Properties of Long Myosin Light Chain Kinase Binding to F-Actin in Vitro and in Vivo*

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Lula Smith‡§, Mojgan Parizi-Robinson‡, Min-Sheng Zhu‡, Gang Zhi, Ryosuke Fukui, Kristine E. Kamm, and James T. Stull¶

From the Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9040

Short and long myosin light chain kinases (MLCKs) are Ca²⁺/calmodulin-dependent enzymes that phosphorylate the regulatory light chain of myosin II in thick filaments but bind with high affinity to actin thin filaments. Three repeats of a motif made up of the sequence DFRXXL at the N terminus of short MLCK are necessary for actin binding (Smith, L., Su, X., Lin, P., Zhi, G., and Stull, J. T. (1999) J. Biol. Chem. 274, 29433–29438). The long MLCK has two additional DFRXXL motifs and six Ig-like modules in an N-terminal extension, which may confer unique binding properties for cellular localization. Two peptides containing either five or three DFRXXL motifs bound to F-actin and smooth muscle myofilaments with maximal binding stoichiometries consistent with each motif binding to an actin monomer in the filaments. Both peptides cross-linked F-actin and bound to stress fibers in cells. Long MLCK with an internal deletion of the five DFRXXL motifs and the unique NH₂-terminal fragment containing six Ig-like motifs showed weak binding. Cell fractionation and extractions with MgCl₂ indicate that the long MLCK has a greater affinity for actin-containing filaments than short MLCK in vitro and in vivo. Whereas DFRXXL motifs are necessary and sufficient for short MLCK binding to actin-containing filaments, the DFRXXL motifs and the N-terminal extension of long MLCK confer high affinity binding to stress fibers in cells.

MLCK, a dedicated protein kinase activated by Ca²⁺/calmodulin, phosphorylates the regulatory light chain of myosin II. This signaling event enhances actin-activated myosin ATPase activity that mediates contraction in smooth muscle and striated muscles as well as nonmuscle cells (3, 5, 11–13). The long MLCK is identical to the short MLCK except for an extended N terminus with two additional putative actin-binding motifs in tandem with the three demonstrated actin-binding motifs plus six additional immunoglobulin-like modules. Four of its five actin-binding motifs contain DFRXXL residues, whereas the most N-terminal motif contains a conservative substitution in one residue, DVRXXL. Because the short MLCK contains only DFRXXL and the conservative substitution of valine for phenylalanine occurs in only one of the five motifs, we will use a generic nomenclature of DFRXXL for all of these motifs.

Although the actin-binding properties of short MLCK have been defined in vitro and in vivo (1, 17, 18), it is not clear whether the five actin-binding motifs of long MLCK share an identical functional role in terms of binding abilities. Poperechnaya et al. (19) found different distributions of the MLCKs in HeLa cells with short MLCK, primarily cytoplasmic, in contrast to long MLCK localized to stress fibers (19). However, short MLCK was localized to stress fibers in fibroblasts and A7r5 cells (1, 18). Additionally, specific localization of the long MLCK to the cleavage furrow in dividing cells required both the N-terminal extension with six Ig-like modules and five DFRXXL motifs. Many actin-binding proteins modulate the function of actin filaments (20, 21). Accordingly, smooth muscle MLCK has been shown to cross-link actin filaments into bundles (22–24). However, the region of the kinase required for
actin bundling has not been delineated.

To address these issues, we expressed peptides containing either three or five actin-binding motifs and examined their biochemical properties in vitro and in vivo. In addition, we investigated binding properties of wild-type and mutated short and long MLCKs in vivo (Fig. 1).

EXPERIMENTAL PROCEDURES

Construction of N-terminal MLCK Peptides—A short MLCK peptide containing residues 1–147 (3DFR-MLCK) was prepared using PCR and the expanded high fidelity system (Roche Molecular Biochemicals). A C-terminal His tag and a phosphorylation site corresponding to the recognition site for protein kinase A (17) was originally constructed by Poperechnaya et al. (19). The full-length MLCK gene to maintain reading frames for the two genes. In cell cultures, eGFP-MLCK210 plasmid was originally constructed by Poperechnaya et al. (19). The full-length coding region for chicken smooth muscle long MLCK without a stop codon was inserted in eGFP-NI vector (CLONTECH Inc.) between the EGFP gene driven by a cytomegalovirus promoter. Two extra guanine nucleotides were introduced immediately after 5718 nt of the long MLCK gene to maintain reading frames for the two genes. In cell cultures, eGFP-MLCK210 was used as wild-type control (long MLCK-GFP). The 5’DFRXXL region (2638 to 3033 nt) of long MLCK was deleted by PCR with the long MLCK-GFP plasmid as a template and using primer pairs 5’-GCAAAAATGAAGATATCTTCACACTG-3’ and 5’-GCTAGCGGCGATCGCGAGTCTTCCTGCTCTTC-3’. In this fragment, an original EcoRV site in the gene remained in the 5’ end and an NheI site was introduced in the 3’ end. After subcloning in TOPO vector (CLONTECH Inc.), a 0.28-kb fragment was produced by EcoRV and NheI digestion and ligated to long MLCK-GFP vector in which a region between 2638 and 3033 nt was removed by EcoRV digestion. The final construct (5’DFR) long MLCK-GFP) was confirmed by sequencing.

To delete the region that contains 5DFR and the full-length short form of MLCK (2638 to 5721 nt), a fragment corresponding to the region 2361 to 2637 nt was first amplified by PCR with primer pairs 5’-GCAAAAATGAAGATATCTTCACACTG-3’ and 5’-GACGTGCACTAGGGATCCCCCTTCCTGCTCTTC-3’ and an EcoRV site and a BamHI site in the 5’ and 3’ termini, respectively. This fragment was subcloned into TOPO vector and confirmed by sequencing. It was removed from TOPO vector by EcoRV and BamHI digestion and ligated with long MLCK-GFP vector that was previously digested with EcoRV and BamHI. The resultant fragment (N-term), long MLCK-GFP, contained the N-terminal six Ig-like motifs of long MLCK.

Protein Expression—Recombinant plasmids harboring 3DFR-MLCK or 5DFR-MLCK were transformed into BL21(DE3) cells. Expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside as described (17). Cells were harvested at 8,000 × g for 10 min, resuspended in 5 ml phosphate-buffered saline, 5 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml pepstatin, and lysed by sonication at 4 °C. The lysed suspension was then centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant fraction was filtered with a 0.45-μm filter (Nalgene) and proteins were purified using histidine affinity column chromatography (Novagen). The purified proteins were dialyzed against 20 mM MOPS, pH 7.5, 10 mM NaCl, 1 mM MgCl2, and 1 mM dithiothreitol and stored at −80 °C. GST fusion peptides containing one, two, or three DFRXXL motifs (GST-30, GST-55, and GST-75, respectively) were expressed and purified as described previously (17).

Phosphorylation of DFR-MLCK Peptides—DFR-MLCKs at 50 μM were phosphorylated at the C-terminal sequence LRRASLG by the catalytic subunit of protein kinase A (5 μg/100-μl reaction) in the presence of 100 μM ATP (15 μCi) in 10 mM Tris-HCl, pH 7.4, 5 mM MgCl2, and 1 mM dithiothreitol and stored at −80 °C. Most of the unincorporated 32P was removed with spin columns containing buffer used in the binding assay.

Extraction of Smooth Muscle Myofilaments—Chicken gizzards were homogenized in 10 mM MOPS, 50 mM NaCl, and 1 mM dithiothreitol at pH 7.5 with a Polytron homogenizer (Brinkmann Instruments). Homogenized tissue was centrifuged at 17,320 × g for 10 min at 4 °C. The pellets were homogenized in 10 ml of 10 mM Tris-HCl, pH 7.5, 2 mM EGTA, 2 mM dithiothreitol, 50 mM MgCl2, and 5% Triton X-100 to dissociate endogenous MLCK. After washing in the detergent-containing buffer five times, the myofilaments were then washed three times in 10 mM MOPS, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol and stored in the same buffer on ice.

Filament Binding—A co-sedimentation assay was used to characterize MLCK binding to smooth muscle myofilaments. Phosphorylated DFR-MLCKs were mixed with 0.70 mg/ml myofilaments in a final volume of 150 μl. The mixture was incubated for 30 min at 4 °C in 10 mM imidazole, pH 7.2, 50 mM KC1, 1 mM MgCl2, 0.1 mM EGTA, 10% glycerol, and 0.2 mg/ml bovine serum albumin. The reactions were centrifuged at 17,320 × g for 5 min at 4 °C. An aliquot of the supernatant fraction was subjected to Scatchard analysis. Binding to F-actin was performed as previously reported (17).

F-Actin Cross-linking—F-actin from rabbit skeletal muscle was prepared (25). Increasing concentrations of DFR-MLCK peptides (1–10 μM) were incubated with 6 μM F-actin at 25 °C, for 1 h in 10 mM MOPS, 100 mM NaCl, 1 mM EGTA, and 1 mM dithiothreitol. The reaction mixture was centrifuged at 12,000 × g for 30 min. The pellet and supernatant fractions were resolved by SDS-PAGE and immunoblotted with an actin
monoclonal antibody. The relative actin content in the pellet and supernatant fractions was quantified by densitometry.

**Cell Culture and Transfection—**FuGENE 6 (Roche Molecular Biochemicals) was used to transfect A7r5 or HeLa cells seeded in 60-mm tissue culture dishes at a density of $6 \times 10^5$ in 5 ml of Dulbecco's modified Eagle's medium, 10% fetal bovine serum, and 1% penicillin-streptomycin. The cells were incubated overnight at 37 °C and 12 μl of FuGENE 6 was mixed with 288 μl of serum-free Dulbecco's modified Eagle's medium and incubated at room temperature for 5 min. The FuGENE/Dulbecco's modified Eagle's medium mixture was added to 3 μg of plasmid DNA. The mixture was then overlaid onto cells at 65–75% confluence. Cells were incubated 48–72 h at 37 °C. For transfection with LipofectAMINE Plus® (Invitrogen), DNA was diluted into serum-free media (4 μg of DNA was diluted into 750 μl of serum-free medium), and mixed with 20 μl of LipofectAMINE Plus reagent for 15 min. The pre-complexed DNA and the diluted LipofectAMINE reagent were mixed and incubated for 15 min and then transfected with A7r5 cells for 3 h, after which an additional 5 ml of complete medium was added to cells. Transfected cells were incubated at 37 °C and 5% CO₂ for 2 days before experiments.

NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO₂. For DNA transfection, NIH3T3 cells were seeded onto 40-mm round coverslips (40 circles-1D; Fisher) in 60-mm Petri dishes at 20–30% confluence 1 day before transfection. LipofectAMINE was used for DNA transfection as described above. COS-7 cells were transfected by similar procedures and harvested between 1 and 3 days.

**Cell Imaging—**Fluorescence imaging was performed as described previously (26). Twelve-bit images were obtained with a CCD camera (Quantix Photometrics, Tucson, AZ) and Oncor-image software (Oncor, Gaithersburg, MD). Narrow bandpass interference filters (Omega, Brattleboro, VT) were used to detect GFP (490 nm excitation and 520 nm emission) and rhodamine (550 nm excitation and 575 nm emission). During image collection, cells were kept at 37 °C. Subsequently, the samples were added to cells. Transfected cells were incubated at 37 °C and 5% CO₂ for 2 days before experiments.

**Actin Filaments in Cells—**Medium was removed from tissue culture dishes and replaced with 10 μM lysophosphatidic acid or 1 μM latrunculin A in serum-free media for 1 h before microscopy. Populations of actin filaments in cells were separated by differential centrifugation (27). Cells were lysed in 10 mM MOPS, pH 7.0, 1.0% Triton-X-100, 10% glycerol, 0.5 mM EDTA, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 30 μg/ml aprotinin, 0.5 mg/ml diisopropyl fluorophosphate, 0.2 mg/ml trans-epoxyoucycyll-t-leucylamido (guanidino)butane). The lysates were centrifuged at 16,000 × g for 2 min. The pellet fraction contains Triton-insoluble cytoskeleton fibers (P₁). The supernatant fraction was centrifuged at 100,000 × g for 20 min. The second pellet fraction contains Triton-soluble filamentous actin (P₂), whereas the supernatant fraction (S) contains soluble G-actin. The three fractions were analyzed by Western blotting. Monoclonal antibodies against smooth muscle MLCK, GFP, or actin were used as primary antibodies and goat anti-rabbit monoclonal secondary antibodies horseradish peroxidase conjugate was used with the ECL system for visualization. Short and long MLCKs were dissociated from cellular actin filaments by MgCl₂. In additional experiments short MLCK, long MLCK, 3DFR-MLCK, (5DFR) long MLCK, and (N-term) long MLCK as GFP fusion proteins were expressed in COS cells. A10 cells containing both short and long MLCKs or transfected COS-7 cells were lysed in 50 mM MOPS, pH 7.5, containing 0.5 mM EDTA, 1% Triton-X-100, and protease inhibitors, 0.5 mM diisopropyl fluorophosphate and 0.5 mM trans-epoxyoucycyll-t-leucylamido (guanidino)butane. In additional experiments, rabbit lung tissue was homogenized in the same buffer (10 mg of tissue was homogenized in 600 μl of buffer). Appropriate aliquots from each sample were incubated in the lysis buffer with different concentrations of MgCl₂ for 20 min on ice. The samples were then centrifuged at 100,000 × g for 20 min at 4 °C. Subsequently, the supernatant fractions were diluted with SDS sample buffer, resolved by SDS-PAGE, and further analyzed by Western blotting.

**RESULTS**

**DFR-MLCK Peptides Bind to Actin Filaments—**We previously showed using a cosedimentation assay that the three DFRXXL motifs in the N terminus of short MLCK constitute high affinity binding of MLCK to actomyosin filaments from smooth muscle (1, 17). Here we focused on the properties of the two additional motifs found in long MLCK. Fig. 2 and Table I show that both MLCK peptides have similar $K_D$ values of about 0.25 μM. The stoichiometry of binding, on the other hand, was significantly different. The maximal amount of 5DFR-MLCK binding was 0.21 mol of peptide/mol of actin, whereas maximal binding for 3DFR-MLCK was 0.33 mol of peptide/mol of actin. Similar results were obtained with F-actin filaments (Table I). These results support a model in which each DFRXXL motif in both long and short MLCK binds per one actin monomer in actin filaments (16, 17). The similarity in the apparent $K_D$ values for 3DFR-MLCK versus 5DFR-MLCK was surprising, so additional experiments were performed with expressed peptides as well as other MLCK fragments to measure binding to actin containing stress fibers (see below).

**Actin Cross-linking—**To assess the ability of the DFRXXL motifs of MLCK to cross-link actin filaments without additional structural modules found in full-length kinases, we conducted a low speed centrifugation assay in which increasing concentrations of peptides were incubated with a fixed concentration of F-actin (6 μM). F-actin was not found in the pellet under these conditions but sediments when proteins cross-link filaments because of multiple actin-binding sites in a single polypeptide (20, 28, 29). As shown in Fig. 3A, the amount of actin obtained in the pellet fraction was similar for both 3DFR-MLCK and 5DFR-MLCK at similar concentrations of peptides. Fig. 3B shows that a GST fusion protein containing one complete DFRXXL motif (GST30-MLCK) did not cross-link actin and did GST itself. MLCK GST fusion peptide containing two DFRXXL motifs within residues 1–55 (GST55-MLCK) cross-linked 50% of the actin compared with ~90% with GST75-MLCK that contains all three actin-binding motifs. The latter value was similar to maximal effects observed with 3DFR-MLCK and 5DFR-MLCK. These results indicate that the peptides containing multiple DFRXXL actin binding sequences and flanking residues are sufficient for cross-linking actin filaments.
In Vivo Localization of 3DFR-MLCK and 5DFR-MLCK—To analyze the localization of 3DFR-MLCK and 5DFR-MLCK in cultured cells, fluorescent proteins were fused to the C terminus of 3DFR-MLCK or 5DFR-MLCK, respectively. At similar levels of expression after transfection, the 3DFR-MLCK peptide localized to stress fibers in A7r5 cells, and 5DFR-MLCK displayed a similar pattern (Fig. 4). However, 5DFR-MLCK appeared to have more prominent fluorescence staining on stress fibers in HeLa cells compared with 3DFR-MLCK. When cells were made permeable with saponin, the fluorescence remained associated with stress fibers, indicating high affinity binding to actin-containing stress fibers. The fluorescence of GFP itself was diffusely distributed throughout the cytoplasm, and treatment with saponin resulted in loss of fluorescence (data not shown) (1). These results show that peptides containing three or five DFRXXL motifs are sufficient for binding to actin filaments in both smooth and nonmuscle cells.

Binding Properties of Long MLCK-GFP in Vivo—To determine the unique motif or module responsible for long MLCK binding to actin filaments in vivo, expression vectors were constructed for expression of GFP fusion proteins. The full-length long MLCK-GFP showed binding to actin-containing stress fibers in cells (Fig. 5A) confirming previous observations on localization (19). Internal deletion of the five DFRXXL motifs in the (-5 DFR) long MLCK-GFP construct resulted in fluorescence associated with stress fibers with similar results obtained in cells expressing only the N terminus of long MLCK with six immunoglobulin modules but no DFRXXL motifs. The five 5DFR-MLCK fragment by itself bound to actin-containing stress fibers in NIH3T3 cells (Fig. 5), similar to results obtained in A7r5 and HeLa cells (Fig. 4). Additional experiments showed that short MLCK containing no DFRXXL motifs did not bind to stress fibers (data not shown), confirming previously reported observations (1, 18, 26). These results suggest that there are at least two components in long MLCK involved in stress fiber binding: the five DFRXXL motifs in addition to the N-terminal extension.

### Table I

**Binding properties of MLCK peptides**

The DFR-MLCK peptides were incubated with detergent-washed smooth muscle myofilaments or F-actin as described under "Experimental Procedures." After centrifugation the amount of bound peptide was measured for determination of $K_d$ and $B_{max}$ values as shown in Fig. 2. Numbers represent the mean ± S.E. for at least 5 measurements.

| Peptide   | Filaments | $K_d$ (μM) | $B_{max}$ (mol peptide/mol actin) |
|-----------|-----------|------------|----------------------------------|
| 3DFR-MLCK | Myofilaments | 0.30 ± 0.01 | 0.33 ± 0.01 |
| 3DFR-MLCK | F-actin    | 0.26 ± 0.01 | 0.30 ± 0.01 |
| 5DFR-MLCK | Myofilaments | 0.25 ± 0.07 | 0.21 ± 0.01 |
| 5DFR-MLCK | F-actin    | 0.16 ± 0.01 | 0.18 ± 0.01 |

**Fig. 3.** Actin cross-linking by DFR-MLCK peptides. A, F-actin filaments (6 μM) were mixed with increasing concentrations of 3DFR-MLCK or 5DFR-MLCK. The reaction was incubated at 25 °C for 1 h to assemble the filaments into bundles. After low speed centrifugation, the pellet and supernatant fractions were analyzed by Western blotting and the actin content was determined by densitometry. Each point corresponds to the mean ± S.E. (n = 3). , 3DFR-MLCK; O, 5DFR-MLCK. B, actin filaments (6 μM) were mixed with 2 μM GST75-MLCK (GST75), GST55-MLCK (GST55), GST30-MLCK (GST30), or GST alone. F-actin alone was also analyzed. The measurements were performed as described in A.

**Fig. 4.** Cellular localization of 3DFR-MLCK and 5DFR-MLCK. A7r5 cells or HeLa cells were transfected independently with plasmids for 3DFR-MLCK or 5DFR-MLCK. The top panels are images of intact cells and the bottom panels are images acquired after the cells were made permeable with saponin as described under "Experimental Procedures." Representative images for at least five experiments are shown.

**MLCK Binding to F-actin**

![Diagram](image-url)
Distributions of Long and Short MLCKs in Cell Fractions—In an effort to determine the relative affinities of MLCK isoforms for thin filaments, concentrations of filamentous actin were affected in transfected A7r5 cells treated with either lysophosphatidic acid or latrunculin B. Lysates of treated cells were subjected to centrifugation (Fig. 6). Following treatment with 10 μM lysophosphatidic acid for 1 h to stimulate actin polymerization, the short form of MLCK was associated primarily with the low-speed pellet P1 and supernatant fractions, although long MLCK was found only in the P1 fraction. In addition, when cells were treated with latrunculin B for 1 h to disassemble actin filaments, the short form was distributed among P1, P2, and S fractions. However, the long MLCK was still associated only with P1. Similar results were obtained with NIH3T3 cells (data not shown).

In contrast to MLCK, the distribution of the actin was altered by 1 h treatment with lysophosphatidic acid and latrunculin B (Fig. 6). When A7r5 cells were treated with lysophosphatidic acid, actin was found primarily in P1, with some remaining in the supernatant fraction (S). However, treatment of cells with latrunculin B resulted in more actin in the supernatant fraction although a significant portion still remained in P1 (Fig. 6B). Similar results were obtained with NIH3T3 cells treated with lysophosphatidic acid or latrunculin B (data not shown). Additionally, the total amount of actin was similar to nontreated cells.

To rule out effects of transfection or overexpression of short and long MLCK on cellular distributions, the binding of endogenous short and long MLCKs was examined in the smooth muscle cell line A10 that contains each isoform. A10 cells in culture were incubated with lysophosphatidic acid or latrunculin B as described above, and MLCK distributions were measured in cellular fractions by Western blotting. Fig. 6C shows that long MLCK was found exclusively in P1 when cells were pretreated with lysophosphatidic acid or latrunculin B. In contrast, short MLCK was distributed among P1, P2, and S when cells were treated with lysophosphatidic acid. This distribution was not altered when the cells were treated with latrunculin B. Thus, there was a difference between the distribution of endogenous long MLCK versus short MLCK in lysates of A10 cells treated with lysophosphatidic acid or latrunculin B. However, the overall pattern is similar to that obtained with transfected A7r5 and NIH3T3 cells. Collectively, these results suggest that long MLCK has a greater affinity for actin-containing filaments in cells.

Stress Fiber Localization of Long and Short MLCK in the Presence of Lysophosphatidic Acid but Not Latrunculin B—To visualize the binding of the two MLCK isoforms to cellular actin filaments, A7r5 cells were transfected with either short or long MLCK-GFP. In intact A7r5 cells pretreated with lysophosphatidic acid, short MLCK-GFP binds to stress fibers. In addition, there was diffuse fluorescence, which disappeared upon saponin treatment (Fig. 7). The phalloidin staining showed stress fibers and confirmed colocalization with short MLCK (Fig. 7). In contrast, when cells were treated with latrunculin B, they became rounded and all fluorescence from short MLCK-GFP disappeared after permeabilization. In addition, phalloidin staining showed no stress fibers, although amorphous actin aggregates were apparent (Fig. 7). Similar results obtained with short MLCK-GFP, the long isoform was bound to stress fibers in intact A7r5 cells pretreated with lysophosphatidic acid; however, a diffuse fluorescence was much less apparent. The kinase remained bound after permeabilization and phalloidin staining of the cells confirmed the colocalization of the kinase with stress fibers. However, in cells pretreated with latrunculin B, there was also no significant amount of fluorescence remaining after permeabilization.

The apparent discrepancy between the biochemical results showing MLCKs bound in the P1 fraction after latrunculin B treatment and the cellular images demonstrating the absence of MLCK in permeable cells pretreated with latrunculin B was investigated. A7r5 cells transfected with short MLCK were pretreated with latrunculin B, and the distribution of the kinase was examined by centrifugation between intact cells and cells that were treated first with saponin and then washed. In permeable cells pretreated with latrunculin B, there was a decrease in the total amount of kinase recovered after saponin treatment and washing compared with intact cells (data not shown). Thus, the washing procedure after saponin treatment appeared to remove MLCK bound to actin filaments that were not anchored to the coverslip.
released into the supernatant fraction (Fig. 8A). However, long MLCK required 25 mM MgCl₂ to be released into the supernatant fraction. Similarly, in rabbit lung tissue homogenates the short MLCK was found in the supernatant fractions at the lower MgCl₂ concentrations (Fig. 8B). Thus, the extraction of MLCK by MgCl₂ shows that the long isoform has an apparent greater affinity for actin filaments than the short form. These results are consistent with results obtained on the distributions of short and long MLCKs in cell fractions after pretreatment with lysophosphatidic acid or latrunculin B.

FIG. 6. Distribution of short and long MLCK-GFPs and actin in cell fractions of A7r5 cells treated with lysophosphatidic acid or latrunculin B. A, A7r5 cells transfected with short or long MLCK-GFP were harvested after 48–72 h with lysis buffer as described under “Experimental Procedures.” The lysates were centrifuged at 16,000 × g to obtain pellet 1 (P₁) and subsequently at 100,000 to obtain pellet 2 (P₂) and supernatant (S) cellular fractions. The cell fractions were resolved by SDS-PAGE (5%) and analyzed by Western blotting with a monoclonal antibody against MLCK. Representative blots for at least five experiments are shown. B, A7r5 cells were transfected with short MLCK-GFP and processed as described in A. Cell samples were subjected to SDS-PAGE (10%) and analyzed by Western blotting with a polyclonal antibody against actin. Representative blots for at least three experiments are shown. C, A10 cells expressing endogenous short and long MLCK were treated with lysophosphatidic acid or latrunculin B and processed for Western blotting as described in A. Representative blots for at least three experiments are shown.

Short and long MLCKs as well as selected fragments were expressed in COS cells that are similar to HeLa cells with less stress fibers than A7r5 or A10 cells. Analysis of cell lysates incubated with different concentrations of MgCl₂ showed a greater sensitivity for release of both short or long MLCKs than results obtained with A10 cells (Fig. 8C). However, the relative sensitivities were similar; the amount of short MLCK released into supernatant fractions at lower MgCl₂ concentrations was greater than long MLCK. A similar relationship existed for 3DFR-MLCK and 5DFR-MLCK. Interestingly, both (-5DFR) long MLCK and (N-term) long MLCK showed some binding at 0 mM MgCl₂ but were completely released at 2 mM MgCl₂, consistent with weak binding to stress fibers. These results are qualitatively consistent with results obtained with images of transfected NIH3T3 fibroblasts (Fig. 5). Because the fragment containing five DFRXXL motifs binds as well as the two fragments from long MLCK without DFRXXL motifs, we propose that two binding regions in long MLCK may confer higher affinity to stress fibers.

DISCUSSION

We previously showed that the three DFRXXL motifs in short MLCK were necessary and sufficient for high affinity binding to actin-containing filaments (1, 16, 17). However, the structural basis of long MLCK binding to actin-containing filaments is not clear and there are differences in cellular distributions between short and long MLCKs (11, 19). The N-terminal extension of long MLCK with the six immunoglobulin-like modules plus two DFRXXL motifs binds to actin-containing filaments (19). However, it was not determined if this binding was because of DFRXXL motifs, the immunoglobulin-like modules, or both types of structures. In this study we constructed peptides of MLCK consisting of either three or five DFRXXL motifs binds to actin-containing filaments (19). However, it was not determined if this binding was because of DFRXXL motifs, the immunoglobulin-like modules, or both types of structures. In this study we constructed peptides of MLCK consisting of either three or five DFRXXL motifs binds to actin-containing filaments (19). However, it was not determined if this binding was because of DFRXXL motifs, the immunoglobulin-like modules, or both types of structures. In this study we constructed peptides of MLCK consisting of either three or five DFRXXL motifs binds to actin-containing filaments (19). However, it was not determined if this binding was because of DFRXXL motifs, the immunoglobulin-like modules, or both types of structures. In this study we constructed peptides of MLCK consisting of either three or five DFRXXL motifs binds to actin-containing filaments (19). However, it was not determined if this binding was because of DFRXXL motifs, the immunoglobulin-like modules, or both types of structures.
lysates were incubated with 0, 2, or 50 mM MgCl₂. Results are expressed as percent of protein in supernatant fractions with 0 or 2 mM MgCl₂.

MLCK also acts as an actin bundling protein (22–24). Hayakawa et al. (24) reported that MLCK had two actin-binding sites at Asp²-Pro⁴¹ and Ser¹³⁸-Met²¹³ that were necessary for bundling. A peptide containing both sites bundled actin, whereas peptides containing only one site (residues 2–41 or 138–213) did not. However, we found that MLCK peptides containing three or five actin-binding motifs, but lacking the immunoglobulin and fibronectin modules present in the larger peptide, were sufficient to bind to purified F-actin and cross-link the filaments. Our MLCK peptide with only one actin-binding motif was defective in assembling actin filaments, in agreement with their results (peptide containing residues 2–41 (24)). However, the peptide that contained two DFRXXL motifs cross-linked actin filaments but to a lesser extent compared with the peptide containing three DFRXXL motifs. The apparent differences in cross-linking may reflect differences in relative binding affinities of the motifs because of interactions of residues surrounding core DFRXXL motifs or because of differences in probability of cross-linking dependent on the distance in spacing between the first and second motif versus the first and third motif. However, it is clear that multiple DFRXXL motifs in short peptides are sufficient for actin cross-linking.

Both 3DFR-MLCK and 5DFR-MLCK bound prominently to actin stress fibers in A7r5 smooth muscle cells. In contrast, the expression of 5DFR-MLCK in HeLa cells had more prominent fluorescence associated with stress fibers compared with 3DFR-MLCK. Poperechnaya et al. (19) demonstrated that short MLCK did not bind prominently to actin stress fibers in HeLa cells, and there was diffuse fluorescence, indicating a cytoplasmic distribution. The differences in localization in different cell types may be because of the relative abundance of actin-containing stress fibers or some unidentified regulatory mechanism, such as phosphorylation (32) or complementary interactions with residues in the extended N terminus of long MLCK.

The long MLCK has a greater affinity for actin-containing filaments than the short MLCK (19, 22). Cell fractionation studies of transfected A7r5 cells and endogenously expressed kinase in A10 cells as well as MgCl₂ extraction of kinase from filaments in A10 cell lysates and lung tissue homogenates support this conclusion. Poperechnaya et al. (19) found in interphase cells that fusion of two additional DFRXXL motifs to short MLCK or fusion of three DFRXXL motifs to the N-terminal extension of the long MLCK fragment promoted binding to stress fibers compared with short MLCK or the N-terminal fragment alone. Additionally, Kudryashova et al. (22) found selective KCl extraction of short versus long MLCKs from detergent-washed cytoskeletal proteins, although the structural basis was not examined. Our results show that the 5DFR-MLCK fragment binds to stress fibers. However, a selective internal deletion of the five DFRXXL motifs in long MLCK did not eliminate binding, and the N-terminal extension of long MLCK without DFRXXL motifs also bound stress fibers. The short MLCK lacking DFRXXL motifs does not bind. Thus, long MLCK binding to stress fibers appears to be because of complementary binding of five DFRXXL motifs plus the N-terminal extension.

Cells in culture were treated with lysophosphatidic acid and...
latrunculin B to promote or disrupt, respectively, stress fiber formation. Lysophosphatidic acid acting through its G protein-coupled receptor promotes stress fiber formation via a Rhod mediately pathway with enhancement of myosin light chain phosphorylation by inhibition of myosin phosphatase (30). Latrunculin B disrupts actin polymerization by sequestering monomeric actin in living cells (31). Morphological and biochemical analyses of stress fiber formation in cells treated with these reagents revealed qualitatively expected results. In cells treated with lysophosphatidic acid, both short and long MLCK were bound to stress fibers, but the short form showed significant amounts of unbound kinase in contrast to the long MLCK. The apparent affinity of greater MLCK binding to filaments in vivo may be because of the two additional DFRXXL motifs plus the N-terminal extension, additional cooperative binding to other proteins such as c-actinin and p60cα (32), or both.

The biochemical results obtained after treating cells with latrunculin B were surprising considering the apparent disruption of stress fibers observed microscopically. Others have noted latrunculin B treatment results in disruption of stress fibers in cells in culture (33, 34). After low-speed centrifugation in cells treated with latrunculin B, actin shifted from being primarily in the pellet fraction to the supernatant fraction. After latrunculin B treatment, the relative distributions of either short or long MLCKs were not generally affected. One possibility is that significant amounts of actin remain in filaments for MLCK binding after latrunculin B treatment. However, these actin filaments are not organized in stress fibers in cells and are not attached to focal adhesions and other structures adhering to the coverglass after detergent treatment. When cells were made permeable with detergent, these actin filaments containing bound MLCKs may have been removed with washing. Because the molar ratio of MLCK to actin in filaments in cells is 1:100 or less (35, 36), disruption of most but not all actin filaments may not be sufficient to dissociate bound MLCKs. Additionally, MLCK may stabilize the actin filaments to which it is bound so they are resistant to depolymerization.

In cells in culture, short MLCK distributes between bound and unbound forms, and it is conceivable that both could phosphorylate myosin in thick filaments. The length of the kinase, 41 to 54 nm for the chicken and rabbit kinases, respectively (37), allows an extension of the catalytic core from the actin filament to the myosin thick filament by structural modules intervening between the actin-binding N terminus and the catalytic C terminus. Bound, nondiffusing short MLCK phosphorylates myosin light chain in a Ca2+/calmodulin-dependent manner in the detergent-insoluble cytoskeleton (18). The long MLCK would be extended similarly, because its larger size results from structural modules N-terminal of the five DFRXXL actin-binding motifs. Thus, bound forms of short and long MLCKs have structural modules placing them in a unique position for Ca2+/calmodulin-dependent signaling that leads to myosin II-based motile events.

In summary, these results show that the cellular distributions of short and long MLCKs are different. Whereas DFRXXL motifs are necessary and sufficient for short MLCK binding to actin containing filaments, both DFRXXL motifs and the N-terminal extension of long MLCK contribute to high affinity binding to actin-containing fibers in cells.

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