doi: 10.5505/tjb.2013.32042

Research Article [Araştırma Makalesi]

Yayın tarihi 30 Eylül, 2013 © TurkJBiochem.com

[Published online 30 September, 2013]

Expression and functional analyses of two essential myosin light chains from the fast white muscle of the spotted mandarin fish *Siniperca scherzeri*

[Benekli mandarin balığı, *Siniperca scherzeri*, dayanıklı beyaz kaslarından iki esansiyel miyozin hafif zincirinin ekspresyonu ve fonksiyonel analizi]*

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Registered: 13 July 2013; Accepted: 8 March 2013
[Kayıt Tarihi: 13 Temmuz 2012; Kabul Tarihi: 8 Mart 2013]

ABSTRACT

Objective: The essential myosin light chains (EMLC) are the major structural and regulatory proteins that control actin-myosin interaction in striated muscles. The purpose of this work is to provide a better understanding about the EMLC properties and their expression pattern during muscle development in the mandarin fish.

Material and Methods: The cDNA library was constructed to isolate and characterize MLC sequences. The nucleotide sequences and inferred amino acid sequences were analyzed. Whole-mount *in situ* hybridization was carried out to investigate the gene expression pattern. To assess their biological function in calcium binding, MLC recombinant proteins were purified to analyze their calcium binding activity *in vitro*.

Results: Two cDNA clones encoding MLC1 and MLC3 were identified from the fast white muscles of the spotted mandarin fish, *Siniperca scherzeri*. Sequence analysis revealed that MLC1 and MLC3 in the spotted mandarin were not produced by alternative splicing as reported in avians and rodents but were encoded by two different genes. MLC1 and MLC3 proteins contained two EF-hand domains, which are helix-loop-helix structural domains found in a large family of calcium-binding proteins.

Conclusion: These two EMLC genes exhibited a muscle-specific expression. Moreover, the distinct patterns of expression of MLC1 and MLC3 had overlapped during the skeletal muscle development in the early embryonic stage. MLC1 and MLC3 recombinant proteins were able to bind Ca^{2+} *in vitro*, which is a typical characteristic of EF-hand superfamily proteins.

Key Words: *Siniperca scherzeri*, essential myosin light chain, white muscle, fish muscle growth, developmental expression

Conflict of Interest: The authors declare that there are no conflicts of interest.

ÖZET

Amaç: Esansiyel miyozin hafif zincirleri (EMLC) çizgili kaslarda aktin-miyozin etkileşimini kontrol eden başlıca yapısal ve düzenleyici proteinlerdir. Bu çalışmanın amacı mandarin balığında kas gelişimi süresince EMLC özelliklerini ve ekspresyonunu daha iyi anlayabilmektir.

Materyal ve Metot: cDNA kütüphanesi MLC sekansını karakterize ve izole edebilmek için düzenlendi. Nükleotit sekansı ve bulunan amino asit sekansı analiz edildi. Gen ifade kalıbını araştırmak için Whole-mount *in situ* hibridizasyon yöntemi kullanıldı. Kalsiyum bağlanmasındaki biyolojik fonksiyonları *in vitro* değerlendirebilmek için MLC recombinant proteinler saflaştırıldı.

Bulgular: MLC1 ve MLC3'ü kodlayan iki cDNA kopyası benekli mandarin balığı, *Siniperca scherzeri,* dayanıklı beyaz kaslarından tanımlandı. Sekans analizi sonucunda benekli mandarin balığında bulunan MLC1 ve MLC3'ün kuşlarda ve kemiricilerde bildirildiği gibi alternatif birbirine eklenmeler ile oluşmadığı gösterildi. MLC1 ve MLC3 proteinleri, kalsiyum bağlayan proteinleri içeren geniş bir aile içinde bulunan, heliks-loop-heliks yapısal bölgesini içeren iki EF-el bölgesi olan proteinlerdir.

Sonuç: Her iki EMLC geni kasa özel ekspresyon göstermiştir. Bunun yanında erken embriyonik evrede, MLC1 ve MLC3'de ekspresyonun belirgin kalıbı olarak çakışmalar belirlendi. MLC1 ve MLC3 rekombinant proteinleri EF-el super ailesi tipik özelliği olan *in vitro* Ca^{2+} bağlayabilmektedir.

Anahtar Kelimeler: *Siniperca scherzeri*, esansiyel miyozin hafif zincirleri; beyaz kas; balık kas gelişimi; gelişimsel ifade

Çıkar Çatışması: Yazarların çıkar çatışması bulunmamaktadır.

Introduction

The myosin heavy chain (MyHC) and light chains (MLC) are the major protein components of teleost muscle tissue [1,2]. The MLCs exist in two forms, the essential light chain (MLC1/3) and the regulatory light chain (MLC2) [3]. The relative content of MyHC and MLC in muscle fiber determines the muscle properties. It has been shown that the fiber type composition of individual muscles and expression of MLC changes dramatically during fish ontogeny [4]. The expression of the MyHC and MLCs is asynchronous during the Zebrafish muscle development [5]. In rainbow trout, none of the MLC transcripts were detected before stage 12 (20 somites). In stage 12 rainbow trout embryos, only MLC1 mRNA is detected and its expression is restricted to the most rostral somites. In contrast, MLC3 expression is first detected in stage 16 embryos and expression is seen in the most rostral somites but not in the newly formed posterior somites of stage 17 embryos [6]. These observations revealed that the muscle related genes including MyHCs and MLCs are activated asynchronously during somitogenesis, and their temporal expression not only reflects physiological function in somite formation, but also can be used as molecular markers for different developmental stages of skeletal muscle formation.

The MLCs belong to the EF-hand superfamily that also include the muscle proteins troponin C and parvalbumin [7]. The EF-hand superfamily is a large group of proteins, which contain EF-hand motif formed by a helix-loop-helix structure and have the ability of binding metal ions or forming dimers. Many EF-hand proteins undergo conformational changes after Ca^{2+} binding, and expose hydrophobic domains to interact with corresponding hydrophobic domains in the effectors. The increase in intracellular Ca^{2+} is a widely established signal controlling a variety of processes in eukaryotic cells partly by the interaction of EF-hand proteins and cellular effectors in a Ca^{2+}-dependent fashion [8, 9]. However, although the structure of these proteins are now known in some fishes, including X-ray crystal structure of parvalbumin [10,11], metal-binding property for these proteins has not been well studied in fish yet.

The Chinese mandarin fish (CMF) is the most important aquaculture species in China as well as in Eastern Asia [12]. CMF includes seven subspecies, but only two of them, *S. chuatsi*, and *S. scherzeri*, have become widely aquaculture species. They have several commercial values, such as good flesh texture, full scale of amino acid and fatty acids, and appealing taste [13]. We decided to use CMF species as a model to study genes involved in controlling muscle flesh quality and muscle structural characteristics. Thus, we assayed CMF skeletal muscle transcriptional profile by cDNA microarray analysis and cDNA-EST sequencing [14,15] and cloned and characterized several muscle structural genes, including myosin heavy chain (MyHC) and myosin light chain (MLC) genes [16,17]. However, we did not observe any available MLC sequence for *S. scherzeri* and the characterization of these genes had not been performed.

Therefore, in the present study, we first isolated cDNA clones encoding skeletal white muscle myosin MLC1 and MLC3 from the spotted mandarin fish, *S. scherzeri*, analyzed the developmental expression of MLCs by whole-mount *in situ* hybridization, and then carried out the functional studies to assay if both MLC1 and MLC3 have the ability to bind Ca^{2+} *in vitro*. Our study provides important insights into the EMLC expression and function in fish muscle development.

Materials and Methods

Sample collection and preparation

The spotted mandarin fish (*Siniperca scherzeri*) was raised at Hunan Aquatic Research Institute, Changsha, Hunan, China. The white muscle was taken from the dorsal skeletal muscles of five adult fish aged about 2 years with an average body weight of about 450g. Embryos were generated by artificial fertilization and cultured in freshwater aquaria at approximately 20°C. Samples from the different development stages were collected from fertilization to hatching and stored in liquid nitrogen for total RNA preparation.

cDNA library construction and EST sequencing

Total RNA was extracted from the dorsal skeletal muscles of five spotted mandarin fish as described. Specifically, 5g of each frozen muscle tissue sample was chopped into fine pieces and homogenized by a hand mortar and then extracted with TRIzol^{R} Reagent (Invitrogen, USA) following the manufacturer's standard protocol. The RNA was then purified on an RNeasy MinElute Cleanup kit (Qiagen 7402, China Branch). Purity of the extracted RNAs was assayed by both the ratio of OD260/OD280 values (1.8-2.1) and 1% agarose gel electrophoresis, in which 5s, 18s and 28s RNA were uniformly present (data not shown). Poly(A)+ mRNA was isolated from total RNA using Oligotex mRNA mini kit (Qiagen, China). Then, poly(T)-primed cDNA was synthesized and directionally inserted into the pBluescript-II-SK(+) vector. Sequencing reactions of more than 6,000 single-pass from 5' end of each cDNA clone were carried out with Dynamics ET Terminator (GE Healthcare) with a T3 primer (5'-AATTAACCCTCACTAAAG-3'). The sequencing was performed on a 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA). The MLC sequences were searched for their similarity to the known MLCs on the non-redundant (nr) database in NCBI with BLASTX.

Expression, purification and gel shift assay of

recombinant proteins

The MLC1 and MLC3 cDNAs were cloned into expression vector pET-32a for making recombinant proteins. The MLC1-specific (Forward primers: 5'-cggatccatggcacccaagaaggac-3' and reverse primers: 5'-cggagctcttacacagacatgatgtgcttg-3')andMLC3-specific (Forward primers: 5'-cggatccatgaccgagttctcagcg-3 and reverse primers: 5'-gcgagctcttacacagacatgatgtgcttgac-3') primers were used to isolate the coding region of the cDNAs and subsequently cloned into the *BamH* I and *Sac* I sites of the pET-32a(+) expression vector (Novagen, Madison,WI). Recombinant proteins were purified by Ca^{2+}-dependent phenyl-sepharose chromatography essentially as described [18]. The electrophoresis mobility shift assay was performed as described [19] with either 5mM $CaCl_2$ or 5mM EGTA added to the recombinant proteins. After the denaturation of protein samples (2 µg each), they were applied to the 15% SDS-PAGE gel containing either 2mM $CaCl_2$ or 2mM EGTA, respectively. Protein migration was assayed by staining with Coomassie Blue.

Whole-mount in situ hybridization

Whole-mount *in situ* hybridization was carried out by using a digoxigenin (DIG)-labeled ribo-probe [20]. The MLC1 and MLC3 plasmid DNAs were respectively linearized by *Sac* I or *Xba* I, followed by in vitro transcription reactions with T7 RNA polymerase for the antisense RNA probe. Embryos of various stages were hybridized with the RNA probe in 50% formamide, 20×SSC, heparin 50 mg/ml, tRNA 50mg/ml, and 0.1% Tween 20 at 65°C, followed by incubation with anti-DIG antibody conjugated with alkaline phosphatase and by staining with substrates nitroblue tetrazolium (NBT) and 5-bromo, 4-chloro, 3-indolil phosphate (BCIP) to produce purple insoluble precipitates.

Bioinformatic analysis

The nucleotide sequences and inferred amino acid sequences were analyzed with DNAstar. The BLAST program was used to identify homologous sequences in the GenBank database. The sequences were aligned with multiple alignment program CLUSTAL W.

Results

Isolation and identification of the EMLC cDNAs

In this study, we had done a systematic sequence analysis of cDNAs expression profile in dorsal skeletal muscles of spotted mandarin. Among those 6000 isolated and sequenced clones from the cDNA library which was prepared from the adult fish white muscles only forty-two clones represented EMLCs. Two essential light chains, MLC1 and MLC3, were identified and fully sequenced. Their mRNA nucleotide sequences were submitted to the NCBI database with accession numbers of GQ282999 for MLC1 and GQ283001 for MLC3. As shown in Fig. 1, the MLC1 and MLC3 have an open reading frame of 579 and 453 nucleotides, respectively. Both MLC1 and MLC2 have same start codon ATG and stop codon TAA.

Amino acid sequences comparison of the EMLCs

The amino acid sequences of the two EMLCs were deduced from their cDNA sequences. Among them, cDNAs of the essential light chains, MLC1 and MLC3, were predicted to encode proteins of 192 and 150 amino acid residues, respectively. The sequence identity was 79.5 % between MLC1 and MLC3 in their overlapping region according to the most probable alignment (Fig. 2). The two helix-loop-helix EF-hand domains, found in a large family of calcium binding proteins, were identified in the MLC1 and MLC3 sequences. The second loop sequence in the EF-hand domain of MLC1 or MLC3 was highly conserved. They also exhibited 100% identity with the homologous regions of fish, Xenopus, mouse and human MLC1 or MLC3 proteins, respectively (Fig. 3).

Differential timing expression of MLC1 and MLC3 during development

The expression profiles of the MLC1 and MLC3 were analyzed at different developmental stages by whole-mount *in situ* hybridization. No expression of MLC1 and MLC3 could be detected in gastrula stage embryos (data not shown). By 7 somite stage, MLC1 expression became detectable and was restricted to the rostral somites (Fig. 4A). MLC1 expression increased significantly during early development. Its expression expanded to all somites at 42 hpf (Fig. 4F). In contrast, MLC3 expression appeared later. MLC3 expression was first detected in the rostral somites at 12 somite stage (Fig. 4G). Like MLC1, expression of MLC3 progressed caudally as the somites were being formed in a rostral to caudal wave (Fig. 4).

The calcium binding ability of MLC1 and MLC3

To determine the calcium binding ability of MLC1 and MLC3, recombinant proteins of the MLC1 and MLC3 were expressed in *E. coli* and purified from the soluble fraction of bacterial extracts by passage over a hydrophobic phenyl-sepharose column. The proteins bound to the column in a Ca^{2+}-dependent manner and were eluted by the Ca^{2+} chelator, EGTA. To confirm the Ca^{2+}-binding properties of the proteins, a SDS-PAGE mobility-shift analysis was performed in the presence of excess Ca^{2+} or EGTA. The data shows that both MLC1 and MLC3 recombinant proteins shifted to a faster-migrating conformation in the presence of Ca^{2+} (Fig. 5), indicating that both proteins are Ca^{2+}-binding proteins.

Discussion

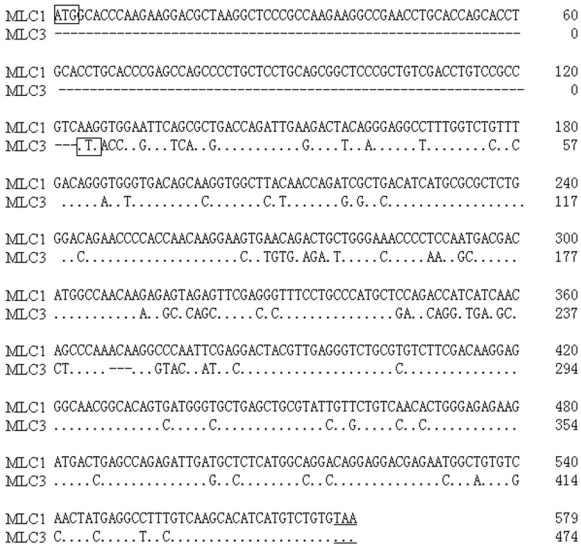

Figure 1. Comparison of nucleotide sequences homology between the MLC1 and MLC3 of *Siniperca scherzeri* "□" indicates start codon; "_" indicates end codon.

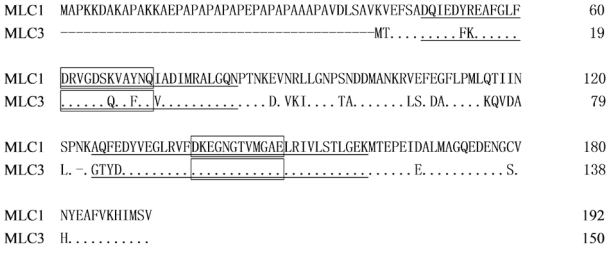

Figure 2. Comparison of the deduced amino acid homology between the MLC1 and MLC3 of *Siniperca scherzeri* "□" indicates the potential Ca^{2+} binding domain; "_" indicates potential EF-hand motif.

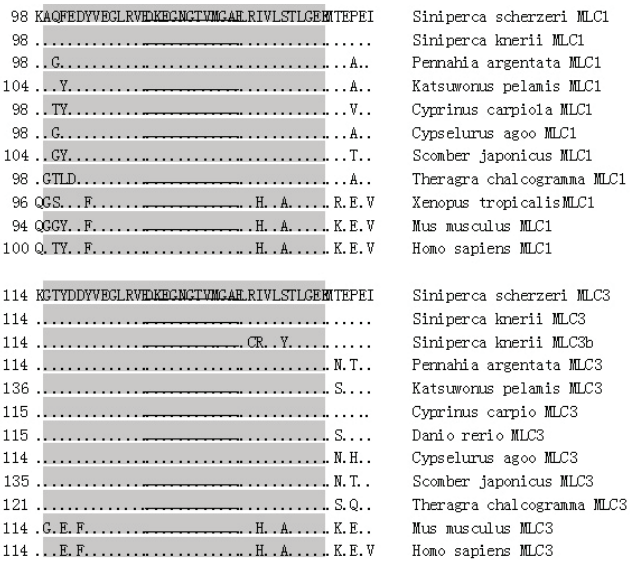

Figure 3. Comparison of the *S. scherzeri* EMLC second EF-hand motif amino acid sequences with those of other species. The shadow indicates the EF-hand motif and the Ca^{2+}-binding domains (underlined).

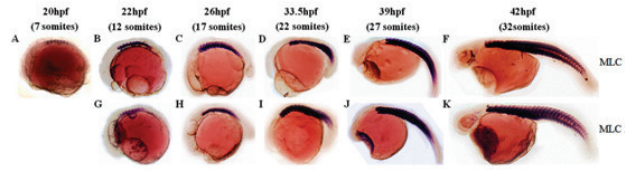

Figure 4. Ontogenic expression of the MLC1 and MLC3 genes as detected by whole-mount in situ hybridization. Embryos were hybridized with anti-sense MLC1 and MLC3 cRNA. Vertical panel columns show embryos at the same stage. Embryos were viewed laterally, anterior to the left, and were at the same magnification.

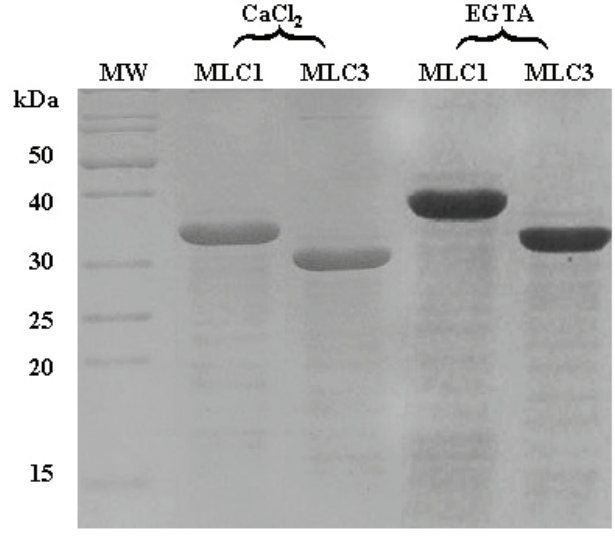

Figure 5. Ca^{2+}-binding ability of the EMLC. Recombinant MLC1 and MLC3 were separated on SDS-PAGE gels in either the presence of EGTA or Ca^{2+}, stained with Coomassie Blue. The position of molecular weight markers (kDa) is shown on the left.

In the present study, we isolated and characterized cDNA clones encoding two essential myosin light chains (MLC1 and MLC3) from *S. scherzeri.* We demonstrated that the MLC1 and MLC3 proteins were encoded by two different genes related with the C-terminal sequences of the two MLCs that were significantly different from each other. This is consistent with a previous report in mullet white skeletal muscle indicating that MLC1 and MLC3 were transcribed from two distinct genes [21]. Similar results were also found in common carp, where MLC1 and MLC3 were encoded by different genes, and their molar ratios were changed following temperature acclimation [22]. In contrast to the findings in fish, MLC1 and MLC3 of mammalian and avian species were produced from a single gene by alternative mRNA splicing after transcription [23, 24]. Moreover,the C-terminal region of the MLC1 and MLC3 of the higher vertebrates, such as mammals, share similar sequences [25], that are different from those observed in *S. scherzeri*.

The essential myosin light chains belong to the EF-hand

superfamily members contain the EF-hand motif formed by a helix-loop-helix structural domain. They also involve in binding to metal ions. It has been suggested that MLC1 and MLC3 evolve from a common ancestor and contain four similar Ca^{2+}-binding domains. The original single Ca^{2+}-binding domain undergoes tandem duplications to produce a protein with such four domains [26]. However, only two EF-hand motifs were found in the *S. scherzeri* MLC1 and MLC3 proteins. Evolutionary substitutions in amino acid sequences suggested the loss of the missing two EF-hand motifs in the MLC1 and MLC3 proteins.

Sequence analysis revealed that the loop region of the second EF-hand motif was well conserved among the essential myosin light chains (EMLCs) of seven fish species. This result is consistent with the earlier reports that the Ca^{2+}-binding domain is the most conserved feature in EMLCs. However, there is a controversial that whether any EMLC binds to Ca^{2+} or not [27-29]. In this study we demonstrated that recombinant MLC1 and MLC3, when expressed in and purified from *E. coli*, displayed altered electrophoretic mobility in the presence of Ca^{2+} which is a common property in EF-hand family members [30]. This finding empirically demonstrates the functionality of at least some of the predicted EF-hand domains in the EMLCs. Although the MLC1 and MLC3 can bind to Ca^{2+} *in vitro*, it should be noted that the electrophoretic mobility assay used in our study is not suitable for testing binding at physiological levels of Ca^{2+} and detailed biophysical analyses are needed to determine whether the EMLCs possess affinities for Ca^{2+} within a physiological range.

The expression patterns of the fish MyHC and MLCs are environmentally and developmentally regulated [31-32]. In this study we showed that MLC1 and MLC3 were differentially expressed in developing somites and skeletal muscles of fish embryos. When we compared MLC1 to MLC3, MLC1 was expressed a few hours earlier, making the MLC1 gene a very early and sensitive marker for fast skeletal muscle differentiation in the spotted mandarin fish. Previously, we applied RT-PCR techniques to detect expression patterns of the MyHC and MLCs in *Siniperca kneri* and *Siniperca chuatsi,* respectively [16, 17]. We demonstrated that the *S. kneri* MyHC gene was expressed from the muscle formation stage onwards. Moreover, the three light chains mRNAs in *S. chuasti* were first detected in the gastrula stage and their expression increased from the tail bud stage to the larval stage. In the present study, the spatio-temporal expression of the *Siniperca scherzeri* EMLCs was analyzed during the developing embryo stage by whole-mount *in situ* hybridization. None of the MLC expression was detected before gastrula stage as assayed by in situ hybridization. However, lower levels of the expression at gastrula and tail bud stages were detected by RT-PCR, but not by in situ. Consistent with this observation, the onset expression of structural genes during the muscle development are expected to be later than that of the myogenic regulatory genes like MyoD or myogenin whose expression begins usually at early gastrula stage or before somitogenesis [33,34].

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 31230076; 31072195), the Key Project of Hunan Province Education Department (10A010) and the State Key Laboratory of Freshwater Ecology and Biotechnology (2012FB01).

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