• BRIEF PEPORT •

Preliminary research on myosin light chain kinase in rabbit liver

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Abstract

AIM: To study preliminarily the properties of myosin light chain kin ase (MLCK) in rabbit liver.

METHODS: **The expression of MLCK was detected by reverse transcription-polymerase chain reaction (RT-PCR); the MLCK was obtained from rabbit liver, and its activity was analyzed by γ-^{32}P incorporation technique to detect the phosphorylation of myosin light chain.**

RESULTS: **MLCK was expressed in rabbit liver, and the activity of the enzyme was similar to rabbit smooth muscle MLCK, and calmodulin- dependent. When the concentration was 0.65 mg·L^{-1}, the activity was at the highest level.**

CONCLUSION: **MLCK expressed in rabbit liver may catalyze the phosphorylation of myosin light chain, which may play important roles in the regulation of hepatic cell functions.**

Subject headings myosin light chain kinase; liver, rabbit; enzyme activity; reverse transcription-polymerase chain reaction

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INTRODUCTION

Protein kinases perform important regulatory roles in response to both intra cellular and extracellular signals. Specific protein kinases are thought to control various cellular functions including glycogen metabolism, muscle contraction , and growth. Myosin light chain kinase (MLCK) is an enzyme of the kinase family, which phosphorylates the light chain of smooth muscle, skeletal muscle and no n-muscle myosin in the presence of Ca^{2+} and CaM, id it requires the C a^{2+} and the Ca^{2+} binding protein CaM for the activity. Phosphorylation of the light chain in skeletal muscle is involved in modulating the tension produced during contract ion, whereas in smooth muscle it appears to be required for initiation of contraction.

Phosphorylation of myosin regulatory light chain (RLC) by smooth muscle myosin light chain kinase is a key event initiating smooth muscle contraction. However, the roles of myosin phosphorylation in non-muscle function is not well understood. However, present researches show that a variety of non-muscle processes are associated with MLCK, including endothelial cell retraction, fibroblast con traction, mast cell secretion, receptor capping in lymphocytes, and platelet aggregation, contraction, etc. That MLCK controls endothelial Ca^{2+} entry in endothelial cells not through myosin light chain phosphorylation suggests its role in vasodilation through its action in endothelial cells[1]. The activation of volume-regulated anion channels in macrovascular endothelium is modulated by myosin light chain phosphorylation through the action of MLCK or myosin light chain ph osphatase[2].

Ma *et al*[3] incubated the filter-grown Caco-2 intestinal monola yers in Ca^{2+} free solution (CFS) and found an increase in tight junction p ermeability of the monolayer. The CFS-induced modulation of tight junction barrier was related with the activation of MLCK activity and centripetal retraction of peri-junctional actin and myosin filaments. Ueno *et al* reported that a Ca^{2+}-CaM- dependent protein kinase purified from rabbit liver phosphorylated the regulator y light chain of hepatocyte myosin. The kinase catalyzed the incorporation of ph osphate into the 22 ku light chains of hepatocyte myosin, which resulted in a 7 - fold activation of the Mg (2+)-ATPase activity by F-actin. MLCK is possibly involved in many Ca^{2+}-dependent activities of monocytes or macrophages[4]. It is found that there are at least two different stress fiber systems in human foreskin fibroblasts including central stress fiber system and peripheral stress fiber system, and the latter system depends on MLCK[5]. MLCK plays an important role in the development of neuron. Preventing calcium influx through blocking of MLCK activity selectively decreased dendritic branching[6]. A novel MLCK cDNA was isolated from a HeLa cell cDNA library. The deduced amino acid sequence was identical to that of a zipper-interacting protein kinase, which mediates apoptosis. With the fragment of the bovine stomach MLCK gene including kinase and calmodulin regulatory domains as a probe. Murata-Hori *et al*. screened a HeLa cell cDNA library. They found that one serine/threonine kinase, HeLa zipper-interacting protein kinase, from non-muscle cells phosphorylated regulatory chain of myosin II[7]. Is there any novel MLCK in hepatocytes? How is MLCK involved in cellular functions of 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 preliminarily studied the expression of the enzyme in the rabbit liver by reverse transcription polymerase chain re action (RT-PCR) and observed some of its properties preliminarily through assaying its catalytic activity of the phosphorylation of myosin light chain by γ-^{32}P incorporation method. Our preliminary research provides basis for our further investigation of functions of MLCK in the liver and its relation with the pathology of some hepatic diseases.

MATERIALS AND METHODS
Reagents and instruments
CaM, rabbit smooth muscle MLCK positive control and myosin

regulatory light chain were the gifts from Dr. Zhi at University of Texas Southwestern Medical Center, USA. EGTA and MOPS were purchased from Sigma Chemical Company, and DTT, phenylmethy sulfonylflouride (PMSF) and proteinase inhibitors from Sino-America n Biotechnological Corp, Shanghai. HEPES was produced by MERCK; EDTA was obtained from Life Technology, GibicoBRL. [γ-^{32}P]ATP (radio activity > 185 PBq ·mol-1·L^{-1}) was purchased from Yahui Biomedical Engineering Corp of Beijing. Other reagents were made in China and they were of analytical purity. Backman LS1701, Liquid Scintillation System was made in USA and DYY-III type-2 electrophoresis and transfer system were made in June 1 Instrument Factory of Beijing and idEA Ideal Sci Co. UV-754 Spectrophometer was made by The Third Factory of Analytical Inst ruments of Shanghai.

Tissue samples and hepatocyte isolation

Fresh liver tissues were obtained from New Zealand white rabbits and hepatocytes were isolated in reference as described elsewhere[8]. For liver tissues they were rinsed with cold Hanks solution (NaCl 137; KCl 5.0; CaCl$_2$ 1.3; MgSO$_4$·7H$_2$O 0.8; Na$_2$ HPO$_4$ 0.6; KH$_2$PO$_4$ 0.4; NaHCO$_3$ 3.0; glucose 5.6 mmol·L^{-1}; pH7. 4). Blood vessels and other tissues were removed carefully. After washing with Hanks solution, the liver was cut into about 1 mm^3 slices and homogenized or stored at -80℃ for further processing.

RT-PCR of MLCK fragments[9, 10]

For RT-PCR, hepatocytes were directly lysed with TRIZOL Reagents (GibcoBRL), and total cellular RNA was isolated according to the manufacturer's instructions. First-strand cDNA was generated in the presence of 0.5 µg·L^{-1} Oligo (dT) 12-18 from 5 µg total RN A with reverse transcriptase (Superscript™ Preamplification System, GibcoBRL). The paired primers to detect endogenous MLCK fragments were designed according to the r eported MLCK sequence, and they are paired primer one: 5'> AAGAATTCGATGTCAGCTGAAC <3' and 5' CTTCTCCAGAAGCTTATAGGA <3', and paired primer two: 5'> CCACTGGTG AAGCTTAAAATC<3' and 5'> TGGAATTCCATGGGGGACGTGAA <3'. The PCR was performed in reference as described elsewhere[9]. The PCR products were examined by 20 g·L^{-1} agarose gel and ethidium bromide staining. The photos were taken for the analysis of the PCR products.

Preparation of MLCK from rabbit liver

Using metho d described by Ramji *et al* and Tang *et al*[11], the liver tissues were obtained. Briefly, the liver slices removed blood vessels and other tissues were put into glass homogenizer with addition of suit amount of tissue buffer for homogenizat ion on the ice block, and the homogenized tissues were thawed for three times (5 min once) after homogenizing. 16 000 r·min^{-1} centrifugation for 30 min at 4℃, and MLCK was prepared by the method described by Wang *et al* and Bartelt *et al*. The purified MLCK was used for activity assay

Assay of calmodulin- and calcium-dependent MLCK activity

Ca^{2+}/CaM-dependent activity of MLCK was measured by rates of [γ-^{32}P]ATP incorporation into myosin light chain as substrate referring to Wang *et al* and Blumenthal *et al*. Briefly, the relation of MLCK activity with time, regulatory light chain and CaM concentrations as well as with MLCK concentrations were analyzed. Maximal activity was determined in the reaction buffer containing 50 mmol·L^{-1} MOPS in mmol·L^{-1} at pH 7.0: magnesium acetate 10, dithiothereitol 1, CaCl$_2$ 0.3; 1 mmol·L^{-1} [γ-^{32}P] ATP (200-300 cpm· pmol^{-1}) ; 1.2 µmol·L^{-1} CaM, 25 µmol·L^{-1} regulatory light chain of myosin, and diluted MLCK at room temperature. MLCK was

freshly diluted in 10 mmol·L^{-1} MOPS (pH 7.0), 1mmol·L^{-1} dithiothreitol, and 1 g·L^{-1} bovine serum albumin and a dded to the reaction mixture. Final MLCK concentrations used in kinetic measurements showed linear phosphorylation rates with respect to time and enzyme concentration in a certain range.

Protein contents determination

Lowry's method was used for the determination of contents of various proteins, in which bovine serum albumin was used as the standard.

Statistical analysis

Data of MLCK activity were collected and processed through analysis of difference of MLCK activity by Student's t test with SPSS 8.0 Windows software. $P<0.05$ or $P<0. 01$ was considered as statistically significant in the difference of MLCK activity.

RESULTS

Confirmation of MLCK expression in rabbit hepatocytes by RT-PCR

Two pairs of specific primers were used to detect the expression of MLCK in freshly is olated hepatocytes. The total cellular RNA was isolated and first-strand cDNA was generated. The results showed that the RT-PCR products were 400 bp and 450 bp respectively, confirming with the expected molecular size (Figure 1), which suggests that there is expression of MLCK in rabbit liver.
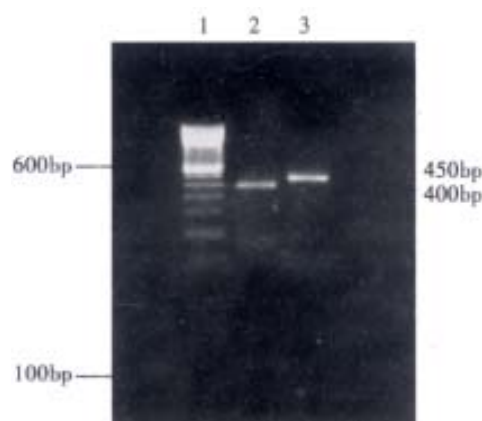

Figure 1 Reverse transcription polymerase chain reaction of cellular total RNA from hepatocytes in New Zealand rabbit. 1.100 bp DNA ladder; 2-3, PCR products: amplified MLCK DNA fragments.

Features of rabbit liver MLCK activity analysis

Comparison of activity of rabbit liver MLCK with that of smooth muscle MLCK To observe the activity level of our isolated MLCK in rabbit liver, we assayed the activity of the MLCK and smooth muscle MLCK. I t was found that both kinds of MLCK had similar catalytic activity, there is no statistically significant difference between two kinds of enzymes ($P<0.01$ £½ by Student's t test (Figure 2 A).

MLCK from rabbit liver was CaM-dependent To confirm the MLCK isolated from the rabbit liver is dependent on CaM, we assayed the effect of phosphorylation of MLC K on regulatory light chain of myosin. It was found that the light chain was obv iously phosphorated when CaM was added into the reaction buffer at the suitable concentration of Ca^{2+}. The MLCK activity increased markedly with CaM added. Ther e is significantly statistical difference when compared with the activity of the MLCK without adding CaM ($P<0.01$) through Student's t test analysis with SPSS 8.0 Windows software. See Figure 2 (B).
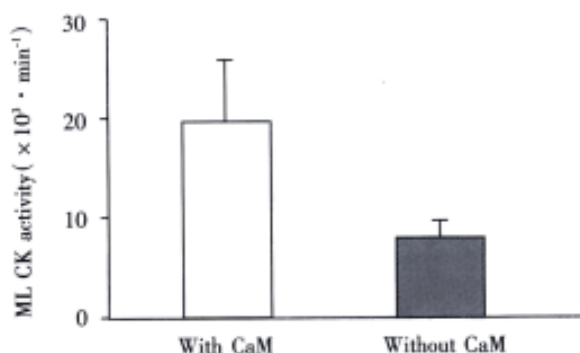
Figure 2 (A) Comparison of enzymatic activity between smMLCK and rabbit liver MLCK ($P>0.05$)
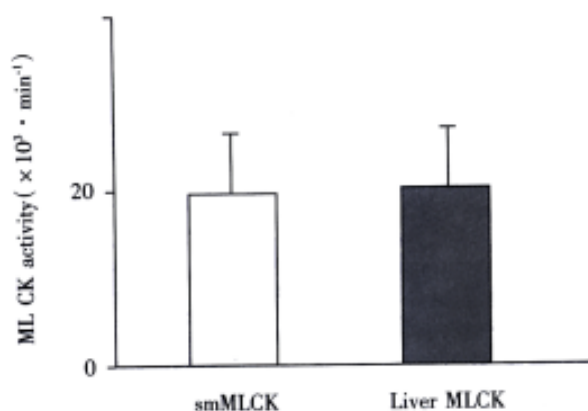

Figure 2 (B) Effect of CaM on MLCK in rabbit liver activity ($P<0.01$)

Action characteristics of MLCK of rabbit liver In the reaction system, enzymatic catalytic action of the MLCK increased gradually at first three min and the extent of phosphorylation of the regulatory light chain of myosin was at the highest level at about three min on the suitable concentrations of substrate, Ca^{2+} and CaM. In addition, the activity of MLCK of rabbit liver changed with the changes of the substrate concentrati on or the concentration of light chain of myosin. When the concentration was 0.65 mg·L^{-1}, the activity of MLCK was at the highest level, the most amount of light chain was phosphorated at this time. Finally, MLCK concentration influenced t he activity of the enzyme itself. On the suitable substrate, CaM and calcium ion concentrations, the activity of MLCK from the rabbit liver was basically at the higher level when MLCK ranged from 15 mg·L^{-1} to 25 mg·L^{-1}.

DISCUSSION

MLCK is the key regulator of cell motility and smooth muscle contraction in higher vertebrates. MLCK expression shows a complex pattern. In undifferentiated myoblasts, 220-kulong or non-muscle form of MLCK is expressed during differ entiation of skeletal muscle. During myoblast differentiation, expression of the 220-ku MLCK declines and expression of this long-form is replaced by 130 ku smooth muscle MLCK and a skeletal muscle-specific MLCK. In fact, 130 ku smooth muscle MLCK is not a smooth muscle-specific protein, it is ubiquitous in all adult tissues[12]. A prerequisite for vertebrate smooth muscle contraction, potentiation of skeletal and cardiac muscle contraction and various non-muscle motile events in response to intracellular Ca^{2+} signaling is the phosphorylation of myosin II RLC at Ser-19 by MLCK. The phosphorylation of RLC increases the Mg^{2+}-ATP activity of myosin, which catalyses cyclic conversion of ATP chemical energy into mechanical work through the reversible actomyosin interactions. Ca^{2+}-calmodulin -dependent phosphorylation of RLC of myosin by the catalytic COOH-terminal half of MLC K activates myosin II in smooth and non-muscle cells. Three-dimensional reconst ructions showed MLCK density on the extreme periphery of subdomain-1 of each actin monomer forming a bridge to the periphery of subdomain-4 of the azimuthally adjacent actin. There is interaction of MLCK-147 close to the COOH terminus of the first actin and near residues 228-232 of the second. The unique location ensure that MLCK binds to actin without interfering with the binding of any other key actin-binding proteins, including myosin, tropomyosin, caldesmon, and calponin[13]. In addition, the unique sequence of MLCK-210 is involved in its interacti on with the microfilaments and contributes to its tighter association with the actin cytoskeleton[14]. Non-muscle cells may use different mechanisms for targe ting the long-form MLCK to actomyosin structures during interphase and mitosis. MLCK and myosin II phosphatase act cooperatively to regulate the level of Ser 19 -phosphorylated myosin II during mitosis and initiate cytokinesis through the a ctivation of myosin II motor activity[15].

Ca^{2+}/CaM forms a ternary complex with MLCK, facilitating in activation of the kinase and phosphorylation of RLC. Ph osphorylation induces a conformational change, which allows myosin crossbridges to along actin filaments. Some non-muscle processes are also regulated by RLC phosphorylation of the myosin, while smooth muscle MLCK plays important roles in contractile-motile processes of a variety of cells[16]. MLCK has an actin-binding activity in addition to its kinase activity, which assembles actin filaments in to bundles morphologically and biochemically. There are two actin-binding sites on MLCK, including calcium- and calmodulin-sensitive site and insensitive site . The cross-linking between this two sites assembles actin into bundles[17]. Recently, it is found that a novel approximately 60 ku MLCK immunogen contributes to the aberrant contractility associated with preterm labour[18], while Sohn *et al* reported that calmodulin and MLCK play a role in Ach-induced lower esophageal sphincter contraction, whereas the classical MLCK may not be the major kinase responsible for contraction and phosphorylation of myosin light chain in esophagus. Esophagus contraction is protein kinase C dependent contraction[19]. Various new functions of MLCK have been found recently, activation of myosin II by MLCK produces force for many cellular processes including mitosis, migration, and other cellular shape changes. Inhibition or potentiation of myosin II activation via over-expression of a dominant negative or wild type MLCK can delay or accelerate tumor necrosis factor-á induced apoptotic cell death in cells [20]. MLCK specific ally mediates agonist-induced sarcomere organization during early hypertrophic response[21]. During vascular injury, the expression of MLCK decreased[22]. In rabbit portal vein myocytes, MLCK mediates noradrenaline-evoked non-selective cation current[23]. In the liver, agents that elevate intracellular free Ca $^{2+}$ concen tration increase tight junctional permeability and stimulate bile canalicular contraction. Myosin phosphorylation is probably responsible for the tight junction al permeabilization caused by elevation of intracellular Ca^{2+} in hepatocytes. Moreover, the integrity of the phosphorylation system of myosin is essential for normal bile flow. In addition, hepatic sinusoidal Ito cells (fat storing cells) play a regulatory role on hepatic sinusoidal blood flow through their contraction, while the integrity of myosin light chain kinase is essential for Ito cell con traction and normal sinusoidal blood flow. However, the roles of myosin phosphor ylation by MLCK in non-muscle tissues is not well characterized but correlates with important activities such as cell division, receptor capping, etc. Recently, the study showed the existence of a 208 ku protein, named embryonic MLCK because its expression can be detected in early embryonic

tissues, stem cells, and in proliferating cultured cells. In the liver, down-regulation of this 208 ku embryonic MLCK is not so dramatic, and the less dramatic decline in the expression of embryonic MLCK may possibly reflect the high regenerative capacity of liver tissue. It is now found that MLCK is associated with non-muscle cells closely[1,2,4-7]. MLCK activation is also a critical step in the cytoskeletal changes causing pseudopod formation during polymorphonuclear leukocyte phagocytosis[24]. MLC K immunoreactivity was found to be colocalized with the insulin granules which suggests that it increases insulin granules in the ready-releasable pool by acting on different steps in the secretory cascade[25]. In 3T3 fibroblasts, MLCK is responsible for phosphorylation of MLC at the cell periphery, showing its unique spatial regulation of myosin RLC[26]. From the gene of vertebrate smooth muscle and non-muscle MLCK there are at least four proteins expressed. Two high molecular weight MLCK splice variants, EC MLCK-1 and EC MLCK-2 (210-214 ku) in human en dothelium are identical except for a deleted single exon in MLCK-2 encoding a 69 - amino acid stretch that contains potentially important consensus sites for phosphorylation by p60 (Src) kinase, while p60 (Src)-mediated tyrosine phosphorylation represents an important mechanism for splice variant-specific regulation of n on-muscle and cascular cell function[27]. MLCK is also associated with the gap fomation and endothelial hyperpermeability of coronary venular endothelial cell monolayers[28]. Our preliminary study revealed that MLCK in the rabbit liver could phosphorate myosin light chain obviously, and was calmodulin-dependent, which may play an important role in maintaining the normal functions of the tissue. Bu t in the liver, what form of MLCK expressed more, long form MLCK (embryonic MLCK) or short form MLCK with the molecular weight about 130-150 ku? What are their exact roles in the liver? What roles it will play in liver regeneration, in liver injury or in hepatic carcinoma? And what are the action mechanisms? These remain to be elucidated in our further study.

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