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Molecular Cloning of a LIM Protein cDNA from the Mulberry Longicorn Beetle, *Apriona germari*

Zhongzheng Gui, Yadong Wei, Hyung Joo Yoon[1], Iksoo Kim[1], Xijie Guo[2], Byung Rae Jin and Hung Dae Sohn*

College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Korea.

[1]Department of Agricultural Biology, National Institute of Agricultural science & Technology, Suwon 441-100, Korea.

[2]Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, China.

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Here we report the molecular cloning of a LIM protein cDNA of the CRP (cysteine-rich protein) family from the mulberry longicorn beetle, *Apriona geramri*. The *A. germari* LIM protein cDNA contains an open reading frame of 276 bp encoding 92 amino acid residues with a calculated molecular weight of approximately 10 kDa. The *A. germari* LIM protein contains the cysteine-rich consensus sequence of LIM domain and the glycine-rich consensus sequence observed in cysteine-rich protein family 1 (CRP1). The potential nuclear targeting signal is retained. The deduced amino acid sequence of the *A. germari* LIM protein cDNA showed 81% identity to both *Bombyx mori* muscle LIM protein (Mlp) and *Drosophila melanogaster* Mlp60A and 77% to *Epiblema scudderiana* Mlp. Northern blot analysis showed that *A. germari* LIM protein is highly expressed in epidermis and muscle, and less strongly in midgut, but not in the fat body.

Key words: *Apriona germari*, cDNA cloning, LIM protein, Mulberry longicorn beetle

Introduction

Myogenesis involves a series of discrete processes beginning with specification and proliferation of the mesoderm, subdivision of functionally distinct muscle differentiation. In muscle development, myoblasts function as muscle stem cells and initially provide a pool of renewable cells.

*To whom correspondence should be addressed.
College of Natural Resources and Life Science, Dong-A University, Busan 604-714, Korea. Tel: +82-51-200-7553; Fax: +82-51-200-7594; E-mail: hdsohn@daunet.donga.ac.kr

The LIM domain defines a zinc-binding motif present in single or multiple copies in a wide variety of eukaryotic proteins that regulate cell growth and differentiation during development (Sadler *et al.*, 1992; Sanchez-Garcia and Rabbitts, 1994; Dawid *et al.*, 1995). The LIM motif was first identified in three developmentally regulated transcription factors, *Caenorhabditis elegans* Lin-11, rat Isl-1, and *C. elegans* Mec-3, from which the name LIM was derived (Way and Chalfie, 1988; Freyd *et al.*, 1990; Karlsson *et al.*, 1990). The LIM motif is defined by a cysteine-rich consensus sequence (Freyd *et al.*, 1990; Karlsson *et al.*, 1990; Sadler *et al.*, 1992) and LIM domain proteins are divided into three evolutionarily conserved members of the CRP (cysteine-rich protein) family, CRP1, CRP2, and MLP (muscle LIM protein)/CRP3 (Liebhaber *et al.*, 1990; Sadler *et al.*, 1992; Weiskirchen and Bister, 1993; Arber *et al.*, 1994; Crawford *et al.*, 1994; Weiskirchen *et al.*, 1995). Members of CRP family of LIM proteins have been implicated in muscle differentiation (Arber *et al.*, 1994).

In insect, LIM domain was well descrbed in *Drosophila melanogaster* (Stronach *et al.*, 1996, 1999; Wheeler and Hynes, 2001). Two muscle-specific LIM proteins of the CRP family in *D. melanogaster*, referred to as muscle LIM proteins (Mlp) were identified and characterized: *Mlp60* encodes a protein with a single LIM domain linked to a glycine-rich region and *Mlp84B* with five tandem LIM-glycine modules (Stronach *et al.*, 1996). In *Drosophila*, the temporal expression and spatial distribution of muscle LIM proteins are consistent with a role for Mlps in myogenesis, late in the differentiation pathway (Stronach *et al.*, 1996). Muscle LIM proteins are associated with muscle sarcomers and require dMEF2 (myocyte enhancer-binding 2 protein) for their expression during *Drosophila* myogenesis (Stronach *et al.*, 1999). In the goldenrod gall moth, *Epiblema scudderiana* (Lepidoptera,

Olethreutidae), furthermore, cold up-regulation of *E. scudderiana Mlp* and the pattern of *E. scudderiana* Mlp levels in the larvae suggest possible roles for the protein, such as in muscle maintenance over the winter or as a preparative function that could facilitate the rapid resumption of development and metamorphosis when environmental temperatures rise in the spring (Bilgen *et al.*, 2001).

In insects, LIM protein genes have been isolated from only two ordinal species such as Diptera, *D. melanogaster* (Stronach *et al.*, 1996), and Lepidoptera, *E. scudderiana* (Bilgen *et al.*, 2001) and *Bombyx mori* (Hwang *et al.*, 2003). The purpose of the present study was to elucidate the LIM protein gene in the mulberry longicorn beetle, *Apriona germari* (Coleoptera: Cerambycidae). In this paper, we report the cDNA cloning and mRNA expression of the *A. germari* LIM protein gene in coleopteran insect for the first time.

Materials and Methods

Insects

The larvae of the mulberry longicorn beetle, *Apriona germari* (Coleoptera: Cerambycidae), were reared on an artificial diet as described previously (Yoon and Mah, 1999).

cDNA library screening, nucleotide sequencing and data analysis

A cDNA library (Kim *et al.*, 2001) constructed using whole bodies of *A. germari* larvae was used in this study. The clones harboring cDNA inserts were randomly selected and sequenced to generate the expressed sequence tags (ESTs) (Kim *et al.*, 2003). The plasmid DNA was extracted by Wizard mini-preparation kit (Promega, Madison, WI). The nucleotide sequence was determined by using a BigDyeTerminator cycle sequencing kit and an automated DNA sequencer (model 310 Genetic Analyzer; Perkin-Elmer Applied Biosystems, Foster City, CA). The sequences were compared using the DNASIS and BLAST programs provided by the NCBI (http://www.ncbi.nlm.nih.gov/BLAST). GenBank, EMBL and SwissProt databases were searched for sequence homology using a BLAST algorithm program. MacVector (ver. 6.5, Oxford Molecular Ltd) was used to align the amino acid sequences of LIM protein. With the four GenBank-registered LIM protein amino acid sequences, phylogenetic analysis was performed using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 (Swofford, 2000). The accession numbers of the sequences in the GenBank are as follows: *Apriona germari* (this study), *Bombyx mori* (BmMlp; AY461436), *Drosophila melanogaster* (DmMlp60A; X91244), *Epiblema scudderiana* (EsMlp; AF206698), and *D. melanogaster* (DmMlp84B; X91245).

RNA isolation and Northern blot analysis

The *A. germari* larva was dissected under the Stereomicroscope (Zeiss, Jena, Germany), individual samples such as midgut, fat body, epidermis, and muscle were harvested, and washed twice with PBS (140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Total RNA was isolated from the midgut, fat body, epidermis, and muscle of *A. germari* larva by using the Total RNA Extraction Kit (Promega). Total RNA (10 μg/lane) from *A. germari* was denatured by glyoxalation (McMaster and Carmicharl, 1977), transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany) and hybridized at 42°C with a probe in a hybridization buffer containing 5 × SSC, 5 × Denhardts solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA. The 618 bp LIM protein cDNA clone was labeled with [α-^{32}P] dCTP (Amersham, Arlington Heights, IL) using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA) for use as a probe for hybridization. After hybridization, the membrane filter was washed three times for 30 min each in 0.1% SDS and 0.2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C and exposed to autoradiography film.

Results and Discussion

cDNA cloning, sequencing and phylogenetic analysis of A. germari LIM protein

In a search of A. germari ESTs (expressed sequence tags), we identified a cDNA showing high homology with pre-

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-77                                                  GGTGGTTGTAGTGAGGA
-60  TAACAAGGGGCCTATTCTCCACCACTTTCGATTCCGAGAAGATCGACCAACTAAATCAAA
  1  ATGCCTTTCAAACCAGTCGAAAACCCAAAATGCCCCAAATGCGGTAAATCAGTATATGCT
  1  M  P  F  K  P  V  E  N  P  K  C  P  K  C  G  K  S  V  Y  A

 61  GCTGAAGAACGTGTGGCTGGAGGTTACAAATTCCACAAATCATGCTTTAAATGCGGACTT
 21  A  E  E  R  V  A  G  G  Y  K  F  H  K  S  C  F  K  C  G  L

121  TGCGGTAAAATGTTAGATTCTACCAATGTAACTGAACACGAAGCTGAACTTTATTGCAAA
 41  C  G  K  M  L  D  S  T  N  V  T  E  H  E  A  E  L  Y  C  K

181  AACTGCCACGCCCGTAAATATGGACCAAAAGGTTACGGTTTTGGCGGTGGTGCTGGTTGC
 61  N  C  H  A  R  K  Y  G  P  K  G  Y  G  F  G  G  G  A  G  C

241  CTTTCCATGGACACCGGTTCCCATCTTCAAGGCAACTAAGAAGGAATTACCAAGAGAGCG
 81  L  S  M  D  T  G  S  H  L  Q  G  N  *

301  GCCCAAAACATCAGCAACAGTGATGGTGTCGTCAAATCTCTACTTTTCCATCCCACTACA
361  GTAATGTGTAATATTTTATAGATTTTATTATCAAATAGAATTAATCTTTATAAATTTTCA
421  TTTTACATGGTAAAATCATTCGATTATTTTGTAATAATTGAATGAATAAACTAAATTTAA
481  GAAAAAAAAAAAAAAAAA
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Fig. 1. The nucleotide and deduced amino acid sequences of the *A. germari* LIM protein cDNA. The start codon of ATG is boxed and the termination codon is shown by asterisk. In the cDNA sequence, the polyadenylation sequence AATAAA is underlined. This cDNA sequence has been deposited in GenBank under accession number AY703842.

viously reported LIM protein genes. The cDNA clone including the full-length open reading frame (ORF) was sequenced and characterized. The nucleotide and its deduced amino acid sequences of the cDNA encoding LIM protein are presented in Fig. 1. The entire length of A. germari LIM protein cDNA is 575 bp containing a complete 276 bp ORF that encodes a polypeptide of 92 amino acid residues with a predicted molecular weight of about 10 kDa.

The alignment of the deduced protein sequence of *A. germari* LIM protein gene with available insect LIM protein sequences is shown in Fig. 2. The alignment result indicates that *A. germari* LIM protein sequence is closely related to *Bombyx mori* LIM protein (BmLIM), *Drosophila melanogaster* muscle LIM protein, DmMlp60A and DmMlp84B, and *Epiblema scudderiana* LIM protein (EsLIM). The deduced protein sequence of *A. germari* LIM protein cDNA is comprised of a single LIM domain linked to a glycine-rich sonsensus sequence [GPKG(F/Y)G(F/Y)GXGAG] observed in CRP1 (Stronach *et al.*, 1996). As described previously in vertebrate CRPs, the *A. germari* LIM domain exhibits the cysteine-rich consensus sequence [$CX_2CX_{17}HX_2CX_2CX_2CX_{17}CX_2C$] that contains a pair of zinc-finger-like structures (Freyd *et al.*, 1990; Karlsson *et al.*, 1990; Sadler *et al.*, 1992; Arber *et al.*, 1994; Michelson *et al.*, 1993). In addition, the potential nuclear targeting signal in *A. germari* LIM protein is retained with one conservative lysine to arginine substitution. Among the insect CRPs, *A. germari* LIM protein bears the highest resemblance to the *Drosphila* muscle LIM protein Mlp60A (Stronach *et al.*, 1996). These similarities led us to unclude that *A. germari* LIM protein cDNA in this

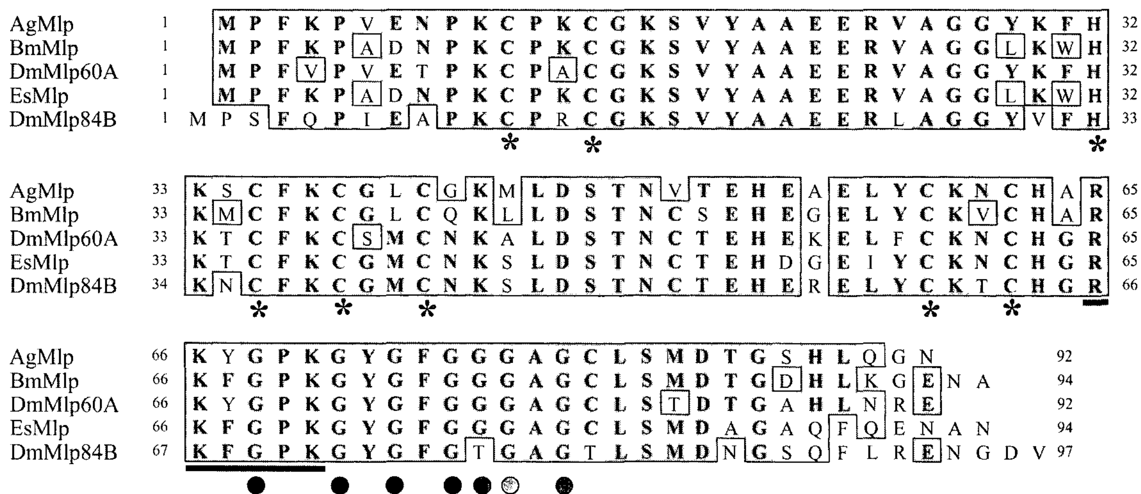

Fig. 2. Alignment of the amino acid sequence of *A. germari* LIM protein with known insect LIM proteins. Residues are numbered according to the aligned insect profilin sequences, and invariant residues are shaded black. The conserved cysteine and histidine residues common to the LIM domain are shown by asterisk. Glycine residues in the glycine-rich region that follows the LIM domain are shown by solid circle. The putative nuclear targeting signal is underlined. The insect LIM protein sequences were taken from the following sources: *Apriona germari* (this study), *Bombyx mori* (BmMlp; AY461436), *Drosophila melanogaster* (DmMlp60A; X91244), *Epiblema scudderiana* (EsMlp; AF206698), and *D. melanogaster* (DmMlp84B; X91245).

Species	GenBank No.	1	2	3	4	5
		Percent similarity				
1. AgMlp	This study		86	88	87	80
2. BmMlp	AY461436	81		81	88	78
3. DmMlp60A	X91244	81	74		85	84
4. EsMlp	AF206698	77	80	75		82
5. DmMlp84B	X91245	69	68	71	71	
		Percent identity				

Fig. 3. Pairwise identities and similarities of the deduced amino acid sequence of *A. germari* LIM protein among insect LIM protein sequences. The insect LIM protein sequences were taken from the following sources: *A. germari* (this study), *B. mori* (BmMlp; AY461436), *D. melanogaster* (DmMlp60A; X91244), *E. scudderiana* (EsMlp; AF206698), and *D. melanogaster* (DmMlp84B; X91245).

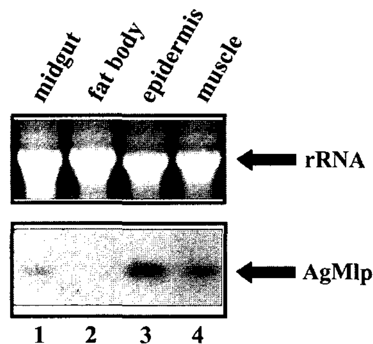

Fig. 4. Northern blot analysis of A. germari LIM protein. Total RNA was isolated from the midgut (lane 1), fat body (lane 2), epidermis (lane 3), and muscle (lane 4) of A. germari larva, respectively. The RNA was separated by 1.0% formaldehyde agarose gel electrophoresis (upper panel), transferred on to a nylon membrane, and hybridized with radiolabelled 618 bp A. germari LIM protein cDNA (lower panel).

study encodes a putative LIM protein of the CRP family.

Fig. 3 also shows the similarity and identity of the deduced protein sequence of *A. germari* LIM protein gene with those of insect LIM protein sequences. The *A. germari* LIM protein sequence was most identical to both *B. mori* Mlp (81% protein sequence identity) and *D. melanogaster* Mlp60A (81% protein sequence identity), and 77% protein sequence identity to the *E. scudderiana* Mlp.

mRNA expression of A. germari LIM protein

To confirm the expression of *A. germari* LIM gene at transcriptional level, Northern blot analysis was performed using mRNA prepared from midgut, fat body, epidermis and muscle, respectively (Fig. 4). Hybridization signal was detected as a single band in mRNA from epidermis, muscle and midgut. The signal of *A. germari* LIM protein transcripts showed a strong band in the epidermis and muscle, while a weak band was found in midgut. In *E. scudderiana* Mlp, intense hybridization signal of the EsMlp transcripts occurred from the body wall (muscle + exoskeleton), but EsMlp transcripts were not found in fat body (Bilgen *et al.*, 2001). Muscle LIM proteins in insects are associated with development and are crucial during myogenesis (Arber *et al.*, 1994; Stronach *et al.*, 1996; Bilgen *et al.*, 2001). Further biochemical and molecular biological studies are necessary to reveal the exact physiological role of *A. germari* LIM protein.

Acknowledgements

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