Identification of a Novel Actin Binding Motif in Smooth Muscle Myosin Light Chain Kinase*

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The activation of smooth muscle contraction is regulated by myosin light chain kinase, a Ca\(^{2+}\)/calmodulin-dependent enzyme (2). The initiation of contraction is triggered by an increase in intracellular Ca\(^{2+}\) which subsequently binds calmodulin. Ca\(^{2+}\)/calmodulin binds to an autoinhibitory regulatory segment in the C terminus, thereby producing an activated catalytic core. Activated myosin light chain kinase phosphorylates the 20-kDa regulatory light chain of myosin which allows myosin cross-bridges to bind to F-actin and produce cell force or shortening. Phosphorylation of myosin is also important in a number of non-muscle contractile processes (3–7).

The binding properties of smooth muscle myosin light chain kinase have been investigated in vitro, demonstrating that the kinase contains distinct binding sites for F-actin and myosin with \(K_d\) values of 4 and 0.8 \(\mu\)M, respectively (8). A peptide composed of amino acids 1–114 of smooth muscle myosin light chain kinase also binds to purified F-actin with a similar affinity (9). Moreover, cleavage of the N terminus of myosin light chain kinase results in the loss of binding. Subsequent studies suggested that regions responsible for binding to F-actin in vitro were contained in residues 1–41 (10, 11).

Although these biochemical studies provide information on the binding properties of smooth muscle myosin light chain kinase to F-actin, the binding affinities are low relative to the apparent high-affinity binding of myosin light chain kinase to detergent-washed myofilaments from gizzard smooth muscle (12), detergent-glycerol skinned smooth muscle tissue strips (13), or actin-containing stress fibers in permeable fibroblasts or smooth muscle cells in culture (1, 14). In all of these preparations, filaments were washed extensively with various aqueous buffers, and the kinase remained bound. Additionally, the C terminus of myosin light chain kinase containing the Ig-like telokin motif bound to myosin-containing filaments (15); however, this C-terminal motif is not necessary for high-affinity binding to detergent-washed myofilaments and actin-containing stress fibers in culture (1, 14). The apparent binding affinities of myosin light chain kinase for detergent-washed myofilaments or purified thin filaments from smooth muscle were greater than to purified F-actin or skeletal muscle myofilaments (14, 16). The reason for these differences in binding affinities to F-actin filaments is not clear at this time.

In this study we have analyzed high-affinity binding of myosin light chain kinase to myofilaments in vitro and actin-containing filaments in vivo by focusing on structural elements in the N terminus of myosin light chain kinase. Three consensus sequences were defined, and their importance for binding was determined in vitro and in vivo.

MATERIALS AND METHODS

Construction of Myosin Light Chain Kinase Mutants—PCR and Expanded High Fidelity System (Roche Molecular Biochemicals) were used to prepare full-length rabbit smooth muscle myosin light chain kinase with a C-terminal flag tag and N-terminal deletion mutants: Δ23-MLCK, Δ39-MLCK, and Δ59-MLCK. The sequences of the oligonucleotide primers corresponding to the coding strand of myosin light chain kinase used to amplify the full-length kinase and the N-terminal deletion mutants are: full-length myosin light chain kinase, 5’-GGGGTACCATGGATTTTCGCTCCGTGCTGGGTAGCA-3’; Δ23-MLCK, 5’-GGGGTACCATGGATTTTCGCTCCGTGCTGGGTAGCA-3’; Δ39-MLCK, 5’-GGGGTACCATGGATTTTCGCTCCGTGCTGGGTAGCA-3’; Δ59-MLCK, 5’-GGGGTACCATGGATTTTCGCTCCGTGCTGGGTAGCA-3’.

The abbreviations used are: PCR, polymerase chain reaction; ΔN23-MLCK, deletion of residues 2–22 in myosin light chain kinase; Δ39-MLCK, deletion of residues 2–38 in myosin light chain kinase; Δ59-MLCK, deletion of residues 2–55 in myosin light chain kinase; CMV, cytomegalovirus; GST, glutathione S-transferase; GFP, green fluorescence protein; MOPS, 3-(N-morpholino)propanesulfonic acid; DMEM, Dulbecco’s modified Eagle’s medium.

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The sequence of the 3′-primer which has the coding sequence for the FLAG tag is 5′-GATCATTCTGTCCTGCTGCTGTACCTCTTCC- 
TCTCCCTTCTCTTCTCCT-3′. The reactions were incubated at 94 °C for 5 min, followed by 30 cycles of denaturing, annealing, and extension (94 °C, 30 s; 60 °C, 30 s; and 68 °C, 2 min). A final incubation at 68 °C for 10 min completed the cycle. The PCR products were then subcloned into pGEX-4T-2 vector (Amerham Pharmacia Biotech) at BamHI and EcoRI sites. A peptide fragment containing residues 1–108 of rabbit smooth muscle myosin light chain kinase as well as the subsequent sequence which encodes a histidine tag was amplified using PCR and subcloned into TOPO vector cloning PCR. The PCR 3.1 expression vector (Invitrogen) under the control of the CMV promoter. The resulting sequences of the recombinant plasmids were obtained by DNA sequencing.

The GST fusion proteins were constructed by amplifying the N-terminal restriction sites (residues 1–30, 1–49, 1–55, 1–60, 1–75, 1–283, or 1–496) of smooth muscle myosin light chain kinase using PCR. The PCR products were then subcloned into pGEX-4T-2 vector (Amerham Pharmacia Biotech) at BamHI and EcoRI sites. A peptide fragment containing residues 1–108 of rabbit smooth muscle myosin light chain kinase as well as the subsequent sequence which encodes a histidine tag was amplified using PCR and subcloned into TOPO vector cloning PCR. The GST fusion proteins were constructed by amplifying the N-terminal restriction site. Primers 1 and 2 were used to amplify product 1, and primers 3 and 4 were used to amplify product 2. Purified products 1 and 2 were mixed, and primers 1 and 4 were added to this mixture to re-amplify the full-length sequence containing the desired mutation. Reactions were incubated at 94 °C for 5 min. PCR was performed for 35 cycles of denaturing, annealing, and extension (94 °C, 30 s; 63 °C, 30 s; and 72 °C, 2 min). The reaction mixtures were then incubated at 72 °C for 7 min. The final PCR products were digested with 7 min and then amplified at 37 °C for 45–72 h. The transfected cells were harvested and lysed at 4 °C in 1% Nonidet P-40, 10 mM MOPS, pH 7.5, 0.5 mM EGTA, 50 mM MnCl2, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/mL leupeptin, 10 μg/mL pepstatin, and 20 μg/mL aprotinin. The lysate was centrifuged at 17,320 × g for 2 min at 4 °C to remove the insoluble fraction. The COS-7 cell lysates were frozen in liquid nitrogen and stored at −80 °C. Western transfer and immunoblotsing was performed with a monoclonal antibody to smooth muscle myosin light chain kinase (does not bind to residues 1–75 of the kinase) or a monoclonal antibody against the FLAG sequence (Eastman Kodak) to detect protein expression.

The GST fusion proteins were obtained by subcloning the various myosin light chain kinase N-terminal sequences amplified by PCR into the GST expression vector pGEX-4T-2. Purification of the sequences was achieved by DNA automated sequencing. The recombinant plasmids were transfected into E. coli BL21(DE3) cells. Cells were grown to 0.6 A260 at 37 °C. Isopropyl-thio-β-galacto- 
topyranoside was added to a final concentration of 0.5 mM for induction of protein expression, and the cells were cultured for an additional 3 h at 37 °C. The cells were harvested by centrifugation at 6,000 g (JA-20 Beckman) for 15 min. The cells were resuspended in 5 mL of 1% phos- 
phate-buffered saline, 5 mM dithiothreitol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg/mL leupeptin, 20 μg/mL aprotinin, and 10 μg/mL pepstatin and then lysed by sonication at 4 °C. After centrifugation at 12,000 × g for 10 min at 4 °C, the supernatant frac- 
tions were filtered with a 0.45-μm filter (Nalgene), and GST fusion proteins were purified using glutathion-Sepharose 4B (Amerham Pharmacia Biotech) affinity column chromatography. Purified proteins were dialyzed against and stored at −80 °C in 50 mM NaCl, 10 mM MOPS, pH 7.0, 1 mM MgCl2, 1 mM dithiothreitol, and 10% glycerol.

In Vivo Transfection of Myosin Light Chain Kinase-GFP Con- structs—A7r5 rat thoracic aorta smooth muscle cells were transfected with the cDNA encoding for the myosin light chain kinase-GFP con- structs (1). For binding experiments in vitro, myosin light chain kinase constructs—FLAG tag is 5′-GATCATTCTGGCTGCTGCTGTACCTCTTCC- 
TCTCCCTTCTCTTCTCCT-3′. The reactions were incubated at 94 °C for 5 min, followed by 30 cycles of denaturing, annealing, and extension (94 °C, 30 s; 63 °C, 30 s; and 72 °C, 2 min). The reaction mixtures were then incubated at 72 °C for 7 min. The final PCR products were digested with 7 min and then amplified at 37 °C for 45–72 h. The transfected cells were harvested and lysed at 4 °C in 1% Nonidet P-40, 10 mM MOPS, pH 7.5, 0.5 mM EGTA, 50 mM MnCl2, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 μg/mL leupeptin, 20 μg/mL aprotinin, and 10 μg/mL pepstatin and then lysed by sonication at 4 °C. After centrifugation at 12,000 × g for 10 min at 4 °C, the supernatant frac- 
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tions were filtered with a 0.45-μm filter (Nalgene), and GST fusion proteins were purified using glutathion-Sepharose 4B (Amerham Pharmacia Biotech) affinity column chromatography. Purified proteins were dialyzed against and stored at −80 °C in 50 mM NaCl, 10 mM MOPS, pH 7.0, 1 mM MgCl2, 1 mM dithiothreitol, and 10% glycerol.

Cell Imaging—Fluorescence imaging was performed as described previously (2). Twelve view changes 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 was used for excitation and 520 nm for emission) and rhodamine (excitation at 550 nm and emission at 575 nm). During imaging, cells were maintained at 37 °C in an open thermal-controlled chamber (Custom Scientific, Dallas, TX).

Preparation of Smooth Muscle Myofilaments—Chicken gizzards (2 g, skinned and ground) were homogenized in 10 ml of 10 mM MOPS, pH 7.5, 50 mM NaCl, and 1 mM dithiothreitol with a polytron homogenizer (Brinkman Instruments). Homogenized tissues were centrifuged at 10,000 × g for 10 min at 4 °C. The pellets were homogenized in 10 ml of an extraction buffer consisting of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 50 mM MgCl2, and 3% Triton X-100. The homogenized suspensions were centrifuged again, and the deter- 
gent washing procedure was performed five times. The myofilaments were then washed three times in 10 mM MOPS, pH 7.5, 50 mM NaCl, and 1 mM dithiothreitol. The myofilaments were resuspended in 6 ml of the extraction buffer and stored at −80 °C.

In Vitro Co-ordinated Binding Assay—Purified myofilaments (0.2–3 mg/mL) were mixed with COS-7 cell lysates (diluted to give 30 nM recombinant protein) expressing either wild-type myosin light chain kinase or the various myosin light chain kinase mutant proteins in a final volume of 100 μl. Protein concentration was estimated using a standard curve of FLAG-tagged bacterial alkaline phosphatase (East- 
man Kodak). The binding buffer contained 50 mM NaCl, 10 mM MOPS,
The conditions for the binding are as described in Fig. 1 with details under “Materials and Methods.” P, pellet; S, supernatant fraction.

**Kinase to Smooth Muscle Myofilaments**—Truncations of residues 2–22 (∆N23-MLCK), 2–38 (∆N39-MLCK), and 2–57 (∆N58-MLCK) were analyzed for myofilament binding (Fig. 2). In this assay, supernatant and pellet fractions prepared in the absence of myofilaments were analyzed to show that full-length and truncated kinases were fully soluble (data not shown). Most of the full-length kinase cosedimented with the smooth muscle myofilaments. However, 50% of ∆N23-MLCK was in the supernatant fraction, similar to GST60-MLCK. Additional truncations in ∆N39-MLCK and ∆N58-MLCK resulted in no significant binding to the smooth muscle myofilaments. These results suggest multiple contributions for binding in residues 2–22 and 23–39.

GST75-MLCK Inhibits Myosin Light Chain Kinase Binding to Smooth Muscle Myofilaments—GST75-MLCK competes with full-length myosin light chain kinase for binding to myofilaments. GST75-MLCK dissociated the endogenous myosin light chain kinase from myofilaments in a concentration-dependent manner (Fig. 3). However, if myofilaments were first preincubated with different concentrations of GST75-MLCK followed by addition of the full-length myosin light chain kinase, the sensitivity for inhibition of association was substantially greater than the sensitivity for dissociation. These results are consistent with high-affinity binding properties with a slow rate of myosin light chain kinase dissociation from myofilaments.

**Identification of a Motif in Myosin Light Chain Kinase Required for Binding to Smooth Muscle Myofilaments**—A comparative sequence alignment of residues 1–75 of smooth muscle myosin light chain kinase among diverse vertebrate species shows that they are almost identical (Fig. 4). Results from deletion mutations in GST fusion proteins as well as sequential truncations of the N terminus of the kinase suggest a core binding sequence in residues 20–40 in the cosedimentation binding assay. Therefore, specific residues within this sequence were replaced with alanine to identify residues that may contribute to binding. Of 10 substitutions, only alanine at residues Asp-30, Phe-31, Arg-32, and Leu-35 produced a mutant GST75-MLCK with decreased binding affinity for myofilaments (Fig. 5). Interestingly, the sequence of 1–75 residues shows that there are three DFRXXL motifs, one located at the extreme N terminus (residues 2–7), a centrally located motif (residues 30–35), and a C-terminal motif (residues 58–63). The contributions of these three motifs to actin-filament binding were examined.

**Alanine Mutations in the DFRXXL Motifs Alter Binding in Full-length MLCK**—To confirm the importance of the central DFRXXL motif identified in GST75-MLCK and to learn if the remaining two DFRXXL motifs are involved in binding of the full-length kinase to actin-containing filaments in *vitro* and *in vivo*, additional mutations were performed in myosin light chain kinase. Myosin light chain kinase mutants in which either D2F3R4, D30F31R32, or D58F59R60 were replaced with alanine all decreased the apparent affinity of myosin light chain kinase for smooth muscle myofilaments (Fig. 6). The relative amounts found in the pellet fractions in the cosedimentation assay were 47, 57, and 34% for ∆AAA1-MLCK, ∆AAA32-MLCK, and ∆AAA40-MLCK, respectively, compared with ≥95% binding for wild-type kinase. These results verify the
In conclusion with the alanine point mutations in the centrally located DFRXXXL motif in GST75-MLCK and show additionally that D2F3R4 and D58F59R60 are also involved in myosin light chain kinase binding. Interestingly, the mutations did not have as great an effect in the full-length kinase compared with the GST-fusion protein. The reason for this difference was not determined but may be related to an effect of GST itself on binding.

To learn if the DFRXXXL motifs are important for binding in living cells, the cDNA encoding GFP was fused to the C terminus of full-length myosin light chain kinases containing the three triple alanine substitutions. Expression of the full-length, wild-type myosin light chain kinase with GFP in A7r5 cells showed fluorescence associated with stress fibers (Fig. 7) similar to previous results (1). The GFP fluorescence remained after permeabilization of the cells with the detergent saponin and was coincident with rhodamine-phalloidin stained F-actin in stress fibers. In contrast, the fluorescence of all three triple alanine myosin light chain kinase mutants, similar to ΔN myosin light chain kinase-GFP, a myosin light chain kinase lacking residues 2–142 (1), was distributed throughout the cytoplasm (Fig. 7). When these cells were made permeable via saponin treatment, the diffuse staining in the cytoplasm disappeared. These results indicate that the kinases containing triple alanine mutations in any one of the three DFRXXXL motifs were released from the cells without high-affinity binding to stress fibers (Fig. 7). These results are qualitatively consistent with the decreased binding of mutant myosin light chain kinases in the cosedimentation assay and indicate that all three DFRXXXL motifs are important for myosin light chain kinase binding in cells.

**DISCUSSION**

In this investigation we have identified a region in the N terminus of smooth muscle myosin light chain kinase that is central to its high-affinity binding to actin-containing filaments by analyzing the binding properties of a series of myosin light chain kinase mutants. The binding assays in vitro show that GST75-MLCK binds to the myofilaments with high affinity and competes with full-length kinase for myofilament binding. This construct contains the three DFRXXXL motifs as well as flanking residues. Disruption of one DFRXXXL motif (GST60-MLCK) reduces but does not eliminate binding. GST30-MLCK does not bind to the myofilaments, indicating that the subsequent deletion of the second DFRXXXL motif prevents binding. ΔN23-MLCK with deletion of the first DFRXXXL motif shows reduced binding compared with wild-type myosin light chain kinase in the cosedimentation assay with myofilaments. The subsequent deletion of a second motif (ΔN39-MLCK) eliminates binding, thus supporting the findings with GST fusion proteins that all three motifs are necessary for high-affinity binding.

*Fig. 3. Effect of GST75-myosin light chain kinase on myosin light chain kinase binding to smooth muscle myofilaments. Smooth muscle myofilaments extracted in the absence of 50 mM MgCl2 were incubated with various concentrations of purified GST75-myosin light chain kinase on ice for 1 h. After centrifugation, the pellet and supernatant fractions were analyzed for the endogenous smooth muscle myosin light chain kinase by Western blotting. Results for dissociation of endogenous myosin light chain kinase are shown by solid squares. Smooth muscle myofilaments in which the endogenous kinase was displaced with 50 mM MgCl2 were preincubated with different concentrations of GST75-myosin light chain kinase. Wild-type rabbit smooth muscle myosin light chain kinase (30 nM) with a C-terminal FLAG-tag was added to the reaction mixture. The pellet and supernatant fractions were obtained after centrifugation and analyzed by Western blot using a monoclonal antibody against the FLAG sequence. The percent myosin light chain kinase bound was calculated after densitometric scanning of immunoblotted bands.*

*Fig. 4. Sequence alignment of the N terminus of myosin light chain kinase among various vertebrate species. Conserved residues are highlighted in bold.*

*Fig. 5. Alanine scanning mutagenesis of GST75-MLCK. Alanine residues were individually substituted at various sites within residues 1–75 of GST75-MLCK. Expressed mutant fusion proteins analyzed for myofilament binding as described in Fig. 1. P, pellet; S, supernatant fraction.*

*Fig. 6. Effect of alanine mutations on the binding of full-length myosin light chain kinase to smooth muscle myofilaments. The myofilament cosedimentation binding assay for the triple alanine mutants in full-length myosin light chain kinase was performed as described under "Materials and Methods." P and S refer to pellet and supernatant fractions, respectively. WT, wild-type kinase; 2AAA, triple Ala mutations in residues 2–4; 30AAA, triple Ala mutations in residues 30–32; 58AAA60, triple Ala mutations in residues 58–60.*
binding. Removal of one motif at either end is sufficient to decrease binding, whereas elimination of two motifs eliminates binding to myofilaments in vitro. Consistent with results obtained with deletion mutagenesis, replacement of DFR with alanines in any one of the motifs of the full-length myosin light chain kinase was sufficient to decrease binding to myofilaments in the cosedimentation assay. Although the three DFR motifs were noted in sequence analysis of the N terminus of smooth muscle myosin light chain kinases from different animal species, their functional importance was not identified (10, 21, 22).

Although in vitro and in vivo assays demonstrate that the triple alanine mutants decrease high-affinity binding of myosin light chain kinase, it is not clear why these mutants show some binding to myofilaments in vitro, whereas there was no significant binding to actin-containing fibers. In cells it is possible that these differences may be because of the extraction procedure for preparation of myofilaments for the cosedimentation assay, temperature differences in the binding assays, etc. Kanoh et al., (1993) noted similarities in the structures of residues 1–114 of myosin light chain kinase to actin-binding proteins gizzard α-actinin and caldesmon. Greatest similarity was found with the N-terminal 134 residues of α-actinin with an identified actin binding site in residues 120–134 (9, 23). However, in searching for similarities related to the DFRXXL sequence, the chicken smooth muscle isoform of α-actinin has two motifs at the N terminus (residues 59–64) and C terminus (residues 771–776), respectively. The number of residues between the two DFRXXL motifs in α-actinin is substantially greater than the distances between respective motifs in smooth muscle myosin light chain kinase. The similarity of the N-terminal DFRXXL motif with its surrounding residues from α-actinin has a greater similarity to the second DFRXXL motif and 20 subsequent residues. The C-terminal motif in α-actinin more closely matches the first motif in myosin light chain kinase and the following 23 residues. Similarities are also noted for a DFRXXL motif in the N terminus of Ca2+/calmodulin-dependent protein kinase I and toward the C terminus of titin. However, a comparative search with three repeat sequences showed no homology to other proteins. Thus, they may represent a novel actin-binding motif in smooth muscle myosin light chain kinase. Interestingly, these motifs are absent in skeletal muscle myosin light chain kinases which do not show high-affinity binding to actin-containing filaments (2, 14, 24).

The unc motifs are repeat sequences exhibiting structural similarities to immunoglobulins and fibronectin and are specifically arranged around the catalytic core of smooth muscle myosin light chain kinase and twitchin kinase and were proposed to be involved in protein-protein interactions (2, 25, 26). However, they are not responsible for high-affinity binding to actin-containing filaments because mutations in the DFRXXL motifs are sufficient to inhibit binding in vitro and in vivo. Furthermore, deletion of residues 1–142 of myosin light chain kinase, while retaining all immunoglobulin-like and fibronectin-like motifs, is sufficient to eliminate binding. The DFRXXL sequences encompass the N terminus of smooth muscle myosin light chain kinase which is highly conserved and not found in skeletal muscle myosin light chain kinase (27–29). Skeletal muscle myosin light chain kinase does not bind to skeletal muscle (24) or smooth muscle myofilaments (14).

Several recent reports show smooth muscle myosin light chain kinase binding to purified F-actin and have defined the F-actin-binding site to residues 2–42 which would encompass the first two DFRXXL motifs (9–11). However, the apparent affinity of myosin light chain kinase to smooth muscle myofilaments is greater than to purified F-actin (14). An unresolved issue is whether these DFRXXL motifs are binding directly to F-actin in smooth muscle myofilaments or in actin-containing stress fibers in cells. It is conceivable that myosin light chain kinase may bind to another thin filament protein or, alternatively, its affinity for F-actin may be increased by associated thin filament proteins. Although this important issue needs additional investigation, the three DFRXXL motifs are important in localizing the kinase to actin-containing filaments in cells. It will be interesting to determine the intracellular dis-
distribution of myosin light chain kinase during dynamic changes in F-actin distribution associated with distinct cell processes.

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