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

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\textsuperscript{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\textsuperscript{2+} levels, light chain phosphorylation and activation of MLCK. Intracellular localization studies performed in mammalian fibroblast cells have localized 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 thin 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 endonuclease, T\textsubscript{4}DNA ligase, dNTPs, amplitaqe 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,
PCR products were inserted into the pBKcmv vector (Figure 1) and then run in 20 g·L\(^{-1}\) and cloned DNA Immunofluorescence detection of MLCK in rabbit livers to the previously described method Polyclonal antibody to human MLCK was prepared according to the anti-MLCK polyclonal antibody preparation. The blocking solution was removed and anti-MLCK polyclonal

Expression and purification of hnmMLCK in E. coli The positive recombinant plasmids were transformed into XL1-blue E. coli competent cells. The single clone was picked up and cultivated overnight at 30 °C with shaking at 250 rpm. The media were diluted (1:100) with Luria-bertani liquid medium and iso-propyl-\(\beta\)-D-thiogalactoside (IPTG) was added into the medium to induce protein expression when OD\(_{600}\) = 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\(^8\). Immuno-fluorescence 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 Nicon fluorescent microscope\(^{9,10}\).

RESULTS

Amplification of human MLCK cDNA The PCR products were detected by 20 g·L\(^{-1}\) agrose 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\(^{-1}\) agrose 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 cytoplasms by immunofluorescence.
Figure 3 DNA sequences of pBK-hnmMLCK were detected by ABI 377 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 ccatgggggaagacgtagaagctggttgcctcgtcacacatttccaaaacctccctcagtgg 98
Sbjct: 118 ccatgggggaagctggttgcctcgtcacacatttccaaaacctccctcagtgg 177
Query: 99 atccctcaagagttgactccatgcccctgacagaggcccctgctttcattttgccccctc 158
Sbjct: 178 atccctcaagagttgactccatgcccctgacagaggcccctgctttcattttgccccctc 237
Query: 159 ggaacctctgcatcaaagaaggagccaccgccaagttcgaagggcgggtccggggttacc 218
Sbjct: 238 ggaacctctgcatcaaagaaggagccaccgccaagttcgaagggcgggtccggggttacc 297
Query: 219 cagagccccaggtgacatggcacagaaacgggcaacccatcaccagcgggggccgcttc 278
Sbjct: 298 cagagccccaggtgacatggcacagaaacgggcaacccatcaccagcgggggccgcttc 357
Query: 279 tgcggattgcggcatccgggggaccttcagccttgtgattcatgctgtccatgaggagg 338
Sbjct: 358 tgcggattgcggcatccgggggaccttcagccttgtgattcatgctgtccatgaggagg 417
Query: 339 acaggggaagatcggctgaagctggttgcctcgtcacacatttccaaaacctccct 458
Sbjct: 418 acaggggaagatcggctgaagctggttgcctcgtcacacatttccaaaacctccct 537
Query: 459 taggggatgattttctagcttc 480
Sbjct: 538 taggggatgattttctagcttc 559

Figure 4 Comparison between sequencing results of hnmMLCK DNA and those published by Genbank.
react with hepatic cells and MLCK was distributed in the peripheral region of hepatic cells. In rabbit portal vein myocytes, MLCK could mediate noradrenaline-activated non-selective cation current\(^{[19]}\). In the liver, agents that elevated intercellular free \(\text{Ca}^{2+}\) concentration could increase tight junctional permeability and stimulate bile canalicular contraction. Myosin phosphorylation is might be responsible for the tight junctional permeability caused by elevation of intercellular \(\text{Ca}^{2+}\) 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 \(\text{CaM}\) was added into the reaction buffer at a suitable concentration of \(\text{Ca}^{2+}\). The activity of MLCK in rabbit livers increased markedly when \(\text{CaM}\) 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.

**REFERENCES**


2. Deng JT, Van Lierop JE, Sutherland C, Walsh MP. \(\text{Ca}^{2+}\)-independent smooth muscle contraction, a novel function for integrin-linked kinase. ] Biol Chem 2001; 276: 16365-16373


Huang ZS, Wang ZW, Liu MP, Zhong SQ, Li QM, Rong XL. Protective effects of polydatin against CCl4-induced injury to primary cultured rat hepatocytes. World J Gastroenterol 2002; 8: 537-539

Chen YM, Qian ZM, Zhang J, Chang YZ, Duan XL. Distribution of constitutive nitric oxide synthase in the jejunum of adult rat. World J Gastroenterol 2002; 8: 537-539


Edited by Zhang JZ and Wang XL