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Full Length Research Paper

Cloning and sequence analysis of translocase of inner mitochondrial membrane 10 homolog (yeast) gene (*TIMM10*) from the giant panda

Yi-ling Hou[1], Yu-jie Du[1, 2], Wan-ru Hou[1*], Cai-quan Zhou[1], Yan-zhe Hao[1] and Tian Zhang[1]

[1]College of Life Science, China West Normal University, 44# Yuying Road, 637002, Nanchong, China.
[2]Zhangjiang Education College, 8# Cunjinsiheng Road, 524048, Zhangjiang, China.

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TIMM10 is a member of the family of small Tim proteins, which is an important translocase located in inner mitochondrial membrane. It is a significant and interesting work to study it. The cDNA of *TIMM10* was cloned successfully for the first time from the Giant Panda (*Ailuropoda melanoleuca*) using RT-PCR technology, which was also sequenced and analyzed preliminarily. The cDNA fragment cloned is 307 bp in size, containing an open reading frame of 273 bp encoding 90 amino acids. Alignment analysis indicated that the nucleotide sequence and the deduced amino acid sequence are highly conserved to other four species studied, including *Homo sapiens*, *Mus musculus*, *Rattus norvegicus* and *Bos taurus*. The homologies for nucleotide sequences of Giant Panda *TIMM10* to that of these species are 94.51, 91.94, 91.21 and 93.77%, respectively, while all the homologies for amino acid sequences are 98.89%. Topology prediction showed there is one cAMP- and cGMP-dependent protein kinase phosphorylation site, two Casein kinase II phosphorylation sites and one Amidation site in the TIMM10 protein of the Giant Panda (*A. melanoleuca*).

Key words: Cloning, sequence analysis, *TIMM10,* the giant panda (*Ailuropoda melanoleuca*).

INTRODUCTION

Import and insertion of hydrophobic membrane proteins into the mitochondrial inner membrane occurs by coordinated actions of preprotein translocases in the outer and inner membranes. The family of small Tim (translocase of inner membrane) proteins belongs to these translocases in the inner membranes, which are located in the intermembrane space of mammalian mitochondria (Bauer et al., 2000; Sirrenberg et al., 1998; Vial et al., 2002). In yeast, during import of metabolite carriers and other integral inner membrane proteins, members of this family, TIMM8, TIMM9, TIMM10, TIMM12, and TIMM13, act along the TIM22 import pathway (Gentle et al., 2007; Mühlenbein et al., 2004). Both TIMM10 (also known as TIM10 or TIM10A) and TIMM9 are ATP-independent chaperones of the mitochondrial intermembrane space and are components of the TIM22 import system and they coassemble into a hexamer complex, Tim9-Tim10 (Curran et al., 2002; Davis et al., 2007; Ivanova et al., 2008; Truscott et al., 2002; Vergnolle et al., 2005; Vergnolle et al., 2007; Vial et al., 2002; Webb et al., 2006), which may prevent aggregation of the unfolded carrier proteins in the aqueous intermembrane space (Curran et al., 2002). Concretely, the Tim9-Tim10 complex has dual roles in mediating the import of inner membrane proteins: like the Tim8-Tim13 complex, the Tim9-Tim10 complex functions as a putative chaperone to guide hydrophobic precursors across the outer membrane, including release from the TOM complex; like membrane-associated Tim12, the complex is the member of the Tim18-Tim22-Tim54 complex and Tim10 is tightly associated with the inner membrane mediating precursor insertion into the membrane (Murphy et al., 2001; Truscott et al., 2002; Vasiljev et al., 2004).

References displayed that Tim10 and Tim9 are themselves imported from the cytosol and organized in specific translocation assemblies in the intermembrane space. Their conformational properties and how these influence the mechanism of assembly remain poorly

*Corresponding author. E-mail: hwr168@yahoo.com.cn. Tel/Fax: +86-0817-2314311.

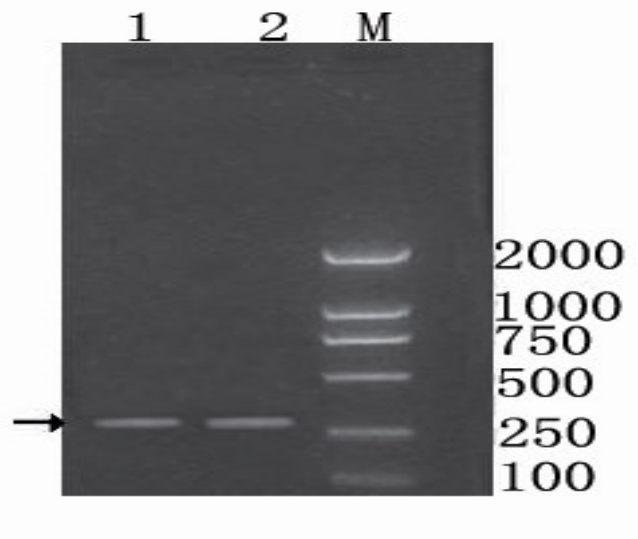

Figure 1. Reverse Transcription Polymerase Chain Reaction Products of *TIMM10* from the Giant Panda (M: Molecular ladder DL2000; 1, 2: the amplified *TIMM10*).

understood, and the three-dimensional structure of the Tim9-Tim10 complex is unknown.(Lu et al., 2004a; 2004b)

The Giant Panda (*Ailuropoda melanoleuca*) is a rare species currently found only in China. It has been declining for thousands of years due to climatic changes and hunting activities. Studies on the declining wild animal are increasingly concerned by the world community. For many years, studies on the Giant Panda have been mainly concentrated on fields such as breeding and propagation, ecology, morphology, taxology, physiology and pathological biochemistry. Recently, researches on genetic diversity, parentage and phylogenesis etc. have been developed, while reports on functional gene are handful (Du et al., 2007; Du et al., 2008; Hou et al., 2007a; 2007b; 2007c; Jennie et al., 1992; Liao et al., 2003a; 2003b; Montali, 1990; Wu et al., 1990;).

This study was conducted using RT-PCR technique to amplify the cDNA sequence of *TIMM10* gene from the total RNAs extracted from the skeleton muscle of the Giant Panda, and then analyzed the sequence characteristics of the protein encoded by the cDNA and compared it with those of human and other mammalian species reported. The study provides scientific data for inquiring into the hereditary traits of the gene from Giant Panda and formulating the protective strategy for the Giant Panda.

MATERIALS AND METHODS

Materials and RNA isolation

Skeletal muscle was collected from a dead giant panda at the Wolong Conservation Center of the Giant Panda, Sichuan, China. The collected skeletal muscle was frozen in liquid nitrogen and then used for RNA isolation.

Total RNAs were isolated from about 400 mg of muscle tissue using the Total Tissue/Cell RNA Extraction Kits (Waton Inc., Shanghai, China) according to the manufacturer's instructions. The total RNAs extracted were dissolved in DEPC (diethypyrocarbonate) water, and kept at -70^{o}C. Primers Design, RT-PCR, Cloning of RT-PCR Products and Sequencing

The PCR primers were designed by Primer Premier 5.0, based on the mRNA sequence of *TIMM10* from *Homo sapiens* (NM_012456), *Mus musculus* (NM_013899), *Rattus norvegicus* (NM_172074) and *Bos taurus* (BC111687). The specific primers of *TIMM10* are as follows:

TIMM10-F: 5′- CACGGTGCTAGGCTGAGATG-3′;
TIMM10-R: 5′-TGAGGTCCCTGTCAGTATAC-3′

Total RNAs were synthesized into the first-stranded cDNAs using a reverse transcription kit with OligodT as the primers followed by PCR amplification according to the manufacturer's instructions (Promega). After amplification, PCR products were separated by electrophoresis in 1.5% agarose gel with 1× TAE buffer, stained with ethidium bromide and visualized under UV light. The expected fragments of PCR products were harvested and purified from gel using a DNA harvesting kit (omega bio-tek, USA), and then ligated into a pUC18 vector at 16^{o}C for 12 h. The recombinant molecules were transformed into *Escherichia coli* complete cells (JM109), and then spread on the LB-plate containing 50 μg/mL ampicillin, 200 mg/mL IPTG and 20 mg/mL X-gal. Plasmid DNA was isolated and digested by *Pst*I and *Sca*II to verify the insert size. Plasmid DNA was sequenced by HuadaZhongsheng Scientific Corporation (Beijing, China).

Data analysis

The sequence data were analyzed by GenScan software (http://genes.mit.edu/GENSCAN.ht ml). Homology research of the Giant Panda *TIMM10* compared with the gene sequences of other species ware performed using Blast 2.1 (http://www.ncbi.nlm.nih.gov/blast/). ORF of the DNA sequence was searched using ORF finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Base usage bias was analyzed by MEGA3.1. Protein structure of the *TIMM10* sequence cloned was deduced using PredictProtein software (http://cubic.bioc.columbia.edu/predictprotein/).

RESULTS

A cDNA fragment of 307 bp was amplified from the Giant Panda with primers TIMM10-F and TIMM10-R (Figure 1). The *TIMM10* sequence has been submitted to Genbank (accession number: EU334856), containing the 5'-untranslated sequence in size of 17 bp and the 3'-untranslated region in size of 17 bp. An ORF of 273 bp encoding 90 amino acids was found in the cDNA (Figure 2). Blast research showed that the cDNA sequence cloned shares a high homology to the *TIMM10* from some mammals reported, including *Homo sapiens*, *Mus musculus*, *Rattus norvegicus* and *Bos taurus*. On the basis of the high identity, we concluded that we had cloned the cDNA encoding the Giant Panda TIMM10 protein.

The molecular weight of the TIMM10 protein from the Giant Panda is 10.3610 kDa and the theoretical pI is

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1     CACGGTGCTAGGCTGAGATG GAT CCA CTC AGG GCC CAG CAG CTG GCT GCG GAG CTG
   GAG
1                          M    D    P    L    R    A    Q    Q    L    A    A
   E    L    E
61   GTG GAG ATG ATG GCT GAT ATG TAC AAC AGA ATG ACC AGT GCC TGC CAT CGG AAG
15    V    E    M    M    A    D    M    Y    N    R    M    T    S    A    C    H
   R    K
121  TGC GTG CCT CCC CAC TAC AAG GAA GCA GAA CTG TCC AAG GGC GAG TCT GTG TGC
40    C    V    P    P    H    Y    K    E    A    E    L    S    K    G    E    S
   V    C
181  CTG GAC CGC TGT GTC TCC AAG TAT CTG GAC ATC CAT GAG CGG ATG GGC AAG AAG
60    L    D    R    C    V    S    K    Y    L    D    I    H    E    R    M    G
   K    K
241  TTG ACA GAG TTG TCT ATG CAG GAT GAA GAG CTG ATG AAG AGG GTG CAG CAG AGC
80    L    T    E    L    S    M    Q    D    E    E    L    M    K    R    V
   Q    Q    S
301  TCT GGG CCC GTG TGAGGTCCCTGTCAGTATAC
100   S    G    P    V    *
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Figure 2. Nucleotide Sequence of cDNA Encoding the Giant Panda *TIMM10* and the Amino Acid Sequence Deduced from Its ORF. Nucleotides are numbered in the 5'-to-3' direction. The predicted amino acid sequence of the gene is shown under the nucleotide.

Table 1. Comparison of nucleotide and amino acid sequences among 5 mammal species.

	A. melanoleuca	***H. sapiens***	***M. musculus***	***R. norvegicus***	***B. taurus***
A. melanoleuca		94.51%	91.94%	91.21%	93.77%
H. sapiens	98.89%		92.67%	92.67%	93.3%
M. musculus	98.89%	100.00%		96.34%	91.58%
R. norvegicus	98.89%	100.00%	100.00%		90.48%
B. taurus	98.89%	97.78%	97.78%	97.78%	

Note: The homology matrix of *TIMM10* encoding sequence is above the diagonal, the homology matrix of protein sequence is below the diagonal.

The protein is composed of 15 negatively charged residues (Asp + Glu), 13 positively charged residues (Arg + Lys) and 62 uncharged residues.

DISCUSSION

Analysis of the homologies for nucleotide sequences and amino acid sequences

Alignment analysis of *TIMM10* among the Giant Panda and the four mammals indicated that both the nucleotide sequence and the deduced amino acid sequence are highly conserved. There is not any deletion and insertion of nucleotide and amino acid residue. Among them, the Giant Panda shares the highest homology for nucleotide sequence from *Homo sapiens*; and the Giant Panda shares the same highest homology for amino acid sequences (Table 1).

Further analysis of base usage bias revealed that the *TIMM10* gene bias some kind of bases. Specifically, the first base of codon biases G, the average content of which is 33.0%; the second base of codon biases A, the average content of which is 37.4%; and the third base of codon obviously biases G, the average content of which is 49.2%, while the site hates A which average content is merely 11.9% (Table 2).

Statistic of base substitution indicated that substitution rate of the third site is far superior to the rate of the first and the second site, which illuminates base substitution rate of the third site is far faster than the rate of the first and the second site. Conclusions that number of base transition is much more than that of transversion can be drawn from the statistic, which shows base transition is the main way for the evolution of *TIMM10* gene (Table 2).

Prediction and analysis of protein functional sites in TIMM10 protein

Some experiments displayed that TIMM10 provides distinct binding surfaces to substrate proteins due to its conserved features, and that a defective N-terminal substrate-binding region is lethal to TIMM10; in contrast, the C-terminal part is related to the formation of the TIMM9-TIMM10 hexamer, which is beneficial but not vital for TIMM10 function (Gentle et al., 2007; Vergnolle et al., 2005; Vergnolle et al., 2007).

Table 2. Compositions and frequencies of base substitution in cDNA of *TIMM10* between *Ailuropoda melanoleuca* and some other mammals.

	The 1st site	The 2nd site	The 3rd site	All sites	The 1st and the 2nd sites
Transition (TS)	1	1	13	15	2
Transversion (TV)	1	0	3	4	1
Total of nucleotide substitution	2	1	16	19	3
Total of bases	91	91	91	273	182
Frequencies of nucleotide substitution (%)	2.20	1. 99	17.58	6.96	1.65
TS/TV	1.2	–	3.9	3.5	2.0
Average content of T/U	16.5	25.5	13.0	18.3	32.0
Average content of C	24.0	19.6	25.9	23.2	43.6
Average content of A	26.6	37.4	11.9	25.3	64.0
Average content of G	33.0	17.6	49.2	33.3	50.6

Table 3. Molecular weight and pI of TIMM10 of the Giant Panda and other four mammals.

	A. melanoleuca	*H. sapiens*	*M. musculus*	*R. norvegicus*	*B. taurus*
Molecular weight(kDa)	10.3610	10.3329	10.3329	10.3329	10.3329
pI	5.89	5.89	5.89	5.89	5.89

Topology prediction revealed there is one cAMP- and cGMP-dependent protein kinase phosphorylation site, two Casein kinase II phosphorylation sites and one Amidation site in the TIMM10 proteins of the five mammalian species. Our analysis indicated that the same number and patter functional sites are located in the same locations in TIMM10 protein of the Giant Panda, *Homo sapiens*, *R. norvegicus*, *M. musculus* and *B. Taurus* (Figure 3). Further analysis detected only two polymorphic sites in the amino acid sequences of the five species compared: the one located at the eighty-third site and the other one located at the ninetieth site, both of which result from the transversion or transition of the corresponding codons. Both polymorphic sites are located at the C-terminal and outside functional sites. The analytic results accounted well for the different functions between N-terminal and C-terminal of TIMM10.

In addition, importantly, it was reported that a striking characteristic of all small Tim proteins is the presence of a strictly conserved “twin CX3C” motif (Cys-X3- Cys) separated by 11–16 residues. It is clear that the twin CX3C is important for the structure and possibly the function of these proteins, but the specific roles of the Cys residues in complex formation, import, or substrate binding remains unresolved. The two distal CX3C motifs are juxtaposed in the folded structure and disulfide-bonded to each other rather than within each other, with an inner cysteine pair and an outer pair. Mutations of the inner Cys are severely affected and form wrong, non-native disulfides, while mutations of the outer Cys that can still maintain the native inner disulfide pair and display weaker functional defects. The facts show that the inner cysteine pair has a more prominent role. This organizing principle is most likely shared by the other members of the small Tim family that form assemblies similar to the TIMM10 complex (Allen et al., 2003; Curran et al., 2002; Lu et al., 2005; Milenkovic et al., 2007).

On the basis of numbers of research findings above, we speculate that the two CX3C motifs are respectively located in the intervals spanning acidic amino residues from 29 to 33 ($C^{29}HRKC^{33}$) and 50 to 54 ($C^{50}LDRC^{54}$) of our predicted TIMM10 protein, and the sixteen acidic amino sequence (VPPHYKEAELSKGESV) separates the “twin CX3C”. Thus, the most important sequence in TIMM10 protein for its structure and function is the sequence:

$C^{29}HRKC^{33}VPPHYKEAELSKGESVC^{50}LDRC^{54}$.

When the sequence is juxtaposed in the folded structure, the Cys^{29} of the first CX3C motif and the Cys^{54} of the second CX3C motif form the outer cysteine pair, and the Cys^{33} of the first CX3C motif and the Cys^{50} of the second CX3C motif make up the inner one. In this way, the short sequence results in a pouch-like structure where the N-terminal, C-terminal, and loop region (the 16-amino acid region between the two CX3C motifs) could be structurally separated (Allen et al., 2003).

The conserved cysteine residues in the 'twin CX3C' motif coordinate zinc and potentially generate a zinc-finger-like structure that binds to the matrix loops of the carrier proteins (Curran et al., 2002).The specific role of Zn2+-binding during the import and assembly of these

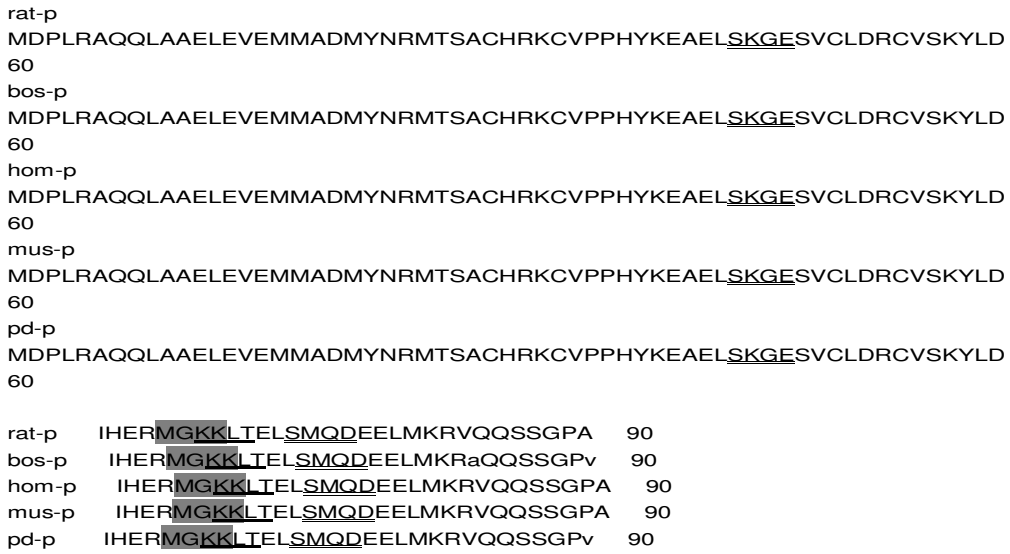

Figure 3. Functional Sites in TIMM10 Among the Giant Panda, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus* and *Bos Taurus.* rat: *Rattus norvegicus*; bos: *Bos Taurus*; homo: *Homo sapiens*; mus: *Mus musculus*; pd: the Giant panda; ___ : cAMP- and cGMP-dependent protein kinase phosphorylation site; ___ : Casein kinase II phosphorylation site; : Amidation site.

proteins is not clear, but some reseachers proposed that Zn^{2+}-binding is essential to maintain the protein in a reduced and import-competent state in the cytosol, and that zinc has to be removed after the protein is imported into mitochondria to initiate protein oxidative folding and assembly (Lu et al., 2005) . Therefor, we can conclude that it is just because the 'twin CX3C' motif is essencial for the function of TIMM10 that the protein sequences are highly conserved among different species. The conserved feature indicated the potential value of TIMM10 in phylogenetic comparison and can be used as candidate markers for phylogeny.

Prediction of the physical and chemical features of TIMM10 protein

Physical and chemical analysis showed that the molecular weight of the putative protein among the five mammalians is very close and that the theoretical pI is exactly identical (Table 3).

In Summary, the complete coding sequence of *TIMM10* gene has been cloned using RT-PCR technology successfully. This is the first report on the *TIMM10* gene from the Giant Panda. The data will not only enrich and supplement the information about *TIMM10* but also allow the isolation of the structural gene from the Giant Panda. Further research on TIMM10 protein isdesirable with a view of further inquiring into the Tim protein family. In addition, it will contribute to the protection for gene resources and the discussion of the genetic polymer-phism of this “Treasure of China”, yet endangered species.

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