

Distribution and expression of non-muscle myosin light chain kinase in rabbit livers

Hua-Qing Zhu, Yuan Wang, Ruo-Lei Hu, Bin Ren, Qing Zhou, Zhi-Kui Jiang, Shu-Yu Gui

Hua-Qing Zhu, Yuan Wang, Ruo-Lei Hu, Qing Zhou, Laboratory of Molecular Biology and Department of Biochemistry, Anhui Medical University, Hefei 230032, Anhui Province, China

Hua-Qing Zhu, Yuan Wang, Ruo-Lei Hu, Qing Zhou, Shu-Yu Gui, Anhui Province Key Laboratory of Genomic Research, Hefei 230032, Anhui Province, China

Bin Ren, Department of Pathology, BIDMC and Harvard Medical School, 99 Brookline, MA 02215, Boston, U S A

Shu-yu Gui, Department of Respiratory Disease, the First Affiliated Hospital of Anhui Medical University, Hefei 230032, Anhui Province, China

Zhi-Kui Jiang, 105 Hospital of PLA, Hefei 230031, Anhui Province, China

Supported by National Natural Science Foundation of China, No. 39870324, Grant for Excellent Young Teachers of Ministry of education of China, No.39870324, National Science Foundation of Anhui Province, No.9904312

Correspondence to: Professor Yuan Wang, Laboratory of Molecular Biology and Department of Biochemistry, Anhui Medical University, Hefei 230032, Anhui Province, China. wangyuan@mail.hf.ah.cn

Telephone: +86-551-5161140

Received: 2003-05-10 **Accepted:** 2003-06-02

Abstract

AIM: To study the distribution and expression of non-muscle myosin light chain kinase (nmMLCK) in rabbit livers.

METHODS: Human nmMLCK N-terminal cDNA was amplified by polymerase chain reaction (PCR) and was inserted into pBKcmv to construct expression vectors. The recombinant plasmid was transformed into XL1-blue. Expression protein was induced by IPTG and then purified by SDS-PAGE and electroelution, which was used to prepare the polyclonal antibody to detect the distribution and expression of nmMLCK in rabbit livers with immunofluorescence techniques.

RESULTS: The polyclonal antibody was prepared, by which nmMLCK expression was detected and distributed mainly in peripheral hepatocytes.

CONCLUSION: nmMLCK can express in hepatocytes peripherally, and may play certain roles in the regulation of hepatic functions.

Zhu HQ, Wang Y, Hu RL, Ren B, Zhou Q, Jiang ZK, Gui SY. Distribution and expression of non-muscle myosin light chain kinase in rabbit livers. *World J Gastroenterol* 2003; 9(12): 2715-2719
<http://www.wjgnet.com/1007-9327/9/2715.asp>

INTRODUCTION

Protein kinase plays an important regulatory role in response to both intracellular and extracellular signals^[1]. Specific protein kinase is thought to control various cellular functions including glycogen metabolism, muscle contraction and growth, etc. Myosin light chain kinase (MLCK) is a Ca^{2+} /calmodulin activated enzyme in the kinase family which catalyses the

phosphorylation of the 20-ku myosin light chain (MLC-20)^[2]. In skeletal muscle, the phosphorylation of MLC-20 correlates with potentiated twitch tension after repetitive stimulation. In smooth muscle cells, this phosphorylation leads to an increase in actomyosin ATPase activity and contraction which appears to be required for initiation of contraction. Phosphorylation of MLC-20 by smooth muscle MLCK is a key event initiating smooth muscle contraction. Although the roles of MLCK in non-muscle cells have not been well defined, a variety of morphological changes such as cellular motility and organelle movement occur concurrently with the increased cytoplasmic Ca^{2+} levels, light chain phosphorylation and activation of MLCK. Intracellular localization studies performed in mammalian fibroblast cells have colocalized MLCK to the spindle apparatus and midbody of mitotic cells. These observations have led to the suggestion that the phosphorylation of MLC-20 by MLCK in non-muscle cells might have a role in cell division and cell motility^[3]. There are at least two different stress fiber systems in fibroblasts including central stress fiber system and periphery stress fiber system and the latter system depends on MLCK^[4]. And at least two distinct classes of MLCK (short and long) phosphorylate the MLC-20 of myosin in thick filaments but bind with high affinity to actin in thin filaments^[5]. But which form of MLCK exists in hepatocytes? How is MLCK involved in cellular functions in hepatocytes? In order to investigate the roles of MLCK in the maintenance of liver functions and its association with some liver diseases in the future study, we prepared polyclonal antibody through expressed MLCK protein in *E. Coli* system, and the antibody was used to detect the distribution and expression of MLCK in hepatocytes with immunofluorescence microscopy. Our research provides the basis for further investigation MLCK functions of in the liver and its relation with the pathology of some hepatic diseases such as hepatocellular carcinoma and hyperlipoproteinemia.

MATERIALS AND METHODS

Reagents and instruments

Plasmid pBKcmv and *E. coli* XL1-blue were from STRATAGENE (La Jolla, CA, USA). The human-non-muscle-MLCK cDNA was a gift from Dr. Stull at University of Texas Southwestern Medical Center, USA. Concept rapid PCR purification system kit was purchased from Life Technology, GibcoBRL. pGEM-T vector system I was purchased from Promega (Madison WI, USA). Restriction endoenzyme, T_4 DNA ligase, dNTPs, amplitaq DNA polymerase, mounting media were obtained from Sigma Chemical Company. PCR primers were synthesized by BioAsia Biotechnology Co., Ltd (Shanghai, China). Other reagents were made in China and were of analytical purity. DYY-III type-2 electrophoresis and transfer system were made by Beijing Instrument Factory. UV-754 spectrophotometer was made by The Third Factory of Analytical Instrument of Shanghai. Nikon eclipse E800 microscope was from Japan.

PCR of hnmMLCK DNA

The primers were designed according to human MLCK cDNA,

prime 1: 5' TGG AAT TCC ATG GGG GAC GTG AA3' containing *EcoRI* restriction site and prime 2: 5' CCA CTG CTG AAG AAG CTT AAA ATC3' containing *HindIII* restriction site. The PCR parameters were pre-denaturation at 94 °C for 5 min before amplification was done for 35 cycles at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, and final extension for 10 min at 72 °C after the last cycle. The PCR products were examined by 20 g.L⁻¹ agarose gel electrophoresis with TAE buffer at 80V for 40 min, visualized with ethidium bromide, and photographed under UV light.

Ligation, transformation and sequence of hnmMLCK DNA^[6]

The PCR products were purified by concept rapid PCR purification system kit and inserted into the T-vector, the resulting ligation products were transferred into XL1-blue *E. coli* competent cells. The positive clone was picked up and the plasmids were prepared. The plasmid and pBKcmv vector were digested with *EcoRI* and *HindIII* and ligated before transferred into XL1-blue *E. coli* competent cells. Individual recombinant white clones were picked up and recombinant DNA was prepared before digested with restrictive endonuclease. The recombinant plasmid sequence was detected with ABI377 auto analytical instrument.

Expression and purification of hnmMLCK in *E. coli*^[7]

The positive recombinant plasmids were transformed into XL-1blue *E. coli*, the single clone was picked up and cultured overnight at 37 °C with shaking at 250 rpm. The media were diluted (1:100) with Luria-bertani liquid medium and isopropyl- β -D-thiogalactoside (IPTG) was added into the medium to induce protein expression when OD₆₀₀=0.6-0.8, and cultured for 4 h. The bacteria were harvested by centrifugation at 5 000 rpm for 10 min and the expressed protein was analyzed by SDS-PAGE. The expressed band was cut from SDS-PAGE gel and electroeluted in transfer buffer for isolation and purification.

Anti-myosin light chain kinase polyclonal antibody preparation

Polyclonal antibody to human MLCK was prepared according to the previously described method^[8].

Immunofluorescence detection of MLCK in rabbit livers

The New Zealand rabbit liver tissues were embedded with O.C.T and frozen sections were prepared. The slices were incubated in 100 % acetone for 10 min at -20 °C and dried in air, then blocked in 5 % non-fat milk in PBS (pH7.4) overnight. The blocking solution was removed and anti-MLCK polyclonal antibody was added, and then incubated in a wet box for 2-3 h. The reactions were incubated with FITC-labeled secondary antibody for 1 h. Finally, the reactions were covered with mounting media before observation with a Nikon fluorescent microscope^[9,10].

RESULTS

Amplification of human MLCK cDNA

The PCR products were detected by 20 g·L⁻¹ agarose gel. The results showed that there was a 450 bp band in the gel (Figure 1), corresponding to the fragment of human MLCK cDNA N-terminate.

Enzymatic and sequence analysis of recombinant plasmid and cloned DNA

The recombinant plasmid was digested with *EcoRI* and *HindIII* and then run in 20 g·L⁻¹ agarose gel, which showed that the PCR products were inserted into the pBKcmv vector (Figure

2). The DNA sequences of pBK-hnmMLCK were detected by ABI377 auto analytical instrument (Figure 3), and compared with Genbank (Figure 4).

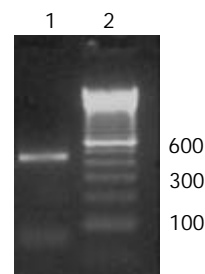


Figure 1 Amplification of hnmMLCK cDNA by PCR. 1. products of PCR amplification, 2. the 100 bp ladder.

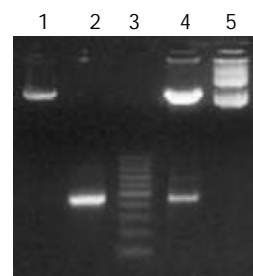


Figure 2 Analysis of human MLCK recombinant plasmids with restriction endonucleases mapping. 1. pBKcmv/*EcoRI-HindIII*, 2. PCR products/*EcoRI-HindIII*, 3. the 100 bp DNA ladder, 4. pBK-hnmMLCK/*EcoRI-HindIII*, 5. pBK-hnmMLCK.

Protein expression and purification

The expressed protein hnmMLCK in *E. coli* was induced with IPTG and bacteria were centrifuged at 5 000 rpm for 10 min. The pellet was resuspended with PBS, and an equal volume of 2×protein loading buffer was added, boiled at 100 °C for 5 min and analyzed by SDS-PAGE. The percentage of the expressed protein was about 21 % by scanning analysis (Figure 5).

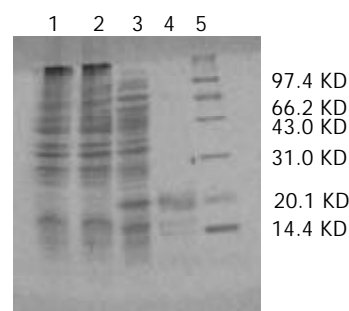


Figure 5 Analysis of pBK-hnmMLCK with SDS-PAGE. 1. pBKcmv in XL1-blue, 2. pBK-hnmMLCK in XL1-blue before induced, 3. pBK-hnmMLCK in XL1-blue after induced, 4. purified expression protein, 5. protein markers.

Antiserum detection by immune double-diffusion

The ratio of antigen to antibody was at least 1:16 (Figure 6), suggesting that the polyclonal antibody could be used for immunofluorescence analysis.

Distribution of MLCK in rabbit livers

MLCK was mainly distributed peripherally in hepatocytes (Figure 7), and was hardly detected cytoplasm by immunofluorescence.

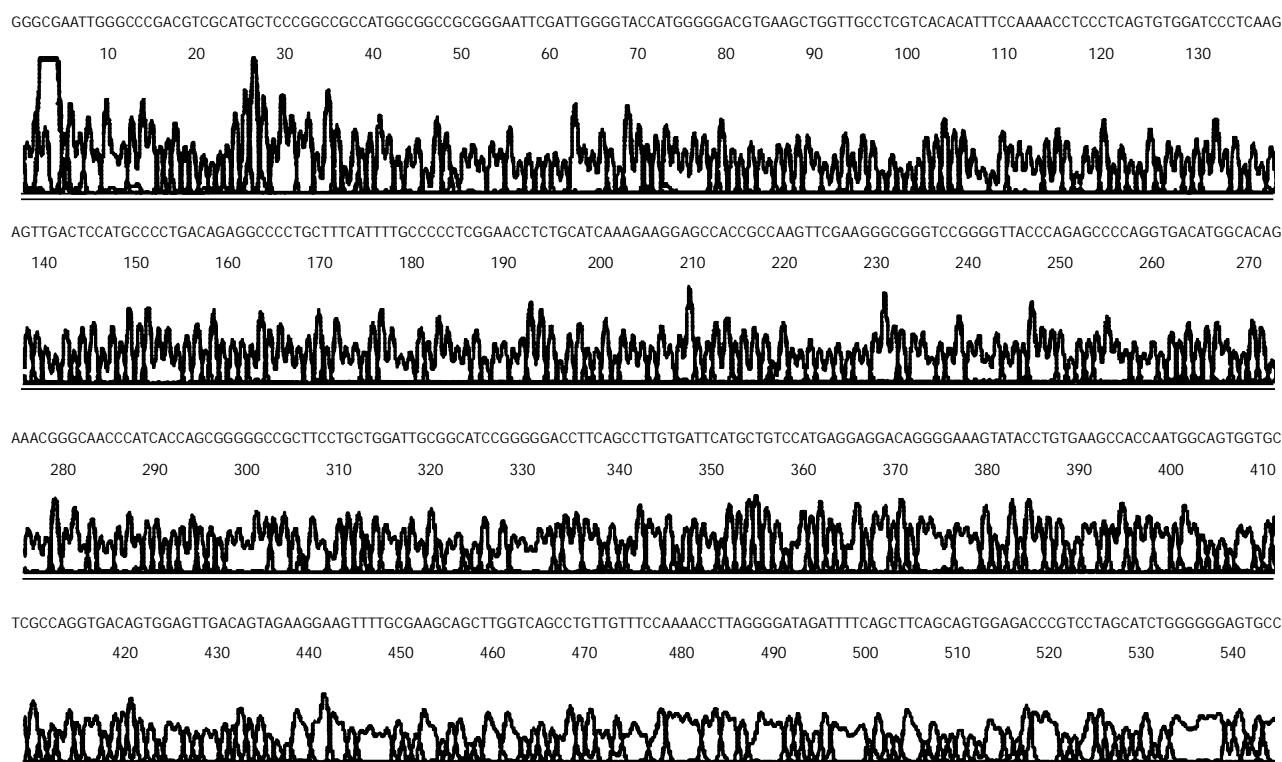


Figure 3 DNA sequences of pBK-hnmMLCK were detected by ABI377 auto analytical instrument.

>gi|7239697|gb|AF069601.2|AF069601 pBKcmv-hMLCK85-144
Homo sapiens myosin light chain kinase isoform 2 (MLCK) mRNA, complete cds
Length = 5719 Score = 852 bits (430), Expect = 0.0
Identities = 439/442 (99%) Strand = Plus / Plus
Query: 39 ccattgggggacgtgaagctggtgcctcgtcacacatttccaaaacctccctcagtggtg 98
|||||
Sbjct:118 ccattgggggatgtgaagctggtgcctcgtcacacatttccaaaacctccctcagtggtg 177
Query: 99 atccctcaagagtgactccatgccctgacagaggccctgtttcattttgccccctc 158
|||||
Sbjct:178 atccctcaagagtgactccatgccctgacagaggccctgtttcattttgccccctc 237
Query:159 ggaacctctgcatcaagaaggagccaccgccaagtcgaaggcggtccggggtacc 218
|||||
Sbjct:238 ggaacctctgcatcaagaaggagccaccgccaagtcgaaggcggtccggggtacc 297
Query:219 cagagccccagtgacatggcacagaaacgggcaaccatcaccagcgggggccgcttc 278
|||||
Sbjct:298 cagagccccagtgacatggcacagaaacgggcaaccatcaccagcgggggccgcttc 357
Query:279 tgctggattgcggcatccggggaccttcagcctgtgtattcatgtgtccatgaggagg 338
|||||
Sbjct:358 tgctggattgcggcatccggggactttcagcctgtgtattcatgtgtccatgaggagg 417
Query:339 acaggggaaagtatacctgtgaagccaccaatggcagtggtgctcgcaggtgacagtg 398
|||||
Sbjct:418 acaggggaaagtatacctgtgaagccaccaatggcagtggtgctcgcaggtgacagtg 477
Query:399 agttgacagtagaagggaagtttgcgaagcagcttggtcagcctgtgtttccaaaacct 458
|||||
Sbjct:478 agttgacagtagaagggaagtttgcgaagcagcttggtcagcctgtgtttccaaaacct 537
Query:459 taggggatagatttaagcttc 480
|||||
Sbjct:538 taggggatagatttaagcttc 559

Figure 4 Comparison between sequencing results of hnmMLCK DNA and those published by Genbank.

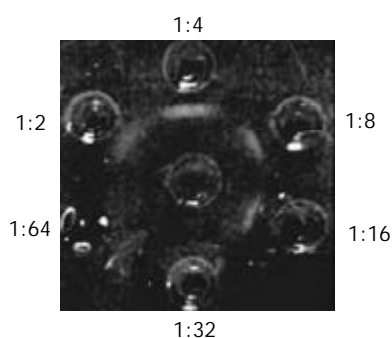


Figure 6 Antiserum titer detected by immuno-double-diffusion.

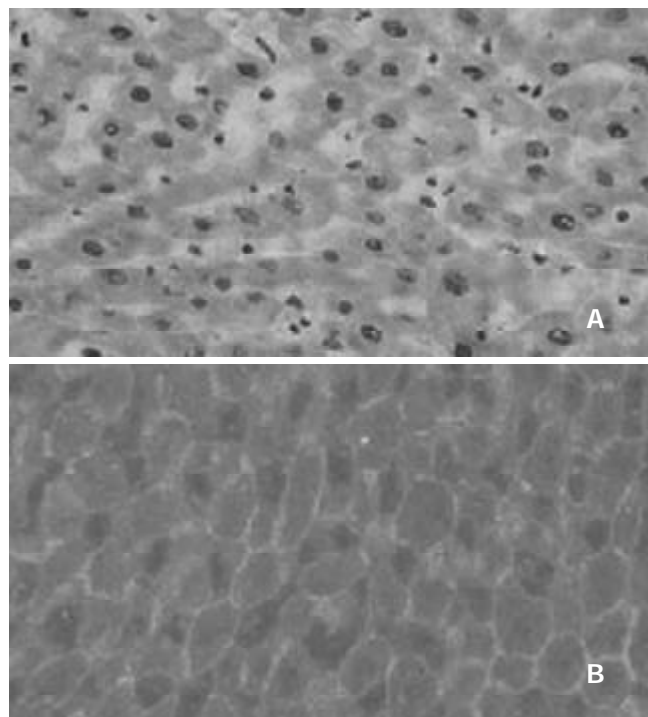


Figure 7 MLCK distribution in hepatocytes of New Zealand rabbits. A: H and E staining, B: MLCK distribution.

DISCUSSION

MLCK is the key regulator of cell motility and smooth muscle contraction in higher vertebrates^[11-13]. MLCK expression shows a complex pattern. In undifferentiated myoblasts, 220-ku long or non-muscle form of MLCK is expressed during differentiation of skeletal muscle. During myoblast differentiation, the expression of 220-ku MLCK declines and expression of this long-term is replaced by 103-ku smooth muscle MLCK and a skeletal muscle-specific MLCK. The two isoforms are products of a single gene^[14], and both short and long MLCKs are expressed in embryonic and adult non-muscle as well as smooth muscle cells^[15,16]. Recently, some study showed the existence of a 208-ku protein, named embryonic MLCK because its expression could be detected in early embryonic tissue stem cells and in proliferating culture cells^[17]. MLCK has several well characterized domains, and they are comprised of an amino-terminal “tail” of unknown function and a central catalytic core that is homologous to the catalytic core of other protein kinases, carboxy-terminal to the catalytic core and calmodulin binding domains, which are involved in the activation of the kinase in responses to increased Ca^{2+} ^[18]. In this study we constructed the expression vector which could express N-terminal MLCK, and used this protein to prepare polyclonal antibody. The results showed that the antibody could

react with hepatic cells and MLCK was distributed in the peripheral region of hepatic cells. In rabbit portal vein myocytes, MLCK could mediate noradrenaline-evoked non-selective cation current^[19]. In the liver, agents that elevated intercellular free Ca^{2+} concentration could increase tight junctional permeability and stimulate bile canaliculi contraction. Myosin phosphorylation might be responsible for the tight junctional permeability caused by elevation of intercellular Ca^{2+} in hepatocytes. Moreover, the integrity of the phosphorylation systems of myosin is essential for normal bile flow. In addition, hepatic sinusoidal Ito cells play a regulatory role on hepatic blood flow through their contraction, while the integrity of MLCK is essential for Ito cell contractions and normal sinusoidal blood flow. However, the role of myosin phosphorylation by MLCK in non-muscle tissues has not been well characterized but correlated with important activities such as cell division, receptor capping^[20,21], etc. It has been found that MLCK was closely associated with non-muscle cells^[16,19,20]. Phosphorylation of myosin light chain by MLCK in non-muscle cells and tissues demonstrated an important physiological function^[22]. For example, myosin light chain phosphorylation has been implicated in secretory vesicle movement, cellular locomotion and changes in cellular morphology^[23]. MLCK activation was a critical step in cytoskeletal changes causing pseudopod formation during polymorphonuclear leukocyte phagocytosis^[24]. MLCK was also associated with the gap formations and endothelial hyperpermeability of coronary venular endothelial cell monolayers^[25]. The preliminary studies showed that the light chain was obviously phosphorylated when CaM was added into the reaction buffer at a suitable concentration of Ca^{2+} . The activity of MLCK in rabbit livers increased markedly when CaM was added, and the activity changed with a substrate concentration or the concentration of light chain kinase^[26]. MLCK immunoreactivity was found to be colocalized with the insulin granules, suggesting that it increased insulin granules in the ready-releasable pool by acting on different steps in the secretory cascade^[27]. In this study, the expressed vector was successfully constructed and MLCK was expressed in *E. coli* system, which lays a good basis for the manufacture and clinical application of the enzyme. The anti-MLCK polyclonal antibody was prepared and used to detect the distribution of MLCK in the cells of rabbit liver. MLCK may play an important role in maintaining the normal functions of tissues. But in the liver, which form of MLCK was expressed, long or short? If there are both forms, which form expresses more? What are their roles in the liver? What roles will it play in liver regeneration, injury or hepatic carcinoma? And what is the mechanism of MLCK activity in the liver? All these remain to be investigated and elucidated in future studies.

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Edited by Zhang JZ and Wang XL