Cell. Mol. Biol. 58 (Supp): OL1791-OL1796
Published on November 15, 2012
DOI 10.1170/211

Cellular & Molecular Biology
http://www.cellmolbiol.com

CLONING AND CHARACTERIZATION OF A cDNA ENCODING PROHIBITIN1 FROM *Lampetra japonica* AND ITS EXPRESSION ANALYSIS

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Abstract

To investigate that prohibitin is probably concerned in B-lymphocyte-like cells mediated signal pathways in Lamprey, a necessary and fundamental plan is firstly conducted. A full-length cDNA encoding the prohibitin1 protein was cloned from *Lampetra japonica* by EST sequence analysis in *L. japonica* leukocyte cDNA library conducted by our laboratory. Prohibitin1 contains a 828bp open reading frame, encoded 275 amino acids residues, and molecular weight is 29.9517KD, isoelectric point is 6.93, consists of 31 negatively charged amino acids residues (Asp+Glu) and 21 positively charged ones (Arg+Lys). The Prohibitin1 gene sequence from *L. japonica* is 71% identical to the ones of other 24 eukaryotic species, which shows the putative prohibitin1 gene is highly conserved. Western blotting analysis results showed the recombinant proteins were the target proteins in prokaryote. Real-time quantitative polymerase chain reaction analysis indicated that the expression of the prohibitin1 gene is significantly up-regulated in leukocyte, heart and gill of *L. japonica* by LPS stress treatment. In conclusion, we have cloned and identified the full-length cDNA of Prohibitin1 in *L. japonica* and found that it was related to adaptive immune response in lamprey for the first time.

Key words: Lamprey, prohibitin, cloning, expression, B-lymphocyte.

Article information

Received on August 8, 2012
Accepted on October 29, 2012

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INTRODUCTION

Lamprey, a living jawless vertebrate, has been regarded as one of the most primitive groups of vertebrates, which fills the gap between invertebrates and vertebrates. Recent archaeological finds indicate that modern lampreys, compared with the ancestors of 3.6 billion years ago, have little change in morphology structure. Indeed, the lamprey is living fossil (3). Thus lamprey is the preferred material for revealing the origin and evolution of vertebrates. At present, most studies have been confined to the morphology structure, ecological distribution and adaptable immunity of the lamprey (5,7,11,15,19), and other aspects of genomics and proteomics have been reported(25), whilst the aspects of functional genes and functional proteins are less known.

Prohibitins (PHB) in eukaryotes consisted of two subunits (PHB1 and PHB2) (2,12). PHB1, a protein of about 280 amino acid residues, was first discovered in mammalian cells during studies of cell senescence (17). PHB1's involvement in a fundamental cellular function is consistent with its amino acid sequence conservation between yeast and mammals, and its apparent expression in all mammalian tissues examined to date (18). PHB1 is a multifunctional and highly conserved protein associated with antiproliferative activity (8), cell cycle regulation (14,23), transmembrane signal transduction and mitochondrial structure, function, and inheritance (1,4,9,10,13,20). Recently, it was found to be over-expressed in breast cancer, gastric cancer and esophageal squamous cell carcinoma (16), and it has been suggested as a biomarker in those diseases.

However, in mouse B-lymphocytes, a small proportion of the cellular PHB and a PHB-like protein was found associated with the IgM antigen receptor (21). A twofold increase in PHB was observed when chronic lymphocytic leukemia B-lymphocytes or normal human B-lymphocytes mature to a plasmacytoid phenotype (6). These studies suggest that increased PHB expression is associated with and may facilitate B-cell maturation. And PHB was probably involved in B-lymphocyte-mediated signal transduction pathways.

Whereas there are no B-lymphocytes but B-lymphocyte-like cells in lamprey (24). Whether are there B-lymphocyte-like cell-mediated signal transduction pathways and whether is PHB expression associated with those pathways in Lamprey? Our laboratory is interested in exploring and discovering the mechanism of lymphocyte-like cell-mediated adaptive immunity in Lamprey. It is very vital and fundamental for the evolution of adaptive immunity. To investigate the connection between PHB and immune response mediated by B-lymphocyte-like cell in jawless vertebrates, a necessary plan is firstly conducted. Therefore, all these above prompted us to clone PHB cDNA from lamprey and basically analyze its expression to focus on the lymphocyte-like cell-mediated adaptive immunity participated by PHB in lamprey in the future.

MATERIALS AND METHODS

Materials and Stress Treatment

L. japonica was harvested at Tongjiang region of Songhua River in Heilongjiang province in December 2009. They were breed at 2-5°C in a fish globe. To investigate the expression of Prohibitin1 gene in different tissues under LPS stress condition, after a week 10 healthy individuals, 35-55 cm length, were immune by each injecting 100ul 100 ug/ml composite antigen into the abdominal cavity twice a week, lasting 2 weeks, and the blood was got by cutting off the tail at the third day after the last injection. Physiological saline treatment was used as the control.

EST analysis

For getting EST sequence data involved in PHB gene, the BLAST sequence analysis was performed for 2,123

valuable ESTs in *L. japonica* leukocytes cDNA library conducted by our laboratory. Then by Tblastx screening the NCBI library we searched the ESTs associated with the CDS region of PHB gene and by sequencher4.2 the ESTs were linked. The linked CDS region of PHB gene was translated and compared with the amino acid sequences of known PHB gene CDS regions of other species by GENEDOC3.0.

cDNA synthesis

Leucocytes were isolated by the Ficoll gradient density centrifugation method (6). Total RNA was isolated by the Trizol of TaRaKa from the leucocytes. Reverse transcription was carried out by TaKaRa 3'-Full RACE Core Set Ver.2.0.

3'-RACE

The 3'end of PHB gene was amplified by RACE-polymerase chain reaction (PCR). The sequences of the forward (F) and reverse (R) primers for 3'-RACE used in the PCR reaction were F1: 5'-CTGCTGCTAAATTCGAACGC-3', R1: 5'-TGCTCCGCCCTTTCCACGAC-3'; F2: 5'-GCGGCCGCTGCCAGGATCCT-3', R2: 5'-CT GTGCTGGGAGCTGGAGC-3'. Outer-PCR was carried out using 150ng each of 3'-RACE Outer Primer and F1 Primer, along with 200μM of each dNTPs and 1.25 U TaKaRa LA Taq DNA polymerase with Reverse transcriptional cDNA as template in a 50μl reaction. PCR conditions were 94°C/30 sec, 55°C/30 sec and 72°C/2 min for 30 cycles. Inner-PCR was carried out using 150 ng each of F1 and R1 Primers, along with 2.5 mM of each dNTPs and 2.5 U TaKaRa LA Taq DNA polymerase with 1 μl Outer-PCR reaction product as template in a 50μl reaction. PCR conditions were 94°C/30 sec, 55°C/30 sec and 72°C/2 min for 30 cycles. The amplification product was purified by 3% gel electrophoresis.

Cloning of CDS region

Cloning of CDS region of PHB gene was carried out by PCR using 150 ng each of F2 and R2 Primers, along with 2.5 mM of each dNTPs and 2.5U TaKaRa LA Taq DNA polymerase with 1 μl 3'RACE-PCR reaction product as template in a 50 μl reaction. PCR conditions were 94°C/30 sec, 55°C/30 sec and 72°C/2 min for 30 cycles. The amplification product was purified by 3% agarose gel electrophoresis and taken back by Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa). The resultant clone was sequenced, screened in NCBI library and found the full-length ORF.

Sequence analysis

Similarity searches for nucleotides and deduced amino acids were conducted using the NCBI BLAST network server (http://www.ncbi.nlm.gov/BLAST). The alignment of the amino acid sequences of PHB with the known ones from 24 species (yeast, mouse, man, et al.) and A. thaliana was carried out using DNAMAN software. And the conserved motifs of them were analyzed using MEME online software. The phylogenetic tree at amino acid level was also drawn using DNAMAN based on the sequence alignment. Signal peptides were predicted with the program SignalP (http://www.cbs.dtu.dk/services/SignalP/). Its isoelectric point (pI) and molecular weight (MW) were analyzed by pI/MW program (available on the ExPaSy Web site at http://www.expasy.org/).

Expression of Prohibitin1 in prokaryotic cells and Western blot

The cDNA was introduced into a bacterial expression vector (pET32a, TaRaKa) and transformed into *E.coli.* BL21 (TaRaKa). Its expression was induced by 1mmol/L IPTG. After the identification by 12% SDS-PAGE, the recombinant protein was detected by Western blot.

*E.coli.*BL21 cells (OD_{600nm}0.4-0.6) were harvested with 1ml ice-cold RIPA lysis buffer and incubated for 1hr at 4°C. The insoluble material was removed by centrifugation at 10,000×g for 10 min at 4°C. Protein content was determined by Folin-phenol method with BSA as a standard substance. The proteins were then placed in diluted SDS sample buffer and denatured for 5 min at 99°C before being subjected to 12% SDS-PAGE. Forty micrograms of total protein was loaded in each lane, subjected to electrophoresis, and subsequently transferred to PVDF membranes (Millipore Corp., Bedford, MA, USA) by electroblotting. The membranes were blocked in TBS buffer (25mM Tris–HCl, 150mM NaCl, pH7.5) containing 0.05% Tween-20 and 5%milk and incubated with a mouse monoclonal antibody against rat PHB (1:3,000) (Bio Basic, Canada), α-tubulin (Boster, China), CoxII (Invitrogen, USA), and cytochrome c (Santa Cruz, USA) in blocking solution. Membranes were washed in TBS buffer containing 0.05% Tween-20. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) reagents (Bio Basic) using a goat peroxidase-conjugated secondary antibody (1:5,000) (Bio Basic). Results of representative chemiluminescence were scanned and densitometrically analyzed using ImageMaster VDS system (Amersham, UK) with the help of the Imagequant TL site program.

Real-Time PCR

The first-strand cDNA was synthesized from 2 μg total RNA using the RevertAid First Strand cDNA Synthesis Kit (TaKaRa, Japan) and oligo (dT) primer. Real-time PCR experiments were performed using Real-Time PCR Master Mix (AppliedBiosystems, USA) and StepOne Real-Time System (AppliedBiosystems, USA) according to the manufacturer's protocols. GAPDH expression was used as an internal standard. Three biological replications were analyzed for each point. PCR was performed using the following primer sets: PHB1, F:5'-TCGGCGAAGGCACTCACT-3', R:5'-GAGGTCGAGAACGGCAGTCA-3'; GAPDH, F:5'-GTGCAAAGCACGTCATCATCTC-3', R:5'-GGGTCGTACTTCTTGTGGTTCAC-3'.

RESULTS

Cloning and Sequence Analysis of PHB1

21 EST sequence data involved in PHB gene were got by the sequence analysis for 2,123 valuable ESTs in *L. japonica* leukocytes cDNA library conducted by our laboratory. While Tblastx screening the NCBI library for the 21 ESTs we have got 5 ESTs associated with CDS region of PHB gene. By sequencher 4.2 the 5 ESTs were linked but when compared with the amino acid sequences of known PHB genes of other species by GENEDOC 3.0, we found this CDS sequence was partial, the missing 3'end of about 50 amino acid residues sequences.

According to the information from cDNA library and the primary analysis of ESTs, an 828 bp full-length cDNA of PHB was successively obtained from the leukocytes of *L.*

japonica (Fig. 1).

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1 ATGGCGGCCGCTGCCAGGATCCTGAACACGGTGGGGAAAGTCGGCCTCGGTCTTGCGGTG
1   M   A   A   A   R   I   L   N   T   V   G   K   V   G   L   G   L   A   V
61 GCCGGAGGAATCGTCAACACGGCTCTGTACAACGTGGAGGGAGGTCATCGTGCGGTCATT
21   A   G   G   I   V   N   T   A   L   Y   N   V   E   G   G   H   R   A   V   I
121 TTCGACCGATTCCGTGGAGTGCAAGATTTGGTGGTCGGCGAAGGCACTCACTTTGTCATC
41   F   D   R   F   R   G   V   Q   D   L   V   V   G   E   G   T   H   F   V   I
181 CCCTGGGTTCAGCGGCCGATCATATTTGACTGCCGTTCTCGACCTCGCAATGTGCCTGTC
61   P   W   V   Q   R   P   I   I   F   D   C   R   S   R   P   R   N   V   P   V
241 GTGACTGGTAGCAAAGATATGCAGAATGTCAACATCACGTTGAGGATCTTGTTCAGGCCC
81   V   T   G   S   K   D   M   Q   N   V   N   I   T   L   R   I   L   F   R   P
301 ATCGCTAGCCAGCTGCCCAAAATCTACATGAGCATTGGTGAGGACTATGATGAACGTGTG
101   I   A   S   Q   L   P   K   I   Y   M   S   I   G   E   D   Y   D   E   R   V
361 CTGCCTTCGATCACCACTGAGGTCCTCAAGGCTGTTGTGGCTCGGTTTGATGCTGGAGAG
121   L   P   S   I   T   T   E   V   L   K   A   V   V   A   R   F   D   A   G   E
421 CTGATCACCCAACGAGAGCTGGTGTCCAGGCAGGTCAGCAATGACTTGATGGAGAGAGCA
141   L   I   T   Q   R   E   L   V   S   R   Q   V   S   N   D   L   M   E   R   A
481 GCCACATTTGGGCTCATCTTGGATGATGTCTCCCTGACTCATCTGACCTTTGGTAGAGAG
161   A   T   F   G   L   I   L   D   D   V   S   L   T   H   L   T   F   G   R   E
541 TTCACACAGGCTGTTGAGGCCAAGCAAGTAGCACAGCAGGAGGCGGAGAGAGCACGCTTC
181   F   T   Q   A   V   E   A   K   Q   V   A   Q   Q   E   A   E   R   A   R   F
601 GTCGTGGAAAGGGCGGAGCAGCAAAAGAAGGCGGCCATCATTGCAGCCGAGGGTGATGCC
201   V   V   E   R   A   E   Q   Q   K   K   A   A   I   I   A   A   E   G   D   A
661 AAGGCTGCACAGCTAATCGCAACAGCGATATCCGAGGCTGGAGAGGGCCTGGTTGAGCTG
221   K   A   A   Q   L   I   A   T   A   I   S   E   A   G   E   G   L   V   E   L
721 CGCAAGCTGGAGGCGGCAGAAGATATCGCCATGCAGCTGTCGAAGTCCCGAGGTGTAGCC
241   R   K   L   E   A   A   E   D   I   A   M   Q   L   S   K   S   R   G   V   A
781 TACCTGCCCTCGGGGCAGCACACGCTGCTCCAGCTCCCAGCACAGTAA
261   Y   L   P   S   G   Q   H   T   L   L   Q   L   P   A   Q   -
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Figure 1. The full-length ORF nucleotide sequence of PHB1 gene. The amino acid sequence is shown in the single letter code below each nucleotide codon. Nucleotide and amino acid numbers are indicated at the left.

Bioinformation Analysis of Amino Acid Sequence Deduced from PHB1

The PHB cDNA encoded 275 amino acids residues. The online homologous search showed that the cDNA of PHB was PHB1 in the known PHB family. As shown in the alignment, amino acid sequences of PHB1 from 24 species were identified consistence to 71% by sequence comparison (Fig. 2). And the result of motifs analysis showed that PHB1 was highly conversed except the motif of 9 was got during the evolution of the gene (Fig. 3). The phylogenetic tree revealed that PHB1 of *L. japonica* had significant homology with ones of other species and the evolution of the gene advanced nearly with Species Evolutionary (Fig. 4). The theoretical molecular mass of the encoded protein was 29.9517KD with a calculated pI of 6.93. The online prediction showed that phosphorylation sites were Serine (3), Threonine (3), Tyrosine (0), no glycosylation sites, a transmembrane structural domain lied in the N-terminal sequence of Prohibitin1, the secondary structure of Prohibitin1 contained the conformation of 47.27% α-helix, 14.18% β-sheet, 38.55% random coil and PHB1 in *L. japonica* was located in cell nucleus, basic cytoplasm and mitochondria which was in conformity with PHB family distribution of other species.

Expression of PHB1 in prokaryote

The cDNA of PHB1 was directionally cloned into the pET32a and transformed into *E. coli*.BL21. SDS-PAGE

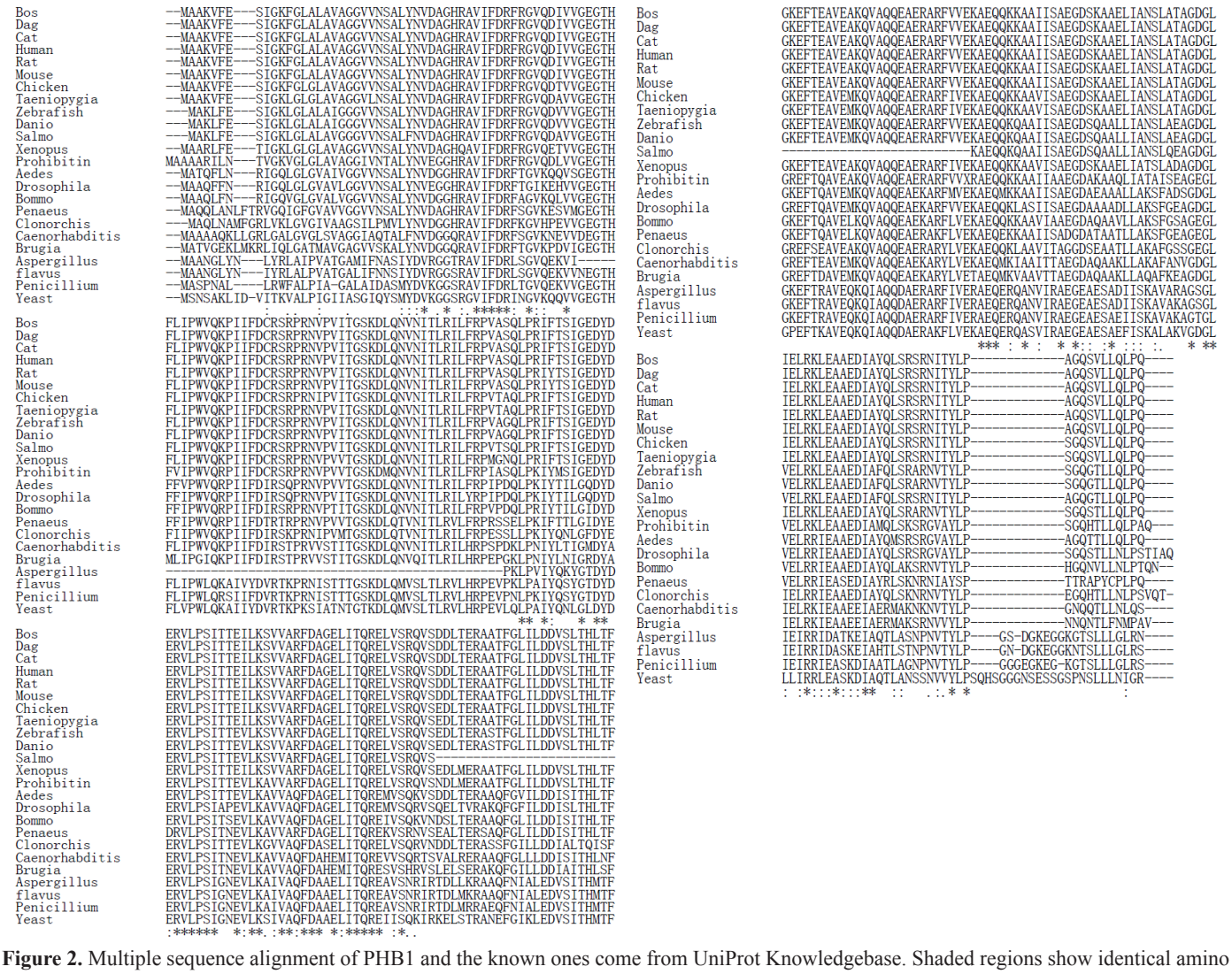

Figure 2. Multiple sequence alignment of PHB1 and the known ones come from UniProt Knowledgebase. Shaded regions show identical amino acids. Conserved amino acids were shown with stars.

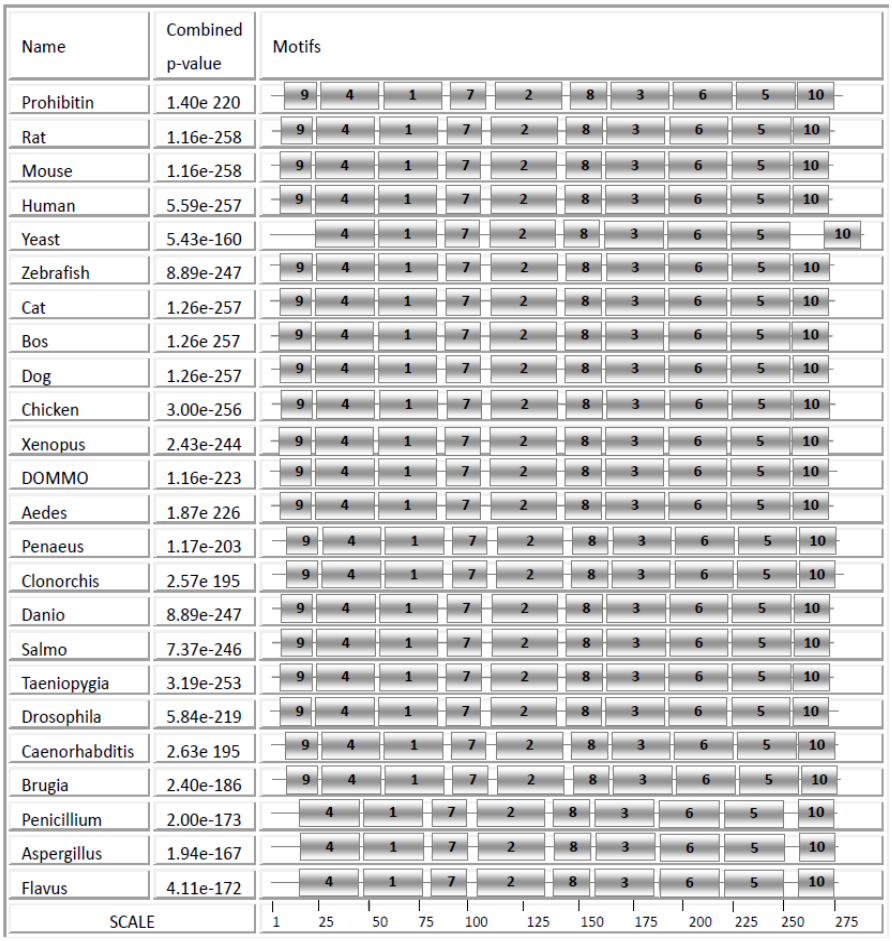

Figure 3. Conserved motifs analysis of PHB1 using MEME online software.

Table 1. Conserved amino acid sequences of PHB1 using MEME online software.

Number of motif	Length (amino acids)	Most probable sequences
1	30	HFLIPWVQKPIIFDCRSRPRNVPVITGSKD
2	30	DYDERVLPSITTEVLKSVVARFDAGELITQ
3	29	SLTHLTFGKEFTEAVEMKQVAQQEAERARF
4	30	SALYNVDAGHRAVIFDRFRGVQDIVVGEGT
5	30	GDGLIELRKLEAAEDIAYQLSRSRNITYLP
6	30	VEKAEQQKKAAIISAEGDSKAAELIANSLA
7	21	NVNITLRILFRPVPDQLPRIY
8	21	SRQVSDDLTERAATFGLILDD
9	21	KVFESIGKFGLGLAVAGGVVN
10	10	SGQSTLLQLPQ

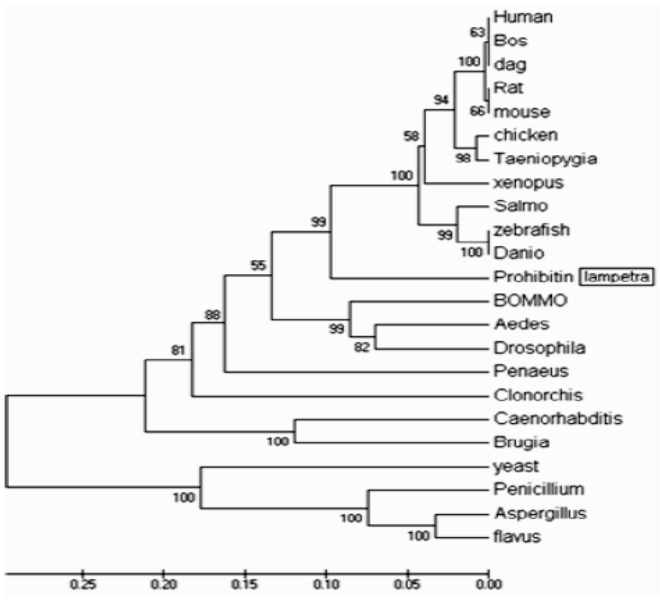

Figure 4. Phylogenetic tree of amino acid sequences of PHB1 is based on the NJ method. The numbers on the branches indicate bootstrap values.

results showed that the recombinant proteins expression was induced by IPTG at 37°C, and the expression content was increased with the extended time from 3hr to 5hr (Fig. 5A). Fig. 5B showed that mouse monoclonal antibody against rat PHB made specific binding with the recombinant proteins. Western blotting analysis results showed the recombinant proteins were the target proteins.

Expression Levels of PHB1 in various tissues

The expression of the PHB1 gene in various tissues after LPS stress treatments was measured by real-time quantitative PCR analysis. Compared with the physiology saline treatment, after 2-week LPS stimulation, PHB1 mRNA contents have a more significant rise in leukocytes, heart and gill of *L. japonica* (Fig. 6). PHB1 gene transcription was strongly up-regulated by LPS. The expression of PHB1 in muscle and intestine increased slightly, but in liver and kidney the expression decreased slightly.

This experiment indicated that PHB1 gene is inducible and can be significantly up-regulated in leukocyte, heart

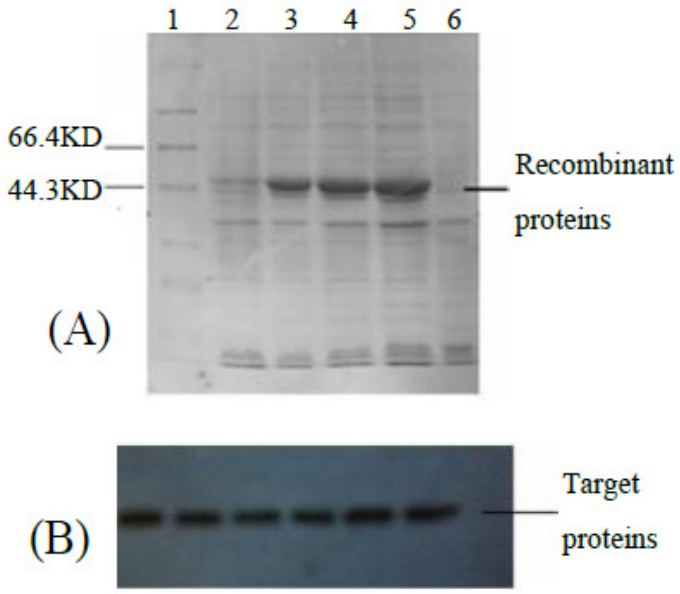

Figure 5. Expression of PHB1 in prokaryote (A) SDS-PAGE analysis results of expression induced by IPTG at 37°C. 1. Protein MW Marker (Low) (TaKaR); 2. Un-induction; 3.3hr; 4. 4hr; 5. 5hr; 6. PET-32a plasmid. (B) Western blotting analysis results.

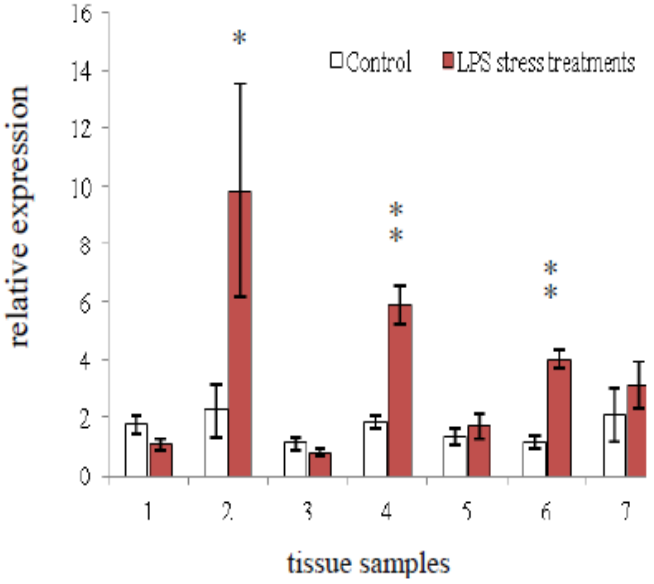

Figure 6. Relative expression of PHB1 in various tissues of L. japonica after 2-week LPS stimulation. 1. Liver, 2. Leukocytes, 3. Kidney, 4. Heart, 5. Intestine, 6. Gill, 7. Muscle. * is $0.01<p<0.05$ and ** is $p<0.01$.

and gill with LPS stimulation.

DISCUSSION

Lampreys occupy a critical phylogenetic position in understanding the origin and evolution of the adaptive immune system. In the present study, we have cloned a full-length cDNA of PHB1 gene in *L. japonica*. Sequence analysis illustrated that the PHB1 shared high homology with other advanced vertebrates. Further identified results showed that the gene coded 275 amino acid residues containing conserved domains from lamprey, salmon, zebrafish, frog, chicken, mouse to human. Real-time PCR assays indicated that mRNA expression of PHB1 was not significantly different in liver, leukocytes, kidney, heart, intestine, gill and muscle. Moreover, after stimulation with LPS, the expression level of PHB1 was obviously increased in leucocytes ($p<0.05$), heart and gill ($p<0.01$). It implied that PHB1 might involve in immune response in *L. japonica*.

PHB was associated with IgM and its expression was increased during maturation in B cells of mammals (6,21). These observations suggested that PHB could have a role in the internalization of the IgM-BCR. Lymphocyte differentiation analysis revealed that there was no evident differentiation of T and B lymphocytes in the leucocytes of *L. japonica* (6). So far it was not also established that there were B-lymphocyte-like cell receptor pathways in Lamprey, the groups being in very important evolution status. Some data suggested the possibility of existing BCR pathways like mammals and PHB playing a role in the pathways in lamprey (22,24). At the same time it was speculated the heart of lamprey might be not only a blood circulation organ but also a vital immune organ. However, it needs to be fully established in times to come that hearts can play a vital immune-regulation role in lower vertebrates. Lamprey, as one of the most primitive vertebrates, represents the many morphological and physiological characters of ancient vertebrates. The knowledge of gene function of those primitive animals will definitely help us to track the road of vertebrate complexity from fish to mammals.

In conclusion, we have cloned and identified the full-length cDNA of PHB1 in *L. japonica* and found that it was related to adaptive immune response in lamprey for the first time. The finding suggests that PHB1 probably is involved in adaptive immunity mediated by B-lymphocyte-like cell in lamprey.

ACKNOWLEDGMENT

This project was supported by the Doctor-Specialized Science Research Fund of the Education Ministry of China under Grant No. 20102136120002 and the Science and Technology Fund of Dalian, P.R.China under Grant No. 2010J21DW018.

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