangon Biotech Co., Ltd. Shanghai, China). First strand cDNA was synthesized using oligo$(dT)_{18}$ primers with QuantScript RT Kit (Tiangen Biotech (Beijing) Co., Ltd., China). Expression of *CiGRP78* mRNAs was assessed in different tissues using quantitative real-time RT-PCR (qRT-PCR) in a Mastercycler ep realplex (Eppendorf) with specific primers: RT-F (5′-GTC ACC TTT GAG ATC GAC GTG-3′) and RT-R (5′-AGA GAG TAG GCG TAG CTC-3′). The PCR program was 1 cycle of 5 min at 94 °C, 28 cycles of 30 s at 94 °C, 30 s at 53 °C, 30 s at 72 °C, and 1 cycle of 10 min at 72 °C. As positive control for RT-PCR, *β-actin* (primers were 5′-CAC TGT GCC CAT CTA CGA-3′ and 5′-CCA TCTCCT GCT CGA AGT-3′) was amplified to determine the concentration of templates. Amplifications were carried out in a final volume of 20 μL, containing 2 μL DNA sample, 10 μL SYBR Premix Ex Taq™ (TaKaRa) and 0.4 μL of each forward and reverse primer. Each sample was run in triplicate. The data were subjected to one-way ANOVA followed by an unpaired, two tailed *t*-test. $p < 0.05$ was considered statistically significant.

2.3. Bacterial cells survival assays

To estimate the growth curves, wild-type *Escherichia coli* DH5α and cells transformed with pET-32a(+)/GRP78 or pET-32a(+) were grown to 1.5 of A_{600} at 37 °C in LB media. Then, cultures were diluted 1:100 into fresh LB media supplemented with appropriate ampicillin. Continuously, optical densities (A_{600}) of these cultures were measured every 30 min, and the means of three experiments were determined (with SD being less than 5%). A single colony of bacteria transformed with pET-32a(+)/GRP78, pET-32a(+) or wild-type *E. coli* DH5α was transferred to 20 mL of LB media containing 50 mg/L of ampicillin. Cells were grown at 37 °C with vigorous shaking until they reached an OD of 0.6–0.8 at A_{600} nm, which were induced by the addition of IPTG to a final concentration of 1.0 mmol/L. After 3 h of growth, the cultures were divided into three aliquots. For heat shock experiments, the first aliquot of cultures was placed at 42 °C. Then 1 mL of the cultures was taken at time-point of 1 h, 1.5 h, 2 h, or 2.5 h respectively, and serial dilution of 1:106 was plated onto LB agar plates containing 50 mg/mL of ampicillin. After incubation overnight at 37 °C, cells viability was estimated by counting the number of colony-forming units. For cold treatments, the second aliquot of cultures was diluted 1:106 and plated onto LB agar supplemented with ampicillin. Plates were incubated at 4 °C for different periods (2 d, 4 d, 6 d, 8 d), and then were cultured overnight at 37 °C. Cell viability was estimated as described above. The third aliquot of cultures was to be a control which were diluted and plated, and then were cultured overnight at 37 °C. For both heat and cold treatments, the means of three experiments were determined (with SD being less than 5% in all cases).

2.4. Cells culture, transfection, and survival assays

The coding region of *Ci*GRP78 was inserted into pEGFP-C1 vector. The pEGFP-C1/GRP78 or empty pEGFP-C1 was transfected into SP2/0 cells (from the Sino-German Joint Research Institute of Nanchang University) and CIK cells (kindly provided by Professor Pin Nie, Institute of Hydrobiology, Chinese Academy of Sciences) respectively. SP2/0 and CIK cells grew overnight to 90–95% confluence in twenty-four-well plates prior to transfection. Transfection was carried out using Lipofectamine 2000 reagent (Invitrogen). For each well, 200 ng of either pEGFP-C1/GRP78 or pEGFP-C1 was mixed with 1 μL of lipofectamine 2000 in 100 μL EMEM without serum. The cells were incubated at 37 °C or 28 °C in a CO_2 incubator for 24 h. These samples were divided into three aliquots for heat (42 °C) or cold (4 °C) treatments as described above. Then cells were washed with PBS and digested by 1 mL 0.25% trypsin for 3–5 min at 37 °C or 28 °C. After detached from culture flask, cells were collected by centrifugation (15K, 15 s) and re-suspended in 1 mL PBS. Cells were counted using a microscope counting chamber. Each experiment was repeated three times.

Pb^{2+} stress was performed only in CIK cells. The cells were grown to confluency at 28 °C in Medium 199 supplemented with 10% FBS, 100 μg/mL penicillin, 100 mg/mL streptomycin. Pb^{2+} treatments were performed by means of adding lead nitrate at different concentrations (10 μmol/L, 25 μmol/L, 50 μmol/L) into Medium 199 nutrient mix for 12 h, 24 h and 36 h respectively. Cells were counted as described above. Each experiment was repeated three times.