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***Clonorchis sinensis*: molecular cloning and localization of myosin regulatory light chain**

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Abstract One cDNA clone was purified from an adult *Clonorchis sinensis* cDNA library, and its deduced polypeptide sequence was found to be homologous with myosin regulatory light chain (MRLC) of invertebrates and vertebrates. Two amino-acid residues, Thr and Ser, were conserved at the phosphorylation sites that regulate the function of MRLCs. Recombinant *C. sinensis* MRLC (rCsMRLC) protein was produced and purified from *Escherichia coli*, and mouse anti-CsMRLC immune sera recognized a protein of molecular weight 24 kDa from a soluble protein preparation of *C. sinensis*. The CsMRLC protein was immunohistochemically localized to the muscle fibers of the subtegumental muscle layer and to the muscles of oral and ventral suckers. However, the rCsMRLC protein proved to be less useful antigen for the serodiagnosis of human clonorchiasis.

Keywords *Clonorchis sinensis* · Myosin regulatory light chain · Muscle fiber

Introduction

The Chinese liver fluke, *Clonorchis sinensis*, inhabits the biliary passages of mammals including those of man. Moreover, clonorchiasis is endemic in China, Korea, Japan, and Vietnam. People are infected by eating raw or inadequately cooked freshwater fish, which harbor infective metacercariae. Infection by this fluke causes epithelial hyperplasia and dilatation of biliary passages

(Rim 1986; Choi et al. 2004), and its clinical symptoms are proportional to worm burden. The common symptoms of acute infection are epigastric pain, malaise, and hepatitis-derived jaundice, whereas in the advanced chronic stage, these include obstructive jaundice, liver enlargement, and toxemia. In addition, it has been reported epidemiologically and experimentally that fluke infections promote the development of cholangiocarcinoma (Flavell 1981; Lee et al. 1994; Shin et al. 1996), and such reports emphasize the importance of early diagnosis and chemotherapy.

The myosins have been less conserved than actins during protein evolution. Myosins of nonmuscular cells form fine, short bipolar filaments as compared with those of skeletal muscle cells. Myosins I, II, and V are found in nonmuscular cells, and participate in organelle movements such as in membrane transport, phagocytosis, and during pseudopodium extension in amoeba (Uchimura et al. 2002). Myosin II is composed of a pair of heavy chains and dissimilar light chains. The essential light chain and regulatory light chain are associated with the head group of myosin heavy chain.

In smooth muscles, the phosphorylation of myosin regulatory light chain (MRLC) stimulates contraction, whereas dephosphorylation causes relaxation (Ikebe and Hartshorne 1985a, b). In MRLC, phosphorylation occurs at several amino acid residues in the N-terminal region of the protein. Phosphorylations of Ser19 and/or Thr18 of MRLC regulate the strength of muscle contraction (Ikebe et al. 1987), and myosin light chain kinase (MLCK) regulates smooth muscle contraction through myosin activation. MLCK is activated by Ca²⁺-calmodulin complex binding, and this activated MLCK complex phosphorylates Thr18 and Ser19 in myosin regulatory light chain (MRLC) and activates the protein (Bruce et al. 2001).

The present study was conducted to characterize *C. sinensis* MRLC by secondary structural analysis of the putative polypeptide deduced from its cDNA sequence, and to localize this entity in the adult fluke and to evaluate its serodiagnostic usefulness.

The nucleotide sequence reported herein was submitted to GenBank and assigned accession number AY519356.

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Materials and methods

cDNA cloning

An expression cDNA library (Hong et al. 2000) of adult *C. sinensis* was immunoscreened using the pooled sera of clonorchiasis patients and several positive clones were purified. Plasmid DNA's were then extracted from positive clones and cDNA inserts were sequenced and searched for in the GenBank database. Of these clones CsMRLC32, encoding a polypeptide similar to MRLC was selected for further characterization.

Expression and purification of recombinant MRLC protein

The coding region of cloned CsMRLC32 cDNA was double-digested with *Bam*HI and *Hind*III endonucleases and electrophoresed in 1% agarose gel. The cDNA fragment was then excised from gel and recovered using a QIAEXTM II gel extraction kit (QIAGEN, Hilden, Germany). It was then directionally subcloned into an expression vector pRSET-A (Invitrogen, Carlsbad, USA), and transformed into *Escherichia coli* (*E. coli*) NovaBlue (Novagen, Madison, USA) using a ligation mixture by the heat-shock method. Plasmid DNA's were extracted from colonies on LB plates containing 50 µg/ml of ampicillin, and sequenced to confirm that the open reading frame (ORF) of the insert cDNA was in-frame with the tag peptide. *E. coli* BL21(DE3)pLysS (Novagen, Madison, USA) was then transformed with recombinant pRSET-CsMRLC plasmid DNA by the heat shock method and the transformed colonies were selected from LB plates containing 50 µg/ml ampicillin and 34 µg/ml chloramphenicol. The rCsMRLC protein was induced by adding 1 mM of IPTG to the culture medium, and the recombinant protein was purified in its native condition by Ni-NTA affinity column chromatography, according to the manufacturer's instruction (QIAGEN) and eluted using a buffer containing 250 mM imidazole.

The quality of the rCsMRLC protein obtained was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The purified rCsMRLC protein was electrophoresed in 12.5% SDS-polyacrylamide gel and stained with Coomassie Brilliant blue or electrotransferred onto nitro cellulose (NC) membranes. The NC membranes were blocked with 5% skim milk and incubated overnight with mouse anti-XpressTM antibody (Invitrogen, CA, USA), reacting with the epitope of tag peptide, at a dilution of 1:5,000 in PBS/Tween-20. After washing, NC membranes were incubated in peroxidase-conjugated antimouse IgG at a dilution of 1:2,000. Color was developed using 4-chloro-1-naphthol.

Production of mouse immune serum

The purified recombinant protein (200 µl) was mixed with an equal amount of complete Freund's adjuvant (Sigma, St. Louis, MO, USA) and injected into the abdominal cavity of each 6 BALB/c mice. Two weeks later, the same amount of recombinant protein was mixed with incomplete Freund's adjuvant and injected into the mice. After another 2 weeks, the mice were boosted with a 20 µl injection of the aqueous recombinant protein into a tail vein. Three days later, blood was taken from a mouse tail vein and checked for antibody production by immunoblotting. Blood was then collected from an orbital vein of antibody-producing mice. Sera were separated and stored in a freezer until required.

Immunoblotting for wild MRLC

To detect wild *C. sinensis* MRLC, a soluble protein extract was prepared with normal saline from adult flukes, subjected to 12.5% SDS-PAGE, and transferred onto NC membranes. Membranes were blocked with 5% skim milk and incubated in mouse anti-CsMRLC immune serum at a dilution of 1:100. Following washing, the NC membranes were incubated with the secondary antibody, alkaline phosphatase (AP)-conjugated antimouse IgG at a dilution of 1:2,000. Color was developed using BCIP/NBT (Sigma).

Immunohistochemical staining

Adult *C. sinensis* flukes recovered from a rabbit liver were fixed in 10% neutral formalin and processed for paraffin embedding. Sectioned flukes in paraffin ribbons, 5-µm thick, were deparaffinized and incubated in the mouse immune serum at dilutions of 1:100 or 1:200. Preimmune mouse serum was employed as a negative control. The ribbons were incubated sequentially in biotinylated antimouse IgG antibody and peroxidase-conjugated streptavidin (Zymed Lab, South San Francisco, CA, USA). Color was developed using di-amino-benzidine followed by Harris' hematoxylin counterstaining.

Immunoblotting of fluke-infected patient sera

The rCsMRLC protein deployed by 12.5% SDS-PAGE was transferred onto NC membranes and blocked with 2% skim milk. The NC membranes were then cut into strips and incubated with fluke-infected human sera at a dilution of 1:200 (Lee et al. 2003). The AP-conjugated antihuman IgG at a dilution of 1:2,000 was used as a secondary antibody, and color was developed using BCIP/NBT. The human sera used in this study were obtained from twenty clonorchiasis patients, eighteen

opisthorchiasis viverrinii patients, seventeen paragonimiasis westermani patients, fifteen fascioliasis patients, and from eleven normal humans as negative controls. Infection by respective flukes was proven by either stool examination or worm recovery.

Results

cDNA and polypeptide sequences of *C. sinensis* MRLC

The cloned cDNA clone CsMRLC32 was 1603-bp long and encoded a putative polypeptide of 204 amino acid residues. The cDNA consisted of a 43-bp 5'-untranslated region and a 922-bp 3'-untranslated region followed by a poly (A₂₃) tail (Fig. 1). Molecular mass and isoelectric point of the CsMRLC32 polypeptide were calculated to be 23.6 kDa and 6.79, respectively. The CsMRLC32 polypeptide showed a high level of sequence identity (45.9–48.8%) with the MRLCs of vertebrates and invertebrates (Fig. 2). The two amino acid residues, Thr19 and Ser20, are highly conserved among animal MRLCs. The CsMRLC32 polypeptide revealed Thr and Ser at the same conserved positions in the N-terminus of MRLC. The secondary structure of the CsMRLC32 polypeptide was predicted to a coiled structure comprising eight helices connected by coiled-coils. The CsMRLC32 had an extended N-terminus peptide se-

Fig. 1 The nucleotide and deduced amino acid sequences of *Clonorchis sinensis* myosin regulatory light chain (CsMRLC) cDNA. Asterisks indicate a translational stop codon. Untranslated regions are represented by lower case letters

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-43 ccgcacacctacggttcggttcaacagaaggcaaacagttcggcc
ATGGGTGACGATAAGAAAGAAAAGAAGGAGAAAAAAGAGAAGAAGGATAAGAAGGAAAAGGAGCACAAAGAAGAAGCGCCAGCCGATGCT 90
M G D D K K E K K E K K E K K D K K E K E H K E E A P A D A
GGAGCCCGCGCAGAGGAGAAAAGGAATCAGGCGGTGGAGGCCAAACGTGCCCAAAGGCCACCTCCAATGTCTTTGGCATGTTTCCACAG 180
G A P A E E K K E S G G G G K R A Q R A T S N V F G M F P Q
AATCAAATCCAAGAATTCAAAGAGGCCCTTCACTCTTATTGATCAAAAACGGGATGGATTTCATTGATATTGAAGATCTGAAGGATATGTAC 270
N Q I Q E F K E A F T L I D Q N R D G F I D I E D L K D M Y
GCATCCTTGGGTGCAACCCCAAGTACGCGGAACCTCAAGGAAATGCTTGATGAATCCCCTGGTCCACTTAACTTACAATGTTTCATCAAT 360
A S L G R T P S D A E L K E M L D E S P G P L N F T M F I N
CTGTTCCGGTGAGAAGTTGAACGGTACCGATCCTGAGGACGCCGTTACGAAAATGCATTTCCGAATGTTTCCGACCCCGGCAACAAAACGAT 450
L F G E K L N G T D P E D A V T K M H F A M F R P R Q Q N D
ATTTGGAGGAGGAATATATTAAGGACCTCCTACAGAATATGGGCGATAATTTTCAGCGCGGAAAGAAATTCGTCAAACCTTGGAAAGAGGCT 540
I W R R N I L R T S Y R I W A I I S A R K E I R Q T W K E A
CCAATCAAGGAAGGCAAACCTGGACTATAACGAATTCGTCACCCTAATCAAACGGGGCAATCAAGACCAACTTTAAactccctgctttctg 630
P I K E G K L D Y N E F V T L I K R G N Q D Q L *
ctgctgcttactactaaaacgcctgcctcacacagcaactgaccaattcgcattcactgtatTTTTTTTcgtgctgtgacgcctcctcacc
tcgaaccacattcacagccgggggtcgttgttgtaactatccccTTTTTcgttttgattcctcatgattatgcacaattaccatttttagc
aaaaccgataaaaatacagattcccgcctaactcggaagcttccaaaccaataacctgaagcctggctttcaaatatcaatcactgttatt
catcttccctTTTTTaaacgaccatagttttgtcaaaaccagtcctcttagaatcaccctagtgatcactctcaccatactactgaata
gcagatacaaaactttgtttaaaaatacgaactttcccgactaccacccctctgcaaaaatctttctgtttctggtttcgtttgggaccag
cactcttccagttcttctgatcaagccgatgatgataagggcattccaaactttgtcttttctggtttgtttgacctgtctcctcaaac
agtcatgtggtgagcagcacacatgtgatgatgatacactttcttatgtggccttatcactgtgtccgtgtccatgatttcagcag
gatgggacgttagacagcaagcagcatggacggctctttcattcttagggagattggtcctaatttttcgaaacctttctccccgtttctgctg
tgactcttgaactggcggagagacgtacaggaaccacccgcttccaaagcatcatggttccctggacagtaaaactgtttccgattttgtt
tgttcaataaaagcattgtttgtttggcagcgtgtatgtttgagctttccagtgatggttacacttccatgtggcaataaccatacga
ttcattgaaaaaaaaaaaaaaaaaaaaaaaa 1560

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quence compared to vertebrate MRLCs, which also had a predicted coiled-coil structure (Fig. 2). Given these results, the cDNA clone CsMRLC32 was considered to encode the MRLC of *C. sinensis* (CsMRLC).

Recombinant and wild CsMRLC proteins and immune sera

The rCsMRLC protein was expressed using the pRSET-CsMRLC construct, as a fusion protein of 32 kDa in *E. coli* BL21(DE3)pLysS strain by IPTG induction, and was purified as a soluble protein to high purity by Ni-NTA affinity chromatography. The fusion protein had a tag peptide of molecular mass about 8 kDa, derived from the expression vector, and an additional peptide from the 5'-untranslated region of CsMRLC32 cDNA. In this respect, the rCsMRLC protein produced had the expected molecular mass of approximately 24 kDa (Fig. 3). Mouse immune sera raised against rCsMRLC protein reacted to the immunogen at dilution exceeding 1:5,000 (data not shown).

The mouse immune sera recognized a protein of molecular mass ca 24 kDa in the soluble protein extract of adult *C. sinensis* (Fig. 4).

Localization of wild-type MRLC

In adult *C. sinensis*, MRLC was localized as a brown staining to the muscle fibers of the subtegumental muscle layer, to those of oral and ventral suckers, and to the muscle fibers of the myoepithelium of intestinal caecae, uterus, and metraterm (Fig. 5).

CsMRLC

MGDDKKEKKEKKEKKDKKEKEHKEEAPADAGA

CsMRLC PAEEKKESGGGGKRAQRAT**TS**NVFGMFPQNQIQEFKEAFTLLIDQNRDGFIDIEDLKDMYASLGR
 Mercenaria DSDKKAKAA**TS**SVLTKFTQNQIQEMKEAFTMIDQNRDGLIDVSDLKEMYSNLGA
 Xenopus MSSKRAKTKTTKKRPQRAT**TS**NVFAMFDQSQIQEFKEAFNMIDQNRDGFIDKEDLHDMLASLGR
 Chicken MSSKKAKTTKTTKKRPQRAT**TS**NVFAMFDQSQIQEFKEAFNMIDQNRDGFIDKEDLHDMLASLGR
 Mouse RPQRAT**TS**NVFAMFDQSQIQEFKEAFNMIDQNRDGFIDKEDLHDMLASLGR
 Pig SSKRAKAKTTKKRPQRAT**TS**NVFAMFDQSQIQEFKEAFNMIDQNRDGFIDKEDLHDMLASLGR

CsMRLC TPSDAELKEMLDESPGPLNFTMFINLFGEKLNLTDPEDAVTKMHFAMFRPRQONDIWRRNILR
 Mercenaria CPQDSVLQAMIKEAP-QLNFTGFLNLFSEKMSGTDPEETLRQA-FQMFDADNTGYIPEEYMKD
 Xenopus NPTDEYLDAMMNEAPGPINFTMFLTMFGEKLNLTDPEDVIRNA-FACFDEEAIGFIQEDNLRE
 Chicken NPTDEYLDAMMNEAPGPINFTMFLTMFGEKLNLTDPEDVIRNA-FACFDEEATGFIQEDYLRE
 Mouse NPTDEYLEGMMNEAPGPINFTMFLTMFGEKLNLTDPEDVIRNA-FACFDEEASGFIHEDHLRE
 Pig NPTDEYLEGMMSEAPGPINFTMFLTMFGEKLNLTDPEDVIRNA-FACFDEEASGFIHEDHLRE

| CsMRLC | <u>TSYRIWAIISARKEIROQWKEAPI</u> -KEGKLDYNEFVTLIKRGNQDQL | Identity (%) |
|------------|---|--------------|
| Mercenaria | LLENMGDNFTKD-EIROQWKEAPI-SGGKVDYNAFVAKIK-GKEQEDA | 45.9 |
| Xenopus | LLTTMGDRFTDE-DVDELFREAPIDKKGNFNNEFTRILKHGAKDKDD | 48.3 |
| Chicken | LLTTMGDRFTDE-EVDELYREAPIDKKGNFNIEFTRILKHGAKDKDD | 48.8 |
| Mouse | LLTTMGDRFTDE-EVDEMYREAPIDKKGNFNVEFTRILKHGAKDK | 47.7 |
| Pig | LLTTMGDRFTDE-EVDEMYREAPIDKKGNFNVEFTRILKHGAKDKDD | 48.3 |

Fig. 2 Multiple alignment of CsMRLC polypeptide with the myosin regulatory light chain (MRLC) peptides of other vertebrates and invertebrates. The conserved threonine and serine residues are indicated by *bold characters*. Predicted helix-forming regions in CsMRLC are *underlined*

6–30% of the sera of patients infected with other flukes (Table 1). Overall, rCsMRLC protein showed a sensitivity of 25% and a specificity of 84% for clonorchiasis.

Antigenicity of rCsMRLC protein

To evaluate serologic antigenicity, rCsMRLC protein was immunoblotted against fluke-infected patient sera. The rCsMRLC protein produced positive reactions with 25% of the sera of clonorchiasis patients, and with

Discussion

Clonorchis sinensis larvae that excyst from metacercariae migrate up through the ampulla of Vater and biliary tracts and grow to adult flukes. They feed on blood cells and epithelial debris detached from biliary mucosa by lacerative movements (Rim 1986). These movements are

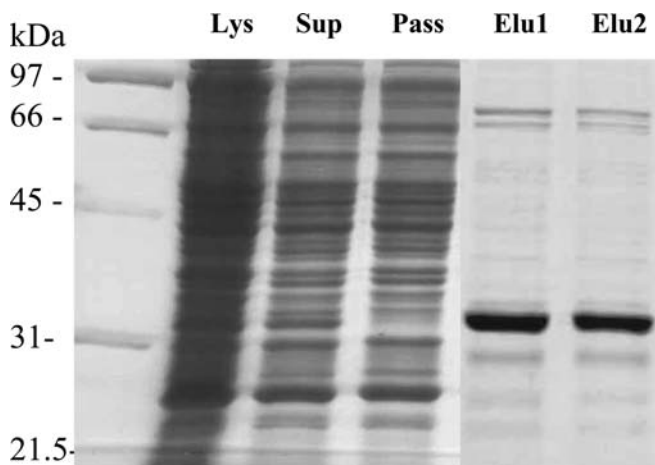


Fig. 3 Purification of recombinant CsMRLC (rCsMRLC) produced in *E. coli* by Ni-NTA affinity chromatography. *Lys*, lysate; *Sup*, supernatant; *Pass*, pass through, *Elu1* and *Elu2*, eluates 1 and 2, respectively

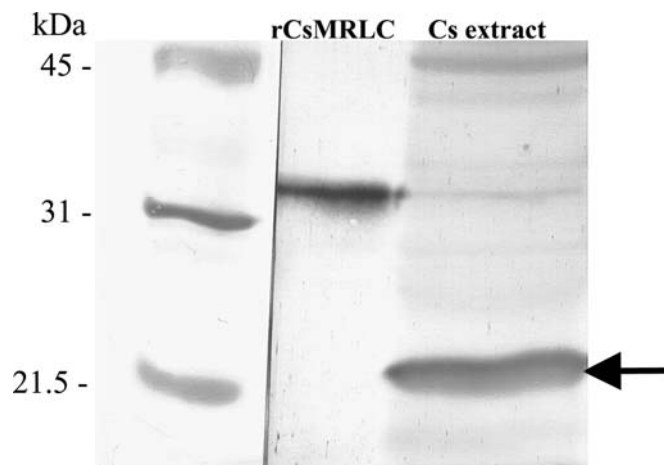


Fig. 4 Native CsMRLC. The CsMRLC (arrow) was detected in *C. sinensis* extract by immunoblotting using a mouse immune serum. rCsMRLC, recombinant CsMRLC

Fig. 5 Localization of MRLC in adult *C. sinensis* tegument. Immunohistochemical staining was done using normal mouse (a) and immune mouse (b) sera. Positively stained are fibrillar structures in subtegumental muscle layer (SM). Ct, cyton; Tg, tegument

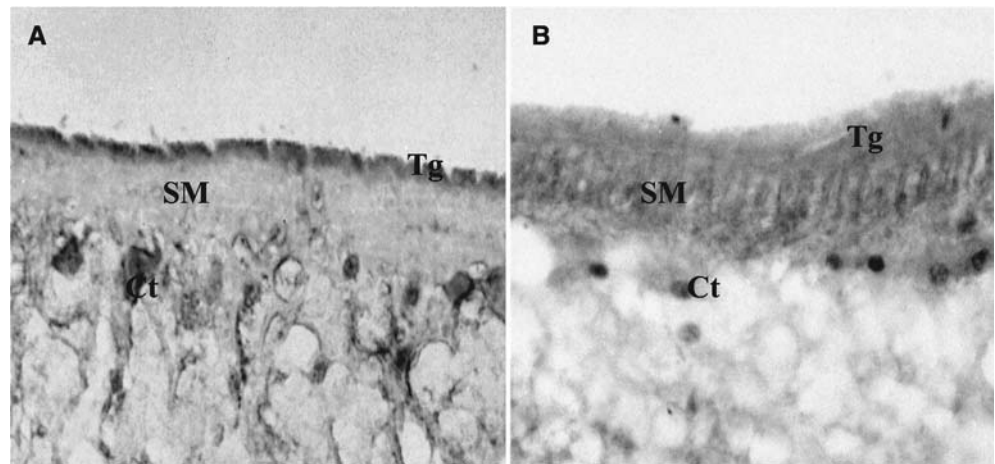


Table 1 Seroreactivity of recombinant CsMRLC (rCsMRLC) protein to trematode-infected patient sera by immunoblotting

| Sera | Clonorchiasis | Paragonimiasis | Fascioliasis | Opisthorchiasis | Control |
|---------------------|---------------|----------------|--------------|-----------------|---------|
| No. of tested | 20 | 17 | 15 | 18 | 11 |
| No. of positive (%) | 5 (25) | 5 (30) | 3 (20) | 1 (6) | 1 (9) |

coordinated by muscle relaxations and contractions, which are regulated in part by the phosphorylation of amino acid residues in MRLCs. Myosin consists of a globular head and a coiled tail, and the globular head region is associated with the essential and regulatory light chains (Devlin 1997).

The CsMRLC polypeptide is conserved at two amino acid residues, Thr19 and Ser20, at the same positions in the N-terminal region of the MRLCs of vertebrates and invertebrates (Ikebe et al. 1994). The muscle-contracting activity of myosin is regulated by the phosphorylation of the Thr18 and Ser19 residues (Ikebe et al. 1998), which is catalyzed by MLCK (Marston and Redwood 1991). Increased cytosolic Ca^{2+} levels stimulate the formation of Ca^{2+} -calmodulin complex and binding to MLCK, which results in MLCK activation. The activated MLCK then catalyses the phosphorylation of target amino residues in myosin light chains (Stull et al. 1997). Rho kinase, activated by Rho protein, catalyzes the phosphorylation of the regulatory light chain of the myosin II isoform, and inhibits the myosin light chain phosphatase (Onishi et al. 1982). The secondary structure of CsMRLC was predicted to be a largely coiled-coil structure to allow it to sit in the helical grooves of the globular myosin head. Based on the conserved positioning of the Thr and Ser residues and the overall coiled-coil secondary structure, it was suggested that these two amino acids are target residues for the regulatory phosphorylation of CsMRLC.

The CsMRLC was found to be a 24-kDa protein, which approximates to its calculated molecular mass. In vertebrates, the MRLCs of smooth muscle and non-muscle cells have a molecular mass of 20 kDa (Trybus and Warshaw 1991; Roger et al. 1991). As compared to vertebrate MRLCs, CsMRLC has an N-terminal

extension of 32 amino acids and an approximate molecular mass of 3.6 kDa. If the N-terminal extension is subtracted, CsMRLC would have a molecular mass 20 kDa, which is comparable with its vertebrate counterparts.

The CsMRLC was found to be localized in the muscle fibers of several muscular tissues in adult *C. sinensis*. The CsMRLC expression, estimated from intensity and extent of staining, was found to be proportional to amount of muscular tissue in adult *C. sinensis* (Hong et al. 2003). Collectively, it is conceivable that CsMRLC play a major role in body movement by controlling muscle contractions.

As serodiagnostic antigens for clonorchiasis, soluble protein preparations from *C. sinensis* have been reported to have high sensitivity but poor specificity. On the other hand, the recombinant proteins of *C. sinensis*, i.e., 26- and 28-kDa glutathione *S*-transferases, and clonin, were found to be highly specific but insensitive serodiagnostic antigens for human clonorchiasis (Kang et al. 2001; Hong et al. 2002; Lee et al. 2003). This was also found to be the case for rCsMRLC protein. The use of a cocktail of antigens of molecularly defined proteins has been proposed to improve sensitivity and retain specificity (Kim et al. 2001).

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