Characterization and Expression Pattern Analysis of the T-Complex Protein-1 Zeta Subunit in *Musca domestica* L (Diptera)

Xuejun Zhao,1 Jiangfan Xiu,1 Yan Li,1 Huiling Ma,1 Jianwei Wu,1 Bo Wang,2 and Guo Guo1,3

1Department of parasitology, School of Basic Medical Sciences, Guizhou Medical University, Guiyang, University City Guiian New District, 550025, China (1109719405@qq.com; xiujiangfan@163.com; 362914845@qq.com; 13294157911@qq.com; wjw1@gmc.edu.cn; 383657226@qq.com), 2Department of Electrochemical Engineering, School of Chemistry and Chemical Engineering, Harbin Institute of Technology, Harbin 150001, China (wangbo19880804@163.com), and 3Corresponding author, e-mail: guoguo@gmc.edu.cn

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Abstract

Chaperonins, belonging to the T-complex protein-1 (TCP-1) family, assist in the correct folding of nascent and misfolded proteins. It is well-known that in mammals, the zeta subunit of the TCP-1 complex (TCP-1ζ) plays a vital role in the folding and assembly of cytoskeletal proteins. This study reported for the first time the cloning, characterization and expression pattern analysis of the TCP-1ζ from *Musca domestica*, which was named as MdTCP-1ζ. The MdTCP-1ζ cDNA is 1,803 bp long with a 1,596 bp open reading frame that encodes a protein with 531 bp amino acids. The analysis of the transcriptional profile of MdTCP-1ζ using qRT-PCR revealed relatively high expression in the salivary glands and trachea at the tissues while among the developmental stages. The highest expression was observed only in the eggs suggesting that the MdTCP-1ζ may play a role in embryonic development. The expression of MdTCP-1ζ was also significantly induced after exposure to short-term heat shock and infection by *Escherichia coli*, *Staphylococcus aureus*, or *Candida albicans*. This suggested that MdTCP-1ζ may take part in the immune responses of housefly and perhaps contribute to the protection against cellular injury.

Key words: T-complex protein-1, chaperonin, *Musca domestica*, microbial challenge, immune response

Protein homeostasis in cells is primarily maintained by molecular chaperones that not only prevent the aggregation of misfolded proteins also assist in the proper folding of nascent proteins. Correct protein folding is executed by three main ATP-dependent chaperone classes which include heat shock proteins 70 and 90 (say Hsp70 and Hsp90), and chaperonin (Hartl et al. 2011). Among these the most complex are the chaperonins, because they facilitate protein folding within their cavity, thereby isolating the protein from its cellular environment (Vanitha et al. 1999, Tang et al. 2006). On the basis of their location, two groups of chaperonins have been characterized: the group I is primarily present in prokaryotes, as well as in specific eukaryotic organelles such as chloroplasts and mitochondria, whereas the group II is in the cytoplasm of archaea and eukaryotes. These two groups of chaperonins have the similar amino acid sequence although their structure and function vary largely.

In mammals, the group II chaperonins have been composed of a hetero-oligomeric double ring complex with eight different subunits per ring, that is, TCP-1ζ, β, γ, δ, ε, ζ, η, and θ (Hynes et al. 1995, Kubota et al. 1995). These are also referred to as the TCP-1 ring complex (TRiC; Horwich et al. 2007). Studies have shown that each of the eight subunits has three domains, two of which specifically bind ATP and unfolded polypeptides, and a third links the first two domains thus facilitating their movement during binding (Carrascosa et al. 2001). TCP-1 has been presumed to play critical roles in regulating the cell cycle and cytoskeleton (Brackley et al. 2009). The reason was that its main substrates were shown to be protein such as cycline, histone, deacetylase, and protein phosphatase PP2A regulatory subunit B (Won et al. 1998, Siegers et al. 2003) and the cytoskeletal proteins, actin, and tubulin (Tian et al. 1995, Farr et al. 1997). TCP-1 integrity is essential for autophagosome degradation in cells or drosophila, which is orchestrated by the actin cytoskeleton (Pavel et al. 2016). The expression of TCP-1 is a vital component of the overwintering defense strategy of many temperate zone insects (Rinehart et al. 2007), however, the TCP-1 group of proteins in insects was less known.

The common housefly, *Musca domestica* (*M. domestica*) is a typical insect, pest of domestic, medical, and veterinary importance. *M. domestica* can be used as an insect vector of many pathogenic
organisms (Malik et al. 2007). Our previous study, constructed a cDNA library from the heat shock-induced third instar housefly larvae, sequenced its structure, and significantly identify the immune-related factors (Liu et al. 2010). This study reported a new type of TCP-1 family (MdTCP-1) identified from the housefly cDNA library. At the same time, a full-length cDNA of MdTCP-1 obtained by the PCR rapid amplification of cDNA ends. The expression profile of gene under the developmental stage, the third instar larval tissues, was investigated using the qRT-PCR method. MdTCP-1 was analyzed in terms of the potential function by exposing houseflies to heat shock and microbial infection. The results provided certain insights into the function of MdTCP-1 and facilitated the understanding of housefly adaptation to extreme environmental stress conditions.

Materials and Methods

Housefly Rearing

M. domestica larvae were reared in a condition-controlled room at 25 ± 1°C and 50-60% RH. The larvae were fed with larval food containing 33 g bran in 100 ml water. The M. domestica adults were fed on a diet comprising water, sugar, milk powder, and were maintained at 25 ± 1°C with 12 h light-to-dark alternating cycles (Codd et al. 2007).

Cloning of MdTCP-1 ORF

First, cDNA was synthesized from the total RNA isolated from the third instar larvae using the Takara Prime Script RT reagent kit (Takara). Then, the cDNA was used as template to amplify the full-length ORF (open reading frame) of MdTCP-1 by means of the primer pair of the (forward primer 5'-ATGCGTCTCAAT TAGTTAATGGAATCC-3', and reverse primer 5'-CTAACTACCCCTT AGACGTTGCTACCAACGCC-3') through rapid amplification of cDNA ends using the DNA kit. The amplified PCR products were cloned into the pMD19-T cloning vector and sequenced.

Sequence Analysis

Nucleotide sequence was analyzed using BLASTN and BLASTP at NCBI. ORF Finder (http://blast.ncbi.nlm.gov/projects/gorf/) was used to analyze the deduced amino acid sequence. Protein motifs were predicted using Simple Architecture Research Tool (http://smart.cnbil-heidelberg.de). The theoretical isoelectric point and molecular weight was calculated with respect to pI/Mw (http://www.expasy.ch/tools/pi-tool.html).

Gene Expression Analysis

Sample Collection. The total RNA was extracted from the different housefly developmental stages and different tissues. The developmental stages included eggs, larvae from the first, second and third instars, pupae, adult females, and adult males. Tissues were respectively the body wall, fat body, midgut, malpighian tubule, trachea and salivary glands taken from the third instar larvae.

Microbial Challenge. In order to investigate the response of the larvae to microbial infection, the third instar housefly larvae were infected with Escherichia coli, Staphylococcus aureus, and Candida albicans, respectively. Each experimental group comprised 200 third instar larvae. These larvae were randomly chosen, and their appropriate weight was 26 ± 5 mg. The infected group larvae were injected with 0.2 μl suspension, that is, ~2 × 10⁶ CFU of bacteria at the 10th segment of segmental venter of larvae using the Auto-Nanoliter injector under a stereomicroscope. After injection, these larvae were raised on sterilized bran and water. At 3, 6, 12, 18, 24, 36, and 48 h postinfection, the larvae were collected, respectively.

Third larvae injected with an equal volume of PBS buffer were used as the negative control group (CK group).

Heat Shock Inducing. The third instar larvae were kept in an incubator at a heat shock temperature of 42 °C (Tang et al. 2012). The control larvae were remained at the ambient temperature. After heat shock, 6–8 larvae were taken for total RNA extraction at different time points, that is, 15 min, 30 min, 1 h, 1.5 h, 2 h.

RNA Extraction and qRT-PCR. The total RNA was extracted from the 6 to 8 larvae collected at each time point after being treated with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction. Then, qRT-PCR was performed using the SYBR Premix Ex Taq II (TaKaRa, Dalian, Liaoning, China) according to the instruction.

The MdTCP-1 mRNA levels were quantified by qRT-PCR performed in a 20 μl reaction volume containing 2 μl of cDNA, 1 μl of each of the forward and reverse primer (10 μM forward MdTCP-1: 5'-CGGATGGATAGTGAAGGT-3' and reverse MdTCP-1: 5'-CTAAAGTAGTACCGTGTTGCTG3'), 10 μl of 2 × SYBR Premix Ex TaqII, 0.4 μl of 50 × ROX Reference Dye (TaKaRa, Dalian, Liaoning, China) (TaKaRa), and 5.6 μl DEPC water. Appropriate controls without cDNA were also run. qRT-PCR was performed on BIO-RAD CX96 with an amplification protocol consisting of an initial 30 s denaturation at 95°C, followed with 40 cycles at 95°C for 5 s and 60°C for 30 s, and a final melting curve analysis with an increase of 0.5°C each cycle from 60 to 95°C. Four biological replicates and three technical replicates were totally implemented.

GAPDH was used as an internal reference, and two primers were utilized to amplify GAPDH: 5'-CATCATCTCCGCTCCATC-3' and 5'-AAAGCATACCAGTGAAGT-3'. These primers were calculated by using the ΔΔCt method where ΔΔCt = ΔCt target - ΔCt reference. The final relative expression was eventually calculated as follows: F = 2−ΔΔCt. Egg sample was used as a calibrator for analyzing the gene expression at the developmental stages. Among the tissues, the larvae not challenged with microbial infection and those not exposed to heat shock were also used as the calibrators respectively in calculating the relative expression ratios after the microbial challenge and heat shock experiments were made.

The statistical significance of the difference in the values obtained was determined by one-way ANOVA posttest Tukey’s multiple comparison tests within SPSS17.0. The level of statistical significance was set at P < 0.05 for all the analysis. GraphPad Prism 5 was employed for creating images.

Results

Cloning and Characterization of MdTCP-1

The nucleotide sequence MdTCP-1 cDNA was submitted to GenBank KM215143. As a consequence, the MdTCP-1 cDNA has a 1,596 bp long ORF that encodes a protein containing 531 amino acids and 120 amino acids with a prediction. The molecular weight of MdTCP-1 is 58,26 kDa with an isoelectric point (pI) of 6.56. The MdTCP-1 protein sequence shares an 89% of amino acid identity with the known homologous proteins from Ceratitis capitata and Bactrocera dorsalis. Two TCP-1 protein family signatures, (35–47) RTNLPGKTMKML, and (84–92) QDDATGDGT, were identified in the predicted MdTCP-1 amino acid sequence.

Tissue-Specific Expression of MdTCP-1 in Housefly Larvae

The expression of MdTCP-1 was investigated in terms of the third instar larval tissues by qRT-PCR. The maximum levels of MdTCP-
The expression of MdTCP-1 mRNA was observed in the salivary glands and trachea, followed by the malpighian tubule and midgut (Fig. 1). In contrast, the lowest levels of MdTCP-1 mRNA were in the body wall.

**Developmental Stage-Specific Expression of MdTCP-1 in Housefly**

In the same way, the expression of MdTCP-1 mRNA in different developmental stages of housefly was analyzed. The data showed that the expression of MdTCP-1 in the larvae, pupae, and adult stages was lower than the levels in the calibrator egg (Fig. 2). The lowest levels were detected in the third instar larvae.

**Expression Profiles of MdTCP-1 After Microbial Challenge and Heat Shock**

Figure 3 shows the temporal change of the levels of MdTCP-1 mRNA of the third instar housefly larvae when infected with *E. coli*. It can be observed that the expression profiles of MdTCP-1 mRNA changed drastically after *E. coli* injection. The levels of MdTCP-1 mRNA gradually increased from the 3 h postinjection to the maximum level at the 24 h postinjection. At the 48 h postinjection, the MdTCP-1 expression appeared to reach a plateau although it was significantly higher, compared with the control.

Similarly, after *S. aureus* and *C. albicans* injections, the MdTCP-1 expression was also up-regulated when compared with the controls. At the 3 h *C. albicans* challenge, the MdTCP-1 expression was up-regulated and continued to increase up to 24 h, then gradually decreased. Interestingly, the expression of MdTCP-1 was slightly lower at the 6 h post *C. albicans* injection. After *S. aureus* challenge, the expression of MdTCP-1 slightly increased until 12 h, however, drastically increased later. This was similar to the expression pattern observed for the *C. albicans* challenge, but significantly lower than that observed for the *E. coli* challenge.

The expression profile of MdTCP-1 gene under heat shock conditions is shown in Figure 4. Compared with the control group, the expression levels of MdTCP-1 were up-regulated, and at 15 min the MdTCP-1 was increased gradually. But at 1 h it was not clearly up-regulated. Particular attention should be paid to the 2 h, at which the expression of MdTCP-1 approached a maximal level. However, heat shock at 42°C >2 h would cause the larvae to die.

**Discussion**

In *M. domestica*, molecular chaperones including sHSP and HSP70 have been reported previously (Tang et al. 2012, Li et al. 2013, Peng et al. 2015). However, the function of the cytosolic chaperonin, TCP-1 was never known. In eukaryotes, TCP-1 is known to be indispensable for cell survival as it participates in the correct folding of cytoskeletal proteins, tumor suppressor proteins and proteins involved in cell cycle regulation (Spieß et al. 2004). In mammals, TCP-1 subunit has been shown to partake in the biogenesis of chicken follicles, growth progression, microfilament and infiltration and metastasis of colon cancer (Wei et al. 2013, Grantham et al., 2006, Han et al. 2011). However, whether or not this gene is present in *M. domestica* and its role was not known before. This study has shown that *M. domestica* expression MdTCP-1 was at all developmental stages and MdTCP-1 can be induced by microbial infections and heat shocks. This revealed that MdTCP-1 may play a critical role as a molecular chaperon in the environmental stress conditions.

The isolated MdTCP-1 cDNA encoded a protein with 531 amino acids and a predicted molecular mass of 58.26 kDa, which was confirmed by separating the protein on SDS-PAGE. Furthermore, MdTCP-1 cDNA had the two characteristic signature sequences of a TCP-1 protein between 35–47 and 84–92 (Kubota et al. 1994, Kim et al. 1994). A high homology of 89% of was observed between the amino acid sequences of MdTCP-1 and homologous proteins from *C. capitata* and *B. dorsalis*.qRT-PCR experiment revealed that MdTCP-1 expression was the highest in the salivary glands and trachea, followed by the malpighian tubule and midgut. The lowest expression level was observed in the fat body and body wall. Salivary gland is known to secrete a number of digestive enzymes that play an important role in an insect’s digestion and also partake in an insect’s defense system (Gao et al. 2015). Previous studies have shown that TCP-1 is important for tubulin folding and microtubule growth at *C. elegans* (Srayko et al. 2005, Lundin et al. 2008). As the salivary glands and trachea (Huang et al. 2007, Li et al. 2002) contain actin and tubulin, it was not surprising that MdTCP-1 was present in high levels in these tissues. In the animal kingdom, RNAi of TCP-1 significantly reduced the intestinal microvilli length leading to the loss of function in most of the apical intestinal areas, causing that the formation of large protein aggregates in the cytoplasm (Saegusa et al. 2014). Therefore, it can be presumed that MdTCP-1 could be involved in regulating various biological processes such as digestion in the housefly.
The temporal expression pattern of MdTCP-1\(_f\) during the developmental stages of *M. domestica* revealed the significantly lower levels of MdTCP-1\(_f\) in the larvae, pupae and adults when compared with the eggs. This result indicated that MdTCP-1\(_f\) may play an important role in embryonic development. The expression level of MdTCP-1\(_f\) in female adult seemed to be far higher than in male adult. TCP-1 is necessary for the beneficial effect on cardiac performance in female *Drosophila* (Gill et al. 2015). CCT6A likely played an important role in sexual maturity (Kang et al. 2012). Because follicle growth and ovulation is regulated by gonadotrophins and steroids (Howles et al. 2000, Hunter et al. 2004, Richards et al. 2010), the expression of CCT6A was interestingly up-regulated in an endometrial adenocarcinoma cell line after treatment with megestrol acetate (Zhang et al. 2006), which implied that its transcription was modulated by progesterone. During mRNA processing, a single gene transcript could be processed differently via alternative splicing and the resulting gene product could perform the same or different functions (Mukherjee et al. 2010). Therefore, the fact may be that the highly conserved eight subunits of the TCP-1 complex may be present in multiple forms due to alternative splicing to participate in various biological processes (Lopez et al. 2016).

Insects live in highly varying environments and may sometimes encounter extreme conditions. Most insects have adapted to these extreme environmental conditions as shown in a number of studies where insects maintain homeostasis using multiple strategies. The most commonly studied extreme environmental condition is the variation in temperature (Gourgou et al. 2010). In addition, exposure of insects to pathogens can also induce changes in an insect’s physiology by increasing the immune responses (Ohta et al. 2006, Xiu et al. 2016). The present results showed that the expression of MdTCP-1\(_f\) was higher in the third instar larvae exposed to heat shock and after infected with three different microbes. These findings were in agreement with the previous studies where high expression levels of TCP-1 were shown in *Delia antiqua* with cold hardiness (Kayukawa et al. 2005). The heat shock-induced the expression patterns indicated that the *Helicoverpa* *zea* HSP70 was heat-inducible (Zhang et al. 2010). Two HSP23 genes in the fruit fly are induced by heat shock in a similar manner. It is well-known that bacterial infection in invertebrates triggers phagocytosis of the pathogen, which also results in the generation of reactive oxygen species (ROX) that lead to protein denaturation and/or proteotoxicity in the host (Ekanayake et al. 2008). In this context, TCP-1 activity may be induced to clear the aggregating denatured proteins to prevent cell death (Nollen et al. 2004, Kitamura et al. 2006). Hence, upregulating the expression of MdTCP-1\(_f\) mRNA may benefit *M. domestica* larvae by reducing the damage caused by the microbial challenge. These results suggested that the involvement of MdTCP-1\(_f\) in the innate immune system could be particularly against microbial infection.

In conclusion, MdTCP-1\(_f\) gene cloned from *M. domestica* encoded a protein with characteristic features of a TCP-1 protein. MdTCP-1\(_f\) mRNA was lower at all developmental stages except for eggs. In the housefly larval tissues, MdTCP-1\(_f\) mRNA constitutively expressed but showed higher expression in the salivary glands and trachea. When *M. domestica* larvae were challenged with microbes or when they received a short-term heat shock, MdTCP-1\(_f\) mRNA expression was higher. This suggested that MdTCP-1\(_f\) may be involved in the immune defense, and thus dedicated contribute to the understanding of the TCP-1 protein in *M. domestica*.

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