# Unique sequence of a high molecular weight myosin light chain kinase is involved in interaction with actin cytoskeleton

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**Abstract Myosin light chain kinase (MLCK) is the key regulator of cell motility and smooth muscle contraction in higher vertebrates. We searched for the features of the high molecular weight MLCK (MLCK-210) associated with its unique N-terminal sequence not found in a more ubiquitous lower molecular weight MLCK (MLCK-108). MLCK-210 demonstrates stronger association with the Triton-insoluble cytoskeletons than MLCK-108, suggesting the role for this sequence in subcellular targeting. Indeed, the expressed unique domain of MLCK-210 binds and bundles F-actin in vitro and colocalises with the microfilaments in transfected cells reproducing endogenous MLCK-210 distribution. Thus, MLCK-210 features an extensive actin binding interface and, perhaps, acts as an actin cytoskeleton stabiliser.**



*Key words:* Myosin light chain kinase; Calmodulin; Cytoskeleton; Microfilament

## 1. Introduction

A prerequisite for vertebrate smooth muscle contraction and various non-muscle motile events in response to intracellular $Ca^{2+}$ signalling is the phosphorylation of myosin II regulatory light chain (RLC) at Ser-19 by a calmodulin (CaM)-regulated protein kinase, myosin light chain kinase (MLCK). The phosphorylation of RLC enhances the $Mg^{2+}$-ATPase activity of myosin, which catalyses cyclic conversion of ATP chemical energy into mechanical work through the reversible actomyosin interactions. The molecular mechanism for transduction of a $Ca^{2+}$ signal into muscle contraction and cell motility changes via the CaM/MLCK-regulated pathway is one of the most extensively characterised signal transduction systems in cell biology, evolving from years of investigation by a number of different groups (for review, see [1,2]).

It is now established that vertebrate MLCK is encoded by a single gene locus that produces two size classes of the enzyme with computed masses of about 108 000 and 210 000 (further referred to as MLCK-108 and MLCK-210). MLCK isoforms are differentially expressed in smooth muscle and non-muscle tissues and during embryonic development [3,4]. Both MLCK-210 and MLCK-108 use a common set of exons to encode the catalytic and kinase-related protein (KRP) segments that are characteristic of most vertebrate MLCKs. Recent reports [5,6] suggest that in mammals, both size classes of MLCK may include multiple splice variants, adding further complexity to the system.

The molecular difference between MLCK-210 and MLCK-108 is the presence of an extended amino-terminal tail region in the former isoform. This unique domain has sequence motifs (e.g. six immunoglobulin C2-type-related folds [3,6]) characteristic of proteins involved in extended protein-protein interactions, including those that interact with actomyosin. Here, we present evidence that the unique sequence of MLCK-210 is, indeed, involved in the interaction of this kinase isoform with the microfilaments and contributes to its tighter, than MLCK-108, association with the actin cytoskeleton.

## 2. Materials and methods

### 2.1. Materials

General reagents of analytical and molecular biology grade were from Sigma and Fisher Scientific. Monoclonal antibody that recognises both MLCK-108 and MLCK-210 (clone K-36) was from Sigma.

### 2.2. Antibody production and immunoblots

Rabbit polyclonal antibodies #859 that recognise both MLCK-210 and MLCK-108 [7], #3007 against KRP domain of MLCK [3] and #3528 against a peptide antigen corresponding to amino acid residues 306–321 of chicken MLCK-210 [3] have been described. Mouse monoclonal antibody 2F4 was produced to the same synthetic peptide as in [3]. 2F4 monoclonal antibody belongs to the IgG1 subtype and recognises MLCK-210 in chicken aortic smooth muscle cells (Fig. 1).

Immunoblots were developed using either enhanced chemiluminescence (ECL) (Phototope-HRP, NEB) or 3,3′-diaminobenzidine. HP-IIcx scanner and NIH Image software were used to quantitate stained gels and immunoblots.

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*Abbreviations:* CaM, calmodulin; GFP, green fluorescent protein; HMM, heavy meromyosin; KRP, kinase-related protein; MLCK, myosin light chain kinase; PMSF, phenylmethylsulfonylfluoride; RLC, $M_r$ 20 kDa regulatory light chain; SMM, smooth muscle myosin


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*2.3. Protein purification and analysis*

MLCK-210 was purified from adult chicken aorta. Briefly, the tissue was homogenised at 4°C and extracted with buffer E (20 mM MOPS, pH 7.4, 2 mM EGTA, 5 mM DTT, 10 mM benzamidine, 0.1 mM *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 0.05 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1 mM phenylmethylsulfonylfluoride (PMSF)) containing 400 mM NaCl, 30 mM $MgCl_2$ and 0.02 mg/ml aprotinin. Clarified extract was diluted 10-fold with buffer E and applied onto a 1 ml DE-52 (Whatman) column. Concentrated crude MLCK-210 was step-eluted with buffer E containing 240 mM NaCl and the concentration of NaCl in eluate was adjusted to 0.5 M. MLCK-210 was finally affinity-purified on #3528 antibody coupled to protein A-Sepharose CL-4B (Pharmacia Biotech). After washing the column with the 1 M NaCl-containing buffer E, MLCK-210 was eluted with 0.2 M glycine, pH 2.2, 0.5 M NaCl, 1 mM DTT, 0.2 mM PMSF and neutralised immediately with 2 M Tris-HCl, pH 8.0. No MLCK-108 contamination was detected in MLCK-210 preparation. MLCK-210 was quantitated by immunoblotting using K-36 monoclonal antibody and turkey MLCK-108 as a standard. Based on the primary structure information [3], the assumption was made that both MLCK isoforms are equally stained by K-36 antibody.

MLCK-210 catalytic activity was measured according to [8] and was shown to be $Ca^{2+}$-CaM-dependent. MLCK-210 had a specific activity of 0.7 and 0.8 μmol Pi/min/mg with heavy meromyosin (HMM) and RLC as substrates, respectively. Specific activity of chicken gizzard MLCK-108 towards RLC was 1.6 μmol Pi/min/mg under the same conditions. Like MLCK-108, MLCK-210 phosphorylated the same peptide as revealed by 1-D tryptic phosphopeptide mapping [9] and conferred to smooth muscle myosin (SMM) the ability to support actin filament movement in an in vitro motility assay (data not shown).

Rabbit skeletal actin [10], turkey gizzard MLCK-108 [11], unphosphorylated chicken gizzard myosin [8] and HMM [12] were prepared as described. The protein concentration was determined spectrophotometrically using the following extinction coefficients and molecular masses: SMM, $A^{1\%}_{280\,nm} = 5.6$, 500 kDa; HMM, $A^{1\%}_{280\,nm} = 6.5$, 330 kDa; G-actin, $A^{1\%}_{290\,nm} = 6.1$, 43 kDa.

Sedimentation analysis was performed as described [13] but in the presence of 100 mM NaCl. Low speed centrifugation was conducted at $12\,000 \times g$ for 30 min at 4°C. For electron microscopy [13], protein solution was diluted immediately before the application on the grids with an ice-cold buffer to achieve the final actin concentration 0.1 mg/ml.

*2.4. Plasmid construction and expression*

The 7n-32-R clone in phage λgt 11 [3] was used as an initial template to design N934 construct containing the unique sequence of MLCK-210 (amino acids 1–934). PCR was performed using Pfu DNA polymerase, 5′-primer 5′-AAAGATCTCATGGGGGATGTTAAAC-3′ containing the *Bgl*II restriction site (underlined) and 3′-primer 5′-TTGGATCCTATTGCTCGGCTGGG-3′ which included stop codon and the *Bam*HI restriction site (underlined). The PCR product was subcloned into pET-30c(+) vector (Novagen). N934 was expressed in BL21(DE3) pLys *Escherichia coli* strain as a fusion protein containing N-terminal 6×His-tag and S-tag and purified using Ni-NTA metal-chelate chromatography (Qiagen).

For the transient expression in cultured cells, the N934 DNA sequence was subcloned into the pFLAG CMV2 vector (Kodak) at the *Bgl*II and *Bam*HI restriction sites to provide expression of N934 with the FLAG sequence and nine additional amino acids (Leu-Ala-Ala-Ala-Asn-Ser-Ser-Ile-Asp) at the N-terminus.

MLCK-210-green fluorescent protein (GFP) construct in pEGFP vector (Clontech) was designed using the MLCK-210 clone described in [3] and kindly provided by Dr Anne Bresnick (Albert Einstein College of Medicine, Bronx, NY, USA).

*2.5. Cell culture and manipulation*

Primary cells from chicken embryo aorta (day 19) were prepared by 0.25% trypsin dissociation. These cells, HeLa cells (ATCC# CCL-2) and A10 cells (ATCC# CRL-1476) were cultured in DMEM supplemented with 10% FBS (HyClone). For immunofluorescence microscopy, cells were fixed in 3.7% formaldehyde and permeabilised with 1% Triton X-100. F-actin was visualised using TRITC-phalloidin (Sigma). Mouse monoclonal antibody to FLAG (Kodak) and FITC-labelled anti-mouse secondary antibodies (Sigma) were used. Cells were examined using Zeiss Photomicroscope III and photographed on Kodak Tri X-pro 400 film.

HeLa cells at 50–70% confluence were transfected using the Maxifectin-21 kit (Pepline, Moscow, Russia) according to the manufacturer's protocol and after 8–24 h of incubation, they were processed for immunofluorescence.

Confluent A10 cells were extracted with the buffer containing 20 mM Tris-Cl, pH 7.4, 125 mM sucrose, 0.05% Triton X-100, 2 mM EGTA, 1 mM PMSF, 100 μM leupeptin, 1 μM pepstatin and 0–0.5 M KCl concentrations. The extracts were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

## 3. Results

*3.1. MLCK-210 and MLCK-108 exhibit differential extraction properties*

We examined the extraction of MLCK-210 and MLCK-108 from cultured cells using established procedures for cytoskeletal protein solubilisation. We used A10 rat aorta-derived smooth muscle cells which express both MLCK-108 and MLCK-210 (Fig. 2). Cells were extracted in buffers containing Triton X-100 and increasing concentrations of KCl and analysed for MLCK presence by immunoblotting. As shown in Fig. 2, MLCK-108 was readily extracted into the soluble fraction at a 100 mM KCl concentration, whereas most of the MLCK-210 remained associated with the Triton-insoluble pellet fraction under these conditions. At 200 mM KCl, MLCK-108 was detected exclusively in the soluble fraction, while MLCK-210 was nearly equally distributed between soluble and particulate fractions. Only at 300 mM KCl and higher, MLCK-210 was localised mostly in the soluble fraction. These results demonstrate that MLCK-210 and MLCK-108 differ in their extraction properties and raise the possibility that the unique N-terminal part of MLCK-210 contributes to its stronger association with the Triton-insoluble particulate fraction.

*3.2. MLCK-210 and its unique amino-terminal segment have actin binding activity*

To directly assess interactions of MLCK-210 with the cytoskeletal elements, we conducted a co-sedimentation assay of

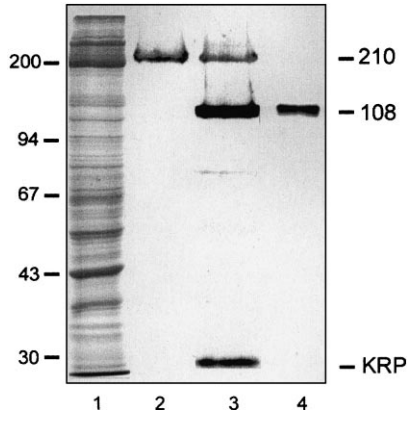


Fig. 1. Specificity of 2F4 monoclonal antibody. Fifteen μg of chicken aorta extract was loaded per each lane, resolved by SDS-PAGE and either stained for proteins (1) or blotted onto PVDF membrane and probed with 2F4 monoclonal antibody (2) and polyclonal anti-KRP antibodies (3). The position of purified turkey gizzard MLCK-108 (20 ng) was also identified with anti-KRP antibodies (4). Positions of MLCK isoforms are marked as 210 and 108 and molecular weight markers are shown on the left.

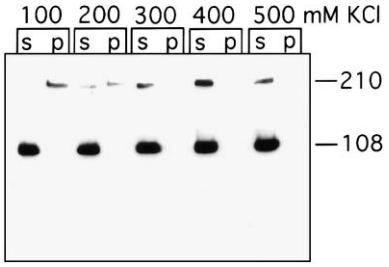


Fig. 2. Differential extraction of MLCK-210 and MLCK-108 from A10 cells. The concentrations of KCl in extraction buffer are indicated on the top of each panel. Triton-soluble supernatant fractions (s) and insoluble particulate fractions (p) were analysed by immunoblotting using polyclonal anti-MLCK antiserum #859. Positions of MLCK isoforms are marked as 210 and 108.

F-actin and SMM filaments with MLCK-210 and N934. Fig. 3A shows that MLCK-210 does not sediment by itself, whereas in the presence of F-actin or SMM, it distributes into the pellet. Inclusion of chicken gizzard KRP in the mixture of MLCK-210 and SMM results in attenuated binding of the kinase to myosin filaments. Fig. 3B demonstrates that N934 binds to actin but its interaction with SMM was not significant when myosin was used at concentrations sufficient to sediment MLCK-210. The presence of smooth muscle tropomyosin, caldesmon and $Ca^{2+}$-CaM in the incubation mixture does not affect N934 interaction with actin (data not shown).

Under low speed sedimentation conditions in which F-actin does not pellet, N934 causes F-actin to sediment (Fig. 4A). This is suggestive of formation of actin filament aggregates. Electron microscopic analysis of mixtures of N934 and F-actin revealed the presence of actin bundles (Fig. 4C), whereas F-actin did not form the bundles in the absence of N934 (Fig. 4B). This suggests that the unique N-terminal tail region of

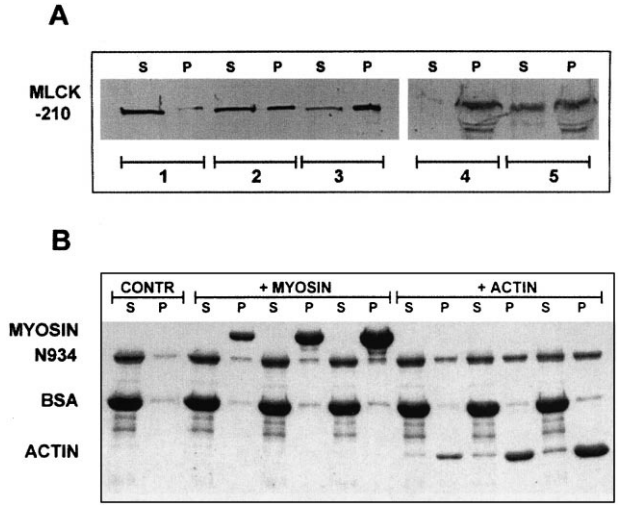


Fig. 3. Co-sedimentation assay of MLCK-210 and its N-terminal segment with actin and myosin. A: Affinity-purified MLCK-210 was incubated without (1) or with 2 μM F-actin (2), 4 μM F-actin (3), 0.6 μM SMM (4), 0.6 μM SMM and 18 μM KRP (5). The supernatants (S) and pellets (P) were immunoblotted and MLCK-210 was visualised with #3528 specific antibody. B: Recombinant N934 (3 μM) was incubated without (CONTR) and with 0.75, 1.5 or 3 μM SMM (+MYOSIN) or with 3, 6 and 12 μM F-actin (+ACTIN). The protein content of supernatants (S) and pellets (P) was assessed by Coomassie staining. BSA, bovine serum albumin.

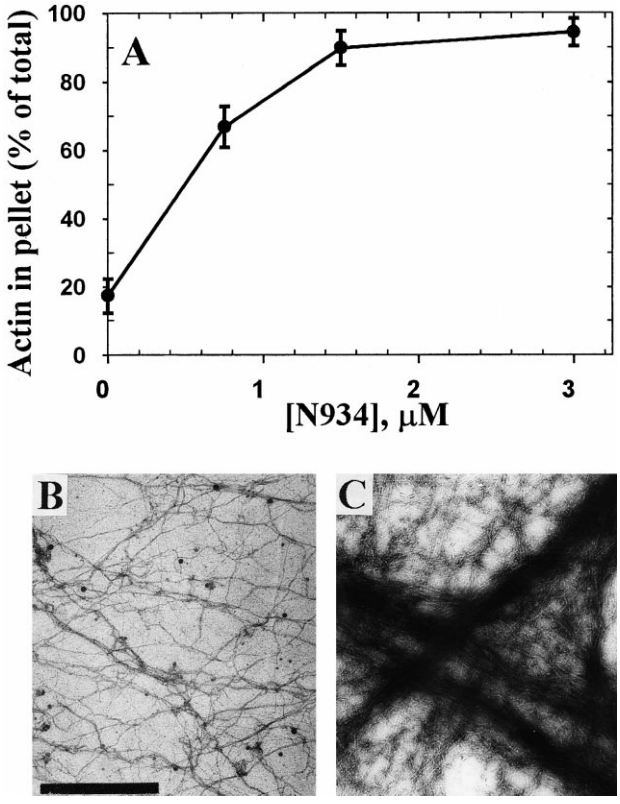


Fig. 4. N-terminal segment of MLCK-210 bundles F-actin in vitro. (A) Ten μM F-actin was mixed with increasing concentrations of N934 in the presence of 100 mm NaCl and subjected to low speed centrifugation in the microfuge. Each point represents mean ± S.D. of four experiments. (B) electron micrographs of F-actin and (C) of F-actin-N934 mixture (7:1 = mol:mol). Scale bar, 1 μm.

MLCK-210 contributes to the association of the kinase with actin filaments and confers actin cross-linking properties to it.

### 3.3. *MLCK-210 and its unique N-terminal segment localise with the microfilaments in cultured cells*

MLCK-210 specific 2F4 antibody stains microfilament bundles in primary chicken embryo aortic cells (Fig. 5A), although there appears to be a somewhat more heterogeneous distribution of staining among cells compared to the actin filament distribution (Fig. 5B). Transient transfection of MLCK-210-GFP construct into HeLa cells produced a pattern similar to the antibody staining, i.e. GFP fluorescence was mainly associated with actin bundles (Fig. 5C,D). To confirm the ability of the N-terminal domain of MLCK-210 to target filamentous actin in living cell, a N934 construct encoding N-terminal FLAG sequence was transiently transfected into HeLa cells. Fig. 5E,F shows that accumulation of N934 on the microfilaments of transfected cells was observed. These results suggest that the amino-terminal sequence of MLCK-210 is involved in subcellular targeting of the enzyme to the microfilaments.

## 4. Discussion

The unique N-terminal domain of MLCK-210 which is not found in MLCK-108 may harbour activities that provide for the differential features of MLCK isoforms in terms of cata-

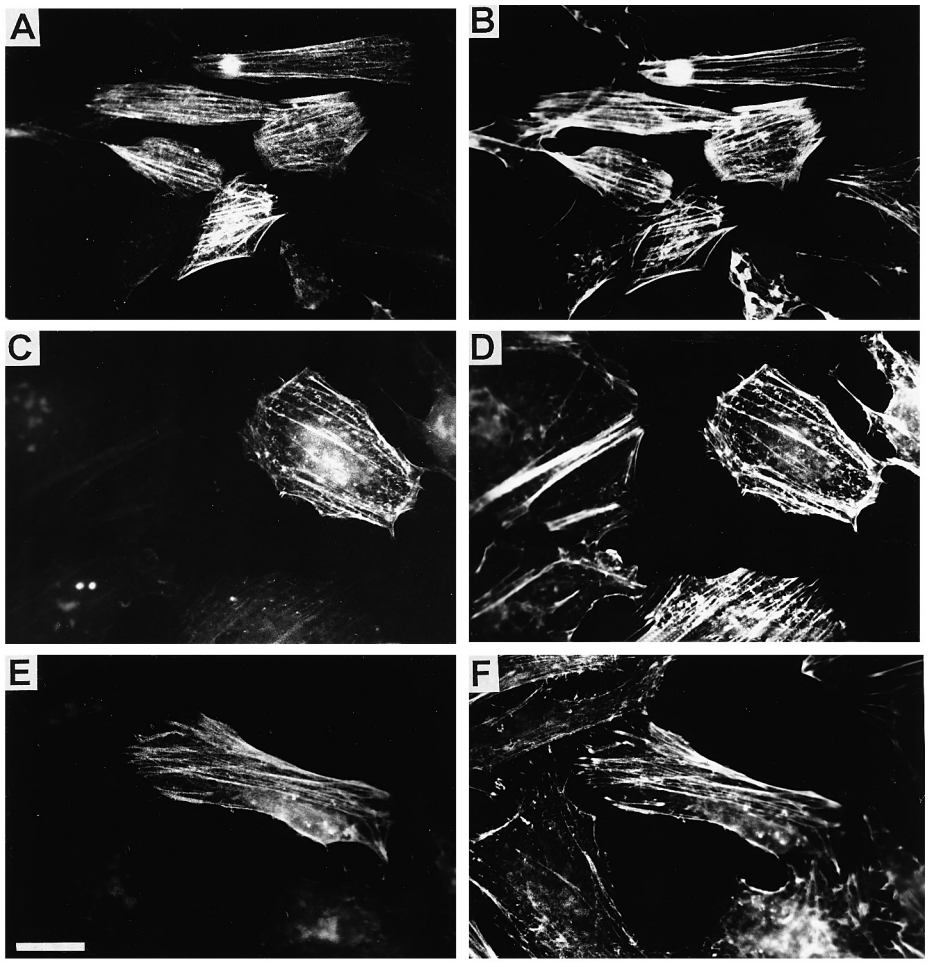


Fig. 5. Association of MLCK-210 and its N-terminal segment with microfilaments in cultured cells. Primary chicken embryo aortic cells were stained with 2F4 monoclonal antibody and FITC-labelled secondary antibody (A) and TRITC-phalloidin to visualise F-actin (B). HeLa cells were transiently transfected with pEGFP-MLCK-210 construct (C,D) or pFLAG-N934 construct (E,F). (C) self-fluorescence of GFP, (D,F) actin visualised by TRITC-phalloidin, (E) anti-FLAG staining as revealed by FITC-labelled secondary antibody. Scale bar, 25 μm.

lytic activity or cytoskeletal interactions. In the study described here, we examined these two hypotheses.

We designed a method to purify MLCK-210 from chicken aorta and this allowed us for the first time to quantitatively compare its specific activity with that of MLCK-108 (see Section 2). With the necessary corrections for the higher molecular weight of MLCK-210, the activity of both isoforms was similar. Hence, the unique tail of MLCK-210 is either not involved in modulation of MLCK catalytic activity or additional modifications such as tyrosine phosphorylation hypothesised by Garcia et al. [14] are required for this to happen.

It is well documented that MLCK-108 binds actin and myosin in vitro and MLCK-108 antibodies as well as MLCK-108 expressed in transfected cells decorate microfilaments [8,15–17]. Similar features were demonstrated in our experiments with regard to MLCK-210 (Fig. 3, 5A,B). We have shown previously that the KRP domain of MLCK-108 contributes to its interaction with myosin [13,18]. The results presented here extend this to MLCK-210 (Fig. 3).

While our studies have been focused on smooth muscle cells where MLCK-210 is associated with the microfilaments, others have reported additional localisation of a high molecular weight MLCK in endothelial cells. Briefly, Verin et al. [19] and Garcia et al. [14] observed a punctate pattern of MLCK staining not coincident with the microfilaments and similar punctation partially overlapping a filamentous actin network in thrombin-stimulated or diperoxovanadate-treated cells. Perhaps, MLCK associates with the cellular structures other than microfilaments in these cells. The nature of potential alternative MLCK-210 intracellular targets would be of great interest and additional studies might reveal their molecular basis.

Cell extraction experiments demonstrated the stronger association of MLCK-210 than MLCK-108 with the detergent-insoluble particulate fraction of A10 aortic smooth muscle cells, suggesting that the amino-terminal sequence of MLCK-210 provides additional linkages to the cytoskeletal elements. The microfilament-associated distribution of MLCK-210 established in this work (Fig. 5) indicates that likely targets for the unique tail domain would be actin, myosin and macromolecules interacting with these proteins. Indeed, we demonstrated that N934 binds F-actin in vitro and colocalises with actin bundles in living cells (Fig. 3B, 4 and 5C,D).

The actin bundling activity of N934 suggests that there is an extended actin binding site or, perhaps, several sites within the MLCK-210 unique sequence. MLCK-108 was also shown to bundle F-actin in vitro [20] by means of the two discrete actin

binding sites located in the N-terminus [21]. Because MLCK-210 incorporates all of the MLCK-108 sequence, MLCK-210 is likely to have a superior actin binding and bundling capacity. This would be consistent with its lower extractability from cultured cells.

The very C-terminal part of MLCK-210 unique sequence contains two DXRXXL motifs similar to three such motifs identified in the N-terminus of MLCK-108 and demonstrated to affect MLCK-108 interaction with microfilaments [22]. The distribution of precisely spaced DXRXXL motifs across the border of the specific and common parts of MLCK sequence suggests the presence of a continuous actin binding region in this area which is entirely used by MLCK-210 and partially by MLCK-108. Altogether, our results indicate that MLCK-210 may serve similar roles in cell function as MLCK-108 and has an additional function as a more efficient microfilament network organiser due to the presence of extra actin binding site(s) in its unique N-terminal sequence.

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