Identification and characterization of the *Bombyx mori* myosin II essential light chain and its effect in BmNPV infection

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Abstract

Myosin, as a type of molecular motor, is mainly involved in muscle contraction. Recently, myosin research has made considerable progress. However, the function of *Bombyx mori* myosin remains unclear. In this study, we cloned the BmMyosin II essential light chain (BmMyosin II ELC) gene from a cDNA library of silkworm, which had an open reading frame (ORF) of 444 bp encoding 147 amino acids (about 16 kDa). After analyzing their sequences, BmMyosin II ELC was similar to the ELCs of 27 other Myosin II types, which contained EFh domain that bound Ca$^{2+}$. In addition, 28 sequences had five motifs, motifs 1 and 3 were relatively conserved. We constructed two vectors with BmMyosin to transfect MGC803 or BmN, monolayer wound healing of cells indicated they can promote cell migration successfully. For three fifth instar silkworms, Bm306, BmNB, BmBC8, we mainly analyzed the change of BmMyosin II ELC from transcription and translation after infecting with nucleopolyhedrovirus (BmNPV). We found that gene expression of resistant strains were higher than susceptible strains at 12 h, while the result of the translation level was opposite that of the transcription level. Through *in vitro* protein interactions, we found BmMyosin II ELC can interact with BmNPV ubiquitin.

Key Words: *Bombyx mori*; cell migration; myosin; ubiquitin

Introduction

Khne (1859) proposed that myosin could be extracted from frog tissue. Myosin, as the unit of myofibril, was composed of many heavy chains and light chains and divided into three areas, spherical head, neck and tail (Kwon et al., 2014). Sweeney et al., 2010 regarded myosin as a kind of molecular motors, which played an important role in muscle contraction, chemotaxis cytoplastic division, cell migration and vesicular transport signal transduction and so on (Andruchov et al., 2006). Myosin is actually a superfamily of chemicals. To date, we have found 24 kinds of myosin, and have divided them into traditional and unconventional myosin based on the myosin source. Specifically, myosin II is considered to be a traditional form of myosin. Unconventional myosin refers to myosin not found in muscle myosin tissue, such as myosin I, III, IV, V, exists only in the muscle cells, while myosin VIII, XI and XII only exist in plants. In recent years, myosin II has been a frequent topic of research, the protein coiled into the shape much like the letter Y. Myosin II has six protein polymers, including two heavy chains of about 220 kDa, two essential light chain of about 17 kDa and two regulatory light chain of about 20 kDa (Santos et al., 2007). According to the study, most myosin light chains were EF-hand superfamily members (Kolodney et al., 1999). Recently, some studies found that myosin plays a crucial role in cell growth and motion (Holmes et al., 2000), and light chain of myosin II has specific functions. Therefore, the light chain of myosin has become a hot research topic. In higher mammal cells, phosphorylation of the myosin light chain can promote cell migration (Straight et al., 2003).

The function of myosin in silkworm (*Bombyx mori*), a representative lepidopteran insect, remains unclear (Wang et al., 2007). Our previous unpublished research found that essential light chain of myosin II was related with nucleopolyhedrovirus (BmNPV). Therefore, we chose the myosin II essential light chain of the *B. mori* (BmMyosin II ELC) infected with BmNPV (T3 strain) to study change of protein expression. Then through protein interactions *in vitro*, we predicted the protein of BmNPV which interacted with BmMyosin II ELC. Simultaneously, we attempted to determine whether BmMyosin II ELC promotes migration in insect and mammalian cells.
Fig. 1 Myosin II essential light chain amino acid sequence homology analysis and the related genbank for 28 species analyzed here. Biston betularia (AEP43792); Antheraea pernyi (AGL33708); Riptortus pedestris (BAN21409); Riptortus pedestris (AGM32403); Zootermopsis nevadensis (KDR17497); Apis cerana (AEY59302); Harpegnathos saltator (EFN85742); Crangon spp (ACR43477); Marsupenaeus japonicus (ADD70028); Procambarus clarkii (APF95338); Drosophila melanogaster (AAA28710); Pseudodiaptomus annandaeli (AGT28473); Lepeophtheirus salmonis (ACO13186); Setaria digitata (ACT15365); Caenorhabditis brenneri (ACD86909); Schistosoma mansoni (AAR99584); Bombyx mori (NP_001040547.1); Pararge aegeria (JAA88715); Anopheles sinensis (KFB36116); Aedes aegypti (ABF18115); Psorophora albipes (JAA94216); Anopheles darlingi (ETN67861); Culex quinquefasciatus (EDS45595); Penaeus monodon (AET87131); Litopenaeus vannamei (ACC78603); Gryllotalpa orientalis (AAW22542); Bombyx mandarina (ACI05250); Papilio polytes (BAM18881). Identical residues are shaded in black, while similar residues are shaded in red, each species contains two EFh domains,* indicates conservative sites.

Materials and Methods

Analysis of bioinformatics on the essential light chain of BmMyosin II

The essential light chain of BmMyosin II was used to find 28 similar protein sequences related to other invertebrates using the NCBI BLAST Network Service (http://au.expasy.org/tools/BLAST/). A neighbor-joining (NJ) phylogenetic tree (Dias et al., 2003) and the schematic distribution of conserved motifs among the defined gene clusters were found using online versions of MEGA5.0 and MEME software.

Cell culture and monolayer wound healing assay

In preparation for TransIT-2020 mediated transfections, MGC803 (human stomach cancer cells) and BmN (insect ovary cells) were plated in a
Fig. 2 Neighbor-joining (NJ) phylogenetic tree and schematic distribution of conserved motifs among the defined gene clusters. The unrooted phylogenetic tree (left side of the figure) from the complete protein sequence was depicted by the MEGA 5.0 program using the NJ method. The tree shows the 28 phylogenetic subgroups (Group I-Group III) with high bootstrap values. Motifs were identified by means of MEME software using the complete amino acid sequence of the 28 invertebrates (right side of the figure).

24-well plate at a confluency of 50 - 70 % (3 - 4×10^4 cells per well) in 500µl plating medium (DMEM or TC-100 medium supplemented with 10 % FBS). Before plating onto this plate, one black line was drawn on the underside of the well with a Sharpie marker. The line would serve as fiducial marks for the wound areas to be analyzed. In preparation for making the wound, the free serum medium was used to prevent cell growth. Two parallel scratch wounds of approximately 300µm width were made perpendicular to the marker line with a yellow P200 pipet tip (Valster et al., 2005). Then vectors were constructed with PCDNA3.0-C-FLAG-Myosin II ELC and PIZ-C-FLAG-Myosin II ELC to transfect corresponding the cells of MGC803 and BmN. The wounds were observed using a microscope. Images were taken at regular intervals over a 0 - 48 h period of both areas flanking the intersections of the wound and the marker lines. Images were analyzed by digitally drawing lines (using Adobe Photoshop) averaging the position of the migrating cells at the wound edges. The cell migration distance was determined by measuring the width of the wound divided by two and by subtracting this value from the initial half-width of the wound (Eccles et al., 2005).

The preparation of BmMyosin II essential light chain polyclonal antibody

The recombinant plasmid PGEX-5X-3-Myosin II ELC was transformed into E. coli BL21, which were incubated at 37 °C in liquid LB culture media containing 50 mg/mL ampicillin. Expression of the GSTtag fusion protein was induced by adding IPTG to a final concentration of 1 mM before another 5-h incubation. Purification of the fusion protein was accomplished using Glutathione-Sepharose beads. Polyclonal antibody of BmMyosin II ELC was prepared by immunizing a laboratory rat (Rattus rattus) using purified Myosin II ELC as antigen (Zhang et al., 2012).
RNA isolation and cDNA synthesis of midguts strains of BmBC₈, Bm306 and BmNB infected BmNPV

Midguts of the 5th instar larvae of strains BC₈, 306 and NB infected BmNPV (1×10⁹ pfu/ml) at 0 h, 6 h, 12 h, 24 h, 48 h, 72 h were collected and washed with cold PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄). Then all samples were frozen immediately in liquid nitrogen and stored at –80 °C until extraction of RNA. Total RNA was extracted from each frozen sample with triol (GENERAY BIOTECH RnaEx). DNA was removed through digestion with RNase-free DNase I at 37 °C for 20 min. The RNA was further purified with phenol-chloroform and precipitated with ethanol. The RNAs dissolved in DEPC-treated ddH₂O were used to synthesize cDNAs with a Thermo Scientific RevertAid First strand cDNA synthesis Kit (Thermo-Fisher Scientific, New York, USA) by following the manufacturer’s instructions (Zhang et al., 2012).

qPCR analysis

qPCR primers were designed using the Primer Select program of the PrimerSelect software. The primer pairs were as follows: (Myosin II essential light chain) Forward primer, 5'-GGACAAAATCCTACAGAG-3', Reverse primer, 5'-CCCTGAGAGTCTTCTTGTC-3'. A SYBR Premix Ex Taq II Kit (Takara Biotechnology Co., Ltd. Dalian, China) was used. qPCR was performed using an ABI 7300 Sequence Detection System (Applied Bio systems, Darmstadt, Germany) under the following conditions: an initial cycle at 95 °C for 30 sec., followed by 40 cycles of 95 °C for 5 sec., 60 °C for 31 sec., one cycle of 95 °C for 15 sec., 60 °C for 1 min., 95 °C for 15 sec., and 60 °C for 15 sec.. Each reaction was performed in triplicate in 96-well plates. The relative expression level was calculated using 2⁻ΔΔCT, where ΔΔCT = ΔCT (target gene) - ΔCT (actin gene), ΔΔCT = ΔCT(target gene)- ΔCT (maximum) (Gareus et al., 2006).

Western blot analysis

The protein of midguts were collected and ground to powder in liquid nitrogen followed by RIPA Lysis Buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 mM PMSF). The protein concentrations were quantitated by the method of BCA, in which BSA was used as the protein standard. Protein samples were equalized and electrophoresed by 12 % SDS-PAGE and electrotransfered to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5 % milk in TBS. Following incubation with purified anti-BmMyosin IgG, the membranes were washed and incubated with HRP-labeled anti-mouse IgG.
The membranes were washed three times with TBST, and scanned using a TMB membrane peroxidase substrate system (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) or was detected by Electro-Chemi-Luminescence (ECL) and exposed to film.

GST pull-down

To directly obtain a function protein, the expression vector PGEX-5X-3 was constructed and used to express the recombinant protein GST-BmMyosin II ELC. The protein was suspended on Glutathione-Sepharose beads in wash buffer (25 mM Tirs-Hcl, 150 mM NaCl, 1 mM EDTA), BmN and BmN infected with recombinant BmNPV (His-Ubiquitin) after two days were collected. Then RIPA Lysis Buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1 % NP-40, 0.5 % sodium deoxycholate, 1 mM PMSF) was used and the recombinant protein was combined. They were pacled in 4°C refrigerator overnight. After washing three times by washing buffer, the beads were loaded on the 12 % SDS polyacrylamide gels. Western blotting was used to analyze the expression of ubiquitin using His tag McAb.

Results

BmMyosin II essential light chain cloning and sequence analysis

Based on the BmMyosin II ELC gene sequence (GenBank: DQ534197) from NCBI, the full length cDNA was cloned. Sequencing showed that its length was 1086bp, ORF was 444bp, encoding protein was 16kDa and PI was 4.49. The sequence found here was the same as the database sequence.

On the base of BmMyosin II ELC, we found 28 similar protein sequences of other invertebrates on the NCBI BLAST Network Service website (http://au.expasy.org/tools/BLAST/) that were used to analyze homology. According to the predicted Myosin II ELC structure domain we found that each species contained two identical EFh domains, which were Ca^{2+} binding sites (Fig. 1).

An un-rooted neighbor-joining (NJ) phylogenetic tree was generated using MEGA5 software based on the alignment of the corresponding Myosin II ELC complete protein sequences. The results showed that it was similar to the sequences of Pararg aegeria. For statistical reliability, we conducted bootstrap analysis with 1,000 replicates. The 28 members of the Myosin II ELC were subdivided into

![Fig. 4](image) The vectors of PIZ/V5-C-Flag-Myosin II ELC and PCDNA3.0-C-Flag-Myosin II ELC transfected BmN and MGC803. Analysis of Myosin II ELC by western blotting using Flag monoclonal antibodies.

![Fig. 5](image) (A) Monolayer wound healing of cells. Phase micrographs of MGC803 human stomach cancer cells at various times after monolayer wounding (left side of the figure). MGC803 showed that the migration of PCDNA3.0-C-Flag-Myosin II ELC transfected MGC803 at 0h, 24 h, and 48 h. Mock showed that the migration of PCDNA3.0 transfected BmN at 0 h, 24 h, and 48 h, there was no obvious migration at any time. Phase micrographs of BmN insect ovary cells at various times after monolayer wounding (right side of the figure). BmN showed that the migration of PIZ/V5-C-Flag-Myosin II ELC transfected BmN at 0 h, 24 h, and 48 h. Mock showed that the migration of PIZ/V5 transfected BmN at 0 h, 24 h, and 48 h. (B) Quantification of cell migration using the monolayer wound healing assay. The means values of three measurements are shown for each point in time and condition.
Fig. 6 (A) Analysis of gene of Myosin II ELC expression was performed by RT-PCR, optical density between myosin and actin on Bm306 at 0 h - 72 h after infecting BmNPV, indicating expression was higher at 12 h than other time. (B) Analysis of gene of Myosin II ELC expression was performed by qPCR at 12 h for three strains of *Bombyx mori* after infecting with BmNPV. Graphs depict the mean relative fold changes and are representative of three replicate plates. (C) Western blotting analysis of the expression levels of Myosin II ELC in three strains of *Bombyx mori* after infecting with BmNPV at 12 h using its polyclonal antibody.

three subgroups, designated Groups I, II and III. BmMyosin II ELC was contained in Group II based on the clade with at least 50% bootstrap support. According to the MEME software (Zhao et al., 2014 http://meme.nbcr.net/meme/cgi-bin/meme.cgi), *Bombyx mori* had five motifs, followed by motif 5 (yellow) corresponding to 3-31 amino acids, motif 2 (blue) corresponding to 32-61 amino acids, motif 4 (pink) corresponding to 65-85 amino acids, motif 1 (light green) corresponding to 90-121 amino acids, and motif 3 (red) corresponding to 126-146 amino acids (Fig. 2). All 28 sequences had motifs 1 and 3, and MEME prediction showed that the amino acid sequences of motif 1 (light green) and motif 3 (red) were relatively conserved (Fig. 3).

**BmMyosin II essential light chain promoted BmN and MGC803 migration after the protein expressing successfully**

We selected two different cell lines, MGC803 and BmN, and constructed two vectors (PCDNA3.0 and PIZ/V5) to encode BmMyosin II ELC protein fused to the Flag epitope tag at the COOH terminus (C-Flag-BmMyosin II ELC) (Clarke et al., 2007). Two days later after transfection, western blotting and Flag specific antibodies can be to detect whether the target protein transfection succeeded (Fig. 4). Monolayer wound healing of cells showed that the target protein can promote cell migration in two cell lines (Shimura et al., 2012 Fig. 5A). Through quantitative calculation, we drew the curve of cell migration and the effect of BmMyosin promotion of BmN became more apparent (Fig. 5B).

**Transcription and expression Levels of the essential light chain of BmMyosin II where Bm306, BmBC8, and BmNB infected BmNPV**

We selected three different silkworm strains, strain Bm306 which is susceptible to NPV and a near isogenic line BmBC8 and BmNB is resistant to NPV (Kang et al., 2011). The 5th instar larvae of BmBC8, Bm306 and BmNB were infected with BmNPV virus and midguts were collected at 0 h, 6 h, 12 h, 24 h, 48 h and 72 h. The RT-PCR results showed that there exits a higher level about BmMyosin II ELC about Bm306 at 12 h (Fig. 6A). To further analyze the BmMyosin II ELC expression after Bm306, BmBC8, BmNB infecting BmNPV at 12h (Zhang et al., 2012), we found the expressed levels in resistant strains BC8 and NB treated with BmNPV were also significantly higher than the susceptible strain 306 by qPCR (Fig. 6B). To further confirm the result of qPCR, antibody against the BmMyosin II ELC protein was used to perform Western blot analysis of BmNPV-infected midgut of *B. mori*. The results showed that a specific band was observed clearly in controls and samples of strains Bm306, while the samples of strain BmNB and
Discussion

The study of myosin, as a kind of superfamily of proteins, has progressed in recent years. The myosin family contained a conserved structure domain of EFh, and a Ca\(^{2+}\) binding site was involved in the domain. Experimental studies have found that when Ca\(^{2+}\) combined with Calmodulin complexes, the serine on the 19\(^{th}\) (Ser19) of the Myosin Light Chain 20 will be phosphorylated, leading to activation of the Myosin head Mg\(^{2+}\)-ATPase, which hydrolysed ATP to produce energy to make the conformation change. The conformation will cause myosin to bind to actin (Spudich et al., 2001), which plays an important function in motion. BmMyosin II ELC had the same EFh domain based on bioinformatics prediction. Recently, the research has shown that cell migration is a process that is critical at many stages of embryonic development, and is essential for tissue repair and immune function. In this article, we proved that BmMyosin II ELC promoted cell migration in BmN and MGC803. In addition, we speculated the EFh domain may be involved in the important functions.

The experiment showed that the heavy chain of myosin was a substrate for MuRF 1 ubiquitin ligase activity, and that the protein of myosin heavy chain was ubiquitinated by MuRF 1 in vitro (Clarke et al., 2007). Previous research in our lab found BmNPV ubiquitin was relevant to BmMyosin II ELC by Co-IP (unpublished data), in this article we chose BmMyosin II ELC as bait to prove an interaction between the target protein and BmNPV ubiquitin via pull down technique.

Of the three different silkworm strains tested here, showed the expression of BmMyosin II ELC had the opposite result in transcription and translation level at 12 h. The expression of BmMyosin II ELC in resistant strain was higher than it was in the susceptible strain at the transcription level, while the result was opposite at the translation level. We speculated that silkworm infected BmNPV at 12 h belonged to late stage, in which DNA replicated and expressed viral structural protein (Faulkner et al., 1997). Insect cells have different abilities to detect the presence of a virus infection and initiate an apoptotic program (LaCount et al., 1997). In addition, baculoviruses are able to interfere with apoptosis by the expression of apoptotic inhibitors (Prikhod'ko et al., 1996), which induce encoding BmMyosin II ELC mRNA of silkworm which was resistant to NPV increase. Research indicated that after AcMNPV infects TN-368 cells, actin moves into nuclei and subsequently is polymerized from G-actin to F-actin, leading to reorganization of the cytoskeleton. When F-actin polymerized, some activators are called Wiskott-Aldrich syndrome proteins (WASPs) and a homolog of WASP (PP78/83) (AcMNPV orf9) is encoded by all lepidopteran NPV genomes combined with WW domain to be involved in nucleating the formation of F-actin filaments. Studies of AcMNPV with mutations of PP78/83 suggested nuclear actin polymerization was required for the coordination of nucleocapsid development. In addition, since PP78/83 is a virion structural protein that is localized to the basal region of the nucleocapsids, it has the ability to cause actin nucleation and subsequent polymerization to release ATP to facilitate the movement of the nucleocapsids through the cytoplasm (O’Reilly et al., 1998). As BmNPV and AcMNPV are closely related to homologous orfs showing ~90 % nt and ~93 % aa sequence identity (Gomi et al., 1999), this shows that the mechanism was approximately the same. There were actin and ATP binding sites in myosin head. When the virus needs a large amount of G-actin, the host was likely to initiate prevention and control mechanisms to reduce the expression of BmMyosin II protein which was resistant to NPV, and uncombined with actin to defend itself against viral infection.

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References


