Journal of Insect Physiology 58 (2012) 1226–1234

Contents lists available at SciVerse ScienceDirect

Journal of Insect Physiology

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

Stress-induced HSP70 from *Musca domestica* plays a functionally significant role in the immune system

Ting Tang [a,b], Chen Wu [a], Jigang Li [a], Guodong Ren [a], Dawei Huang [a,b,*], Fengsong Liu [a,*]

[a] College of Life Sciences, Hebei University, Baoding 071002, China
[b] Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

ARTICLE INFO

Article history:
Received 13 April 2012
Received in revised form 13 June 2012
Accepted 14 June 2012
Available online 28 June 2012

Keywords:
Musca domestica
Heat shock protein 70
Heat shock
Cadmium stress
Bacterial challenge
RNA interference

ABSTRACT

As important molecular chaperones, members of the 70 kDa heat shock protein (HSP70) family play essential roles in stress tolerance and innate immunity in organisms. The full-length complementary DNA (cDNA) of a novel inducible *HSP*70, named as *MdHSP*70, was isolated from *Musca domestica*. The cDNA clone consisted of 2411 bp with a 1956 bp open reading frame which encodes 651 amino acids. Using real-time quantitative polymerase chain reaction (qPCR), we investigated the transcriptional profile of the gene under heat shock, cadmium stress and in response to bacteria. Increased expression of *MdHSP*70 was observed in response to both heat shock and Cd stress. The expression of *MdHSP*70 was significantly induced by *Escherichia coli* or *Staphylococcus aureus* stimulation. Larvae were fed bacteria expressing dsRNA targeting the *MdHSP*70 gene. Our results showed high mortality in larvae treated with dsRNA of *MdHSP*70 at heat shock, Cd stress and bacterial invasion, suggesting that *MdHSP*70 is potentially involved in the stress and immune responses of the house fly and perhaps contributes to protection against cellular injury.

1. Introduction

Environmental stressors, such as hyperthermia, hypoxia, and toxic metals, can induce the expression of intracellular stress proteins such as heat shock proteins (HSPs) (Sanders, 1993; Williams and Morimoto, 1990). Stress-based changes in HSPs are based on the type of inducers (Sanders, 1993). The environment is complex, and an insect's health is greatly affected by environmental variations in temperature, pollutant content and bacterial pathogens. Heat shock or cadmium, which is harmful to most organisms, can induce immediate changes in protein conformation both in insects and humans. These changes have more harmful effects on small-bodied insects such as house flies. Most insects have a limited physiological ability to regulate their body temperature. They use multiple compensating strategies to ensure homeostasis when facing fluctuations of environmental temperature (Bale and Hayward, 2010; Gourgou et al., 2009). Cadmium causes pleiotropic effects on organisms at both the cellular and molecular levels. It binds to cysteine residues of proteins and induces oxidative stress (Planelló et al., 2010). These two inducers link to short-term responses that impact an individual's fitness. Besides abiotic factors, many biotic stressors can cause various disorders. Bacteria or viruses, which frequently occur in the culture medium may cause drastic and catastrophic losses. Intervening pathogenic stimulation can cause a series of immune responses in organisms to maintain cells in a physiological balance, signal normal conduction and immune related gene expression, which can improve an organisms' ability to resist pathogens.

Heat shock protein 70 (HSP70) is a highly conserved family of universal cytosolic chaperones; it plays an essential role in protein metabolism under normal and stressed conditions (Su et al., 2010). It maintains protein homeostasis by regulating the folding of damaged proteins and the degradation of those that are beyond repair (Hartl and Hayer-Hartl, 2002; Kokolakis et al., 2009; Mayer and Bukau, 2005; Nollen and Morimoto, 2002). This protein is also an essential factor for activating many signal proteins such as steroid hormone receptors, cell cycle kinase Cdk4, serine/threonine kinases (Fu et al., 2011) and important immune defense reaction.

Musca domestica (Diptera: Muscidae), commonly called the house fly, is a well-known cosmopolitan pest that is one of the most widely dispersed nonbiting insects in the world. As a prominent model in insect physiology and biochemistry, the transcriptome of *M. domestica* was sequenced in our laboratory (Li et al., 2011). *HSP*70 homologue, designated as *MdHSP*70, was cloned from *M. domestica*. The molecular features and functional mechanisms of *HSP*70 from *M. domestica* have not been extensively investigated. The purpose of this paper is to detect the expression profile

* Corresponding authors. Address: College of Life Sciences, Hebei University, Baoding 071002, China; Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China (D. Huang), College of Life Sciences, Hebei University, Baoding 071002, China (F. Liu). Fax: +86 312 5079362.
E-mail addresses: huangdw@ioz.ac.cn (D. Huang), liufengsong@hbu.edu.cn (F. Liu).

0022-1910/$ - see front matter Crown Copyright © 2012 Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jinsphys.2012.06.007

of *MdHSP*70 under heat, cadmium stress and bacterial challenge. Furthermore, RNAi-mediated gene silencing was explored to investigate the roles of *MdHSP*70 in the house fly. These studies were designed to gain more information about this stress gene related to its function in the cell and its possible adaptation to different environments.

2. Materials and methods

2.1. House fly strain and treatment

The house fly strain used in this study was a gift from Miss Fengqin He, Institute of Zoology, Chinese Academy of Sciences. Larvae were raised in a climate room at 25 °C, relative humidity 75% and medium made of bran (55 g), heat-inactivated yeast (3 g), water (150 mL) until pupation. After eclosion, adult flies were fed on water, sugar, and milk powder. Flies were maintained at 25 °C under 12 h light/12 h dark cycles (LD12:12) (Codd et al., 2007).

The heat resistance and cadmium tolerance assay were performed according to methods described previously (Tang et al., 2011). For heat shock, the 3rd instars larvae were exposed at 42 °C for 1 h and untreated larvae were sampled as a control group. After 42 °C treatment, larvae were transferred to 25 °C for post-stress recovery. Samples were randomly collected at 15, 30 min, 1 h during heating, then transferred to 25 °C and sampled at 1 h, 2 h and 4 h.

In order to learn the response of the house fly to cadmium stress, 3rd instars larvae were reared on a medium in which water was replaced with $CdCl_2$ solutions that ranged from 0 mM to 30 mM for 24 h. Controls received the normal medium. Samples were collected at 3 h, 6 h, 12 h and 24 h after exposure to 30 mM.

For septic injury infection experiments, the 2nd instars larvae were challenged according to the method described previously (Dong et al., 2011). Septic injury was produced by pricking abdomens of larvae which were previously dipped into a concentrated culture of *Escherichia coli* or *Staphylococcus aureus*. Larvae were sampled for RNA extraction at 0 h, 3 h, 6 h, 12 h, 24 h, 36 h, 48 h, and 60 h after infection.

Six larvae were randomly sampled at each time point after exposure to stress and infection. Triplicates were performed at each time point. Samples were collected from exposed treatment, then frozen in liquid nitrogen. Total RNA from each treatment and control sample were extracted from whole bodies and subjected to qPCR testing.

2.2. Reverse transcription PCR and RACE amplification

Total RNA was extracted from larvae of *M. domestica* with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Total RNA was treated with RQ1 Rnase-Free DNase (Promega, USA) to remove the contaminated DNA. RNA quality was assessed by electrophoresis detection on 1% agarose gel, combined with measuring the $OD_{260/280}$ value by spectrophotometer (GeneQuant pro, Amersham Biosciences, Freiburg, Germany). The first-strand cDNA was made using an universal forward primer AOLP [5′-GGCCACGCGTCGACTAGTAC$(T)_{16}$(A/C/G)-3′]. A reverse transcription (RT) reaction was performed with 2 μg RNA as a template in RT buffer containing 10 mM dNTP 1 μL, 10 μM of universal primers 2 μL, 1 mM DTT 1 μL and one unit of M-MLV polymerase (Promega, USA) in 25 μL total volume. As negative control, RT-PCR was performed in the absence of reverse transcriptase (data not shown). The mixture was incubated at 42 °C for 1 h, terminated by heating at 95 °C for 5 min, and subsequently stored at −20 °C.

To amplify the partial cDNA fragment of the *MdHSP*70, two degenerate primers MdhspF1 (5′-TATTTCAACGATTCCCAGCG-3′), MdhspR1 (5′-TGGTGATGGTGATCTTGTTTTC-3′) were designed based on the sequence of the transcriptomic database (Liu et al., 2012). A 25 μL PCR reaction volume contained 2.5 μL 10× PCR buffer, 1.5 μL $MgCl_2$ (25 mM), 2.0 μL dNTPs (2.5 mM), 1.0 μL of each primer (10 μM), 15.8 μL dd H_2O, 0.2 μL Taq polymerase (5 U/μL) (TaKaRa, Dalian, Liaoning, China) and 1 μL of cDNA mix. The PCR reaction was an initial 4 min predenaturation at 94 °C, 35 cycles of 30 s at 94 °C, 40 s at 58 °C, 60 s at 72 °C, 10 min at 72 °C.

To obtain full-length cDNAs, we used the rapid amplification of cDNA ends (RACE) method. For 3′ end RACE PCR, a gene specific primer MdhspF2 (5′-ACTTACTCCGACAACCAACC-3′) was designed based on the obtained partial cDNA fragment sequences. The PCR was performed with gene specific primer MdhspF2 and anchor primer AP (5′-GGCCACGCGTCGACTAGTAC-3′). To amplify the 5′ end of *MdHSP*70, the cDNA was reverse transcribed according to the instruction of the SMART™ PCR cDNA Library Construction Kit (BD Biosciences Clontech). The cDNA was used as template for PCR amplification with 5′ SMART primer and a specific primer MdhspR2 (5′-TTGTCCAAGCCGTAGGCAATGG-3′). To confirm cDNA sequences, a gene-specific primer pair, MdhspF3 (5′-CACAAGATGTCTAAAGCTCCCGC-3′) and MdhspR3 (5′-CAGCGGCGAATGATTTAGTCGAC-3′) were designed basing on the assembled sequences by overlapping initial amplified fragments. The PCR reaction was an initial 4 min predenaturation at 94 °C, 35 cycles of 30 s at 94 °C, 40 s at 55 °C, 60 s at 72 °C; 10 min at 72 °C. The PCR products were cloned into the pMD18-T vector (TaKaRa, Dalian, Liaoning, China) and sequenced in both directions. The sequencing results were verified and then subjected to cluster analysis.

2.3. Sequence analysis

The cDNA clones were sequenced and the full-length *MdHSP*70 cDNA was assembled. The sequence similarity and the deduced amino acid sequence comparisons were carried out using BLAST at NCBI (http://www.ncbi.nlm.nih.gov/blast). Multiple alignments of MdHSP70 amino acid sequences of *M. domestica* with those of other insects were performed by Clustal W program and then a dendrogram was constructed using MEGA 4.1. The molecular features of MdHSP70 were analyzed with the Expert Protein Analysis System (http://www.us.expasy.org/).

2.4. qPCR and statistical analysis

The mRNA levels of *MdHSP*70 under stress treatments were measured by qPCR amplification. PCR amplifications were performed in 25 μL reactions containing 2.5 μL cDNA, 2.5 μL, 10 μM each of gene-specific forward MdhspF4 (5′-TACCCCCTTGTCTTTGGGTATTGAAACC-3′) and reverse primer MdhspR4 (5′-GGTCTGGGTTTGCTTAGTGGGGATGGTG-3′), 12.5 μL of 2× SYBR Premix Ex Taq (TaKaRa) and 7.5 μL ddH_2O. Nuclease-free water was used as a qPCR negative control instead of cDNA templates. The qPCR was performed by Miniopticon (Bio-rad). The amplification reaction protocol was as followed: initial denaturation at 95 °C for 3 min; followed by 35 cycles of 95 °C 20 s, 60 °C 30 s, finally the protocol for the melting curve with an increase of 0.5 °C between each cycle from 55 °C to 95 °C.

β-Actin was used as the reference gene for normalization of target gene expression. Expression levels of the target gene were calculated by comparing the cycle threshold value (Ct) to the reference gene *β-actin*. The relative quantification (comparative method) was calculated using the ΔΔCt method (Livak and Schmittgen, 2001). All samples were normalized to the ΔCt value of a reference gene to obtain a ΔΔCt value (ΔCt target−ΔCt reference). The final relative expression was calculated using the following formula: $F = 2^{-(\Delta Ct\ target\ -\ \Delta Ct\ reference)}$. A randomly selected untreated control sample was used as the calibrator for calculating relative expression ratios. All statistics were determined by