Binding of Myosin Light Chain Kinase to Cellular Actin-Myosin Filaments*

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Pei-ju Lin, Katherine Luby-Phelps‡, and James T. Stull§

From the Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75235

Myosin light chain kinase binds to the actomyosin-containing filaments in smooth and nonmuscle cells. However, the region of the kinase necessary for this high affinity binding in vivo is not known, although it has been proposed that the N and C termini bind to actin and myosin in vitro, respectively. Truncated myosin light chain kinases containing the catalytic core and calmodulin-binding domain but lacking N (amino acids 1–655) and/or C (amino acids 1004–1147) termini were expressed in the baculovirus system and purified. All enzymes were catalytically active and Ca\(^{2+}\)/calmodulin-dependent. The C-terminal truncated myosin light chain kinase bound to detergent-washed smooth muscle contractile proteins similar to recombinant full-length myosin light chain kinase or enzyme purified from smooth muscle. The apparent affinity of the full-length kinase was greater for the actomyosin-containing filaments with associated proteins than for purified smooth muscle F-actin or actomyosin filaments from skeletal muscle. In contrast, truncations at the N terminus alone or at both N and C termini resulted in no significant binding. Similar effects were observed by two other assays: binding of fluorescently labeled myosin light chain kinases to actin-containing stress fibers in detergent-treated fibroblasts and localization of fluorescently labeled kinases after microinjection into primary smooth muscle cells in culture. The full-length and the C-terminal truncated myosin light chain kinases, but not myosin light chain kinases truncated at the N terminus or both N and C termini, associated with filaments in cells. Thus, the N terminus and not the C terminus of myosin light chain kinase is necessary for high affinity binding to actomyosin-containing filaments in smooth and nonmuscle cells.

Phosphorylation of myosin regulatory light chain by smooth muscle myosin light chain kinase, a Ca\(^{2+}\)/calmodulin-dependent protein kinase, is a key event initiating smooth muscle contraction and a variety of nonmuscle processes, including endothelial cell retraction (1–3), fibroblast contraction (4, 5), mast cell secretion (6), receptor capping in lymphocytes (7), and endothelial cell retraction (1–3), fibroblast contraction (4, 5), and fibronectin type III motifs, respectively). The C-terminal region of smooth muscle myosin light chain kinase, which is homologous to immunoglobulin and fibronectin (immunoglobulin C2 and fibronectin type III motifs, respectively). The C-terminal region of smooth muscle myosin light chain kinase, which is homologous to immunoglobulin C2 motif, is expressed as an independent protein, telokin (16, 17). Truncation of the C terminus (telokin) of smooth muscle myosin light chain kinase decreases its affinity for myosin but not actin (18). In addition, telokin is able to compete with purified myosin light chain kinase for binding to myosin. Thus, the primary contribution of the C-terminal region of smooth muscle myosin light chain kinase is binding to myosin in vitro (18). Limited proteolysis of smooth muscle myosin light chain kinase at the N terminus results in loss of F-actin-binding, and the purified N-terminal peptide (amino acids 1–114) has a similar actin binding affinity as the full-length myosin light chain kinase (19). Thus, the N-terminal region of smooth muscle myosin light chain kinase is important for F-actin binding in vitro. It is not clear which of these two regions is important for myosin light chain kinase binding in vivo.

Evidence indicates that smooth muscle myosin light chain kinase may be more tightly bound to contractile protein filaments than predicted from \(K_d\) values obtained with purified actin or myosin. In the purification of myosin light chain kinase from smooth muscle tissues actomyosin-containing filaments are washed extensively with detergent and low salt buffers before dissociating the kinase with 50 mM MgCl\(_2\) (20). Smooth muscle fibers made permeable with 1% Triton X-100 and 50%
glycerol for several weeks still retain contractile function in the presence of Ca\(^{2+}\)/calmodulin and Mg\(^{2+}\) ATP due to myosin regulatory light chain phosphorylation (21, 22). The concentration of smooth muscle myosin light chain kinase in these permeable muscle strips (3.2 μM) was similar to the concentration in intact muscle tissue (3.4 μM) (21). Thus, smooth muscle myosin light chain kinase binds to myofilaments with an apparent affinity greater than that predicted from the K\(_d\) values reported for myosin or actin. Localization studies by immunocytchemistry suggest high affinity binding to stress fibers in non-muscle cells (11, 12). Exogenously supplied myosin light chain kinase can bind to stress fibers in permeable fibroblasts extracted with Triton X-100 and high salt and restore Ca\(^{2+}\)/calmodulin-dependent contractility (4). As mentioned earlier, skeletal muscle myosin light chain kinase, which is known not to bind to the myofibrillar proteins (13), does not contain the N- and C-terminal regions present in smooth muscle and non-muscle myosin light chain kinases. It is not clear whether the N terminus, C terminus, or both are responsible for the high affinity binding of the kinase to myofilaments in smooth muscle cells or stress fibers in nonmuscle cells in vitro. In this study, we address this question by using purified, full-length, and N- and/or C-terminal truncated smooth muscle myosin light chain kinase proteins expressed in Sf9 insect cells.

EXPERIMENTAL PROCEDURES

Expression of Full-length and Truncated Smooth Muscle Myosin Light Chain Kinases in Baculovirus Expression System—Oligonucleotide primers were designed for syntheses of DNA fragments of truncated rabbit smooth muscle myosin light chain kinase designated as primers N1, N2, C1, and C2. The DNA fragments of truncations at the C terminus (ΔC), N terminus (ΔN), and both N and C termini (ΔNC) of smooth muscle myosin light chain kinases were synthesized by polymerase chain reaction using 20 units/μl Vent DNA polymerase (New England BioLabs). Reaction mixtures contained the cDNA fragment of the full-length myosin light chain kinase as a template and oligonucleotide primer pairs N1/C1, N2/C2, and N2/C1 for synthesis of ΔC, ΔN, and ΔNC myosin light chain kinases, respectively. After incubation at 94°C for 5 min, reactions proceeded by 35 cycles of denaturing, annealing, and extension (94°C, 1 min; 55°C, 2 min; and 72°C, 3 min). DNA fragments (ΔC, 3.0 kilobase pairs; ΔN, 1.5 kilobase pairs; and ΔNC, 1.1 kilobase pairs) from the polymerase chain reaction and the cDNA fragment of full-length myosin light chain kinase (3.6 kilobase pairs) were ligated by XbaI and BamHI into baculovirus expression vectors pVL1393 (Clontech) for the full-length and ΔNC myosin light chain kinases or transposition vector pFastBac1 (Life Technologies, Inc.) for ΔC and ΔN myosin light chain kinases. Procedures for myosin light chain kinase expression in baculovirus expression system were according to the manufacturer’s instructions (BacPAC\textsuperscript{TM} baculovirus expression system from Clontech and Bac-To-Bac\textsuperscript{TM} baculovirus expression system from Life Technologies, Inc.). In brief, recombinant baculoviruses of the full-length and ΔNC myosin light chain kinases were obtained by cotransfection of baculovirus genome DNA BacPAC6 (Clontech) and transfer vectors pVL1393 carrying DNAAs for the full-length and ΔNC myosin light chain kinases and then followed by baculovirus purification. Recombinant baculoviruses of ΔC and ΔN myosin light chain kinases were obtained by transposition of pFastBac1 carrying DNA fragments for ΔC and ΔN myosin light chain kinases into bacmid DNA and then followed by transfection. Sf9 cells (purchased from Life Technologies, Inc.) were grown in culture in Grace’s medium plus 10% fetal bovine serum or SF900 II serum-free medium (Life Technologies, Inc.) were grown in culture in Grace’s medium plus 10% fetal bovine serum or SF900 II serum-free medium (Life Technologies, Inc.). Sf9 cells in suspension culture were lysed on ice for 20 min in 30 mM MOPS at pH 7.0 (for full-length and ΔN myosin light chain kinases) or at pH 7.5 (for ΔC and ΔNC myosin light chain kinases), 30 mM (for full-length and ΔC myosin light chain kinases) or 5 mM (for ΔN and ΔNC myosin light chain kinases) MgCl\(_2\), 0.5 mM EGTA, 1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, and protease inhibitors (100 μg/ml phenylmethanesulfonyl fluoride, 20 μg/ml leupeptin, 30 μg/ml aprotinin, 60 μg/ml tosyllysyl chloromethyl ketone, and 60 μg/ml tosylphenylalanyl chloromethyl ketone). Cell lysates were clarified by centrifugation at 7,000 rpm (JA-10, Beckman) for 10 min and applied to a DEAE-Sephael column (Bio-Rad), which had been equilibrated in buffer A (20 mM MOPS at pH 7.0 (for full-length and ΔN myosin light chain kinases) or at pH 7.5 (for ΔC and ΔNC myosin light chain kinases), 30 mM EGTA, 10% glycerol, 1 mM dithiothreitol, and 100 mM MgCl\(_2\)/calmodulin-dependent contractility (4). Thus, smooth muscle myosin light chain kinase was performed at 4°C if not specified. Cell pellets harvested by centrifugation were lysed in buffer A by 30 min at 30°C. Myosin light chain kinase was freshly diluted in 10 mM MOPS, 10 mM MgCl\(_2\), 1 mM CaCl\(_2\), and 1 mM dithiothreitol at pH 7.0. Myosin light chain kinases were eluted by a buffer containing 30 mM MOPS, 10 mM MgCl\(_2\), 5 mM EGTA, and 1 mM dithiothreitol at pH 7.0 plus protease inhibitors. Purified myosin light chain kinases were dialyzed against 10 mM MOPS, 10 mM MgCl\(_2\) 10% glycerol, and 1 mM dithiothreitol at pH 7.0 containing protease inhibitors and stored at −80°C.

Myosin Light Chain Kinase Activity Assays—Ca\(^{2+}\)/calmodulin-dependent activity of myosin light chain kinase was measured by rates of \(^{32}\)P incorporation into regulatory light chain (27). Maximal activity was determined in the reaction containing 50 mM MOPS at pH 7.0, 10 mM magnesium acetate, 1 mM EGTA, 0.5 mM CaCl\(_2\), 1 mM ATP, 200–300 cpm/pmol, purchased from ICN), 1.2 μM calmodulin, 25 μM regulatory light chain, and diluted myosin light chain kinase at 30°C. Myosin light chain kinase was freshly diluted in 10 mM MOPS, pH 7.0, 1 mM dithiothreitol, and 1 mM mg bovine serum albumin and added to the reaction mixture. Final concentrations of myosin light chain kinase used in kinetic measurements showed linear phosphorylation activity and respect to enzyme concentrations. K\(_{cat}\) values (μM) and V\(_{max}\) values (pmol/min/μmol) were determined from Lineweaver-Burk double-reciprocal plots by varying concentrations of calcium and nonradiolabeled substrate, and the kinase activity was assayed.

Additional measurements used bacterially expressed human regulatory light chains. K\(_{cat}\) values represent the calmodulin concentration (nm) required for half-maximal activation and were determined from sigmoidal fit curves by varying calmodulin concentrations from 0.5 to 8 μM (28).

Extraction of Myofilaments from Smooth Muscle Tissues and Myofibrils from Skeletal Muscle—Skinned and ground turkey gizzard and rabbit skeletal muscle tissues (2 g) were homogenized in wash buffer (10 mM MOPS, 50 mM NaCl, and 1 mM dithiothreitol at pH 7.1) with a Polytron homogenizer (Brinkmann Instruments). Homogenized tissues were centrifuged at 12,500 rpm (JA-20, Beckman) for 10 min at 4°C. The pellet fractions (smooth and skeletal muscle myofilaments) were collected and homogenized in buffers without or with 50 mM MgCl\(_2\) (to remove endogenous myosin light chain kinase from the smooth muscle proteins (20)) as follows. Pellet fractions were homogenized in 12 ml of 10 mM Tris-Cl, 50 mM NaCl, 2 mM EGTA, 1 mM dithiothreitol, and 5% Triton X-100, with or without 50 mM MgCl\(_2\) at pH 7.4 with a Polytron homogenizer. The rehomogenized pellet fractions were centrifuged at 12,500 rpm (JA-20, Beckman) for 10 min at 4°C. After repeating this procedure eight times, myofilaments were washed in the wash buffer twice. The final washed myofilament pellets were resuspended in wash buffer without 50 mM NaCl and stored at 0°C.

Binding Assay in Vitro—Binding of myosin light chain kinases to...
actin filaments in vitro was measured by a co-sedimentation procedure according to Sellers and Pato (14). Binding of myosin light chain kinases to smooth muscle myofilaments in vitro was also measured by co-sedimentation according to Sellers and Pato (14) but with some modifications. The binding reaction (50 μl) contained myofilament protein, 50 μM or 200 μM purified myosin light chain kinases in 10 mM MOPS at pH 7.0, 50 mM NaCl, 2 mM dithiothreitol, 1 mg/ml bovine serum albumin, 1 mM MgCl₂ in the presence of either EGTA or Ca²⁺/calmodulin (concentration varied, see details in figure legends). After incubation at room temperature for 10 min, samples were centrifuged at 15,000 × g for 20 min. The supernatant fractions were removed, and the pellet fractions were washed with wash buffer once and either resuspended in 50 μl of SDS-PAGE sample buffer for immunoblot analysis or extracted with 50 μl of MgCl₂-containing buffer (10 mM Tris-Cl at pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, and 50 μM MgCl₂) followed by centrifugation at 15,000 × g for 20 min, from which the extracted fractions were subsequently assayed for myosin light chain kinase activity. The amounts of myosin light chain kinase in the supernatant and pellet fractions were compared by measurements of the activity or by quantitative immunoblotting.

Cy3 Labeling of Smooth Muscle Myosin Light Chain Kinases—Cy3-OSu (Amersham Life Sciences) was dissolved in dry dimethyl formamide (29). The concentrations of dye in the stock solution and Cy3-labeled proteins were calculated by dividing the concentration of Cy3 by that of the protein. Cyanine 3 dye. After dialysis, Cy3-labeled myosin light chain kinases were resuspended in 50 mM Tris-Cl at pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, and 50 mM MgCl₂ containing 1 mg/ml bovine serum albumin. The supernatant fractions were compared by measurements of the activity or by quantitative immunoblotting.

Cy3 Labeling of Smooth Muscle Myosin Light Chain Kinases—Cy3-OSu (Amersham Life Sciences) was dissolved in dry dimethyl formamide (29). The concentrations of dye in the stock solution and Cy3-labeled protein samples were determined by measuring the absorbance of the dye solution in phosphate-buffered saline at A₅₀₀ (A₅₀₀ value for concentration of Cy3 (μl × length (cm) × e₈₀₀ (15,000 × g)) (29)). Labeling reactions contained 200 μM MOPS at pH 8.0, 30 mM magnesium acetate, 100 mM NaCl, 1 mM ATP, 1–4 mg/ml myosin light chain kinase, and Cy3 (molar concentration 10–15-fold greater than myosin light chain kinase). After incubation at room temperature for 20 min, reactions were centrifuged at 15,000 × g for 10 min at 4 °C to remove any aggregated protein. The supernatant fractions were subsequently dialyzed by Slide-A-Lyzer™ dialysis cassettes (Pierce) overnight at 4 °C against injection buffer (10 mM MOPS, 30 mM magnesium acetate, 100 mM NaCl, and 1% sucrose at pH 7.1) to remove free Cy3 dye. After dialysis, Cy3-labeled myosin light chain kinases were concentrated to 2–8 mg/ml by Aqueous probe (Calbiochem), centrifuged at 15,000 × g for 10 min, and stored at −80 °C. The ratio of Cy3 to protein was calculated by dividing the concentration of Cy3 by that of myosin light chain kinase for Cy3-labeled myosin light chain kinase. Cy3-labeled myosin light chain kinases were compared with unlabeled myosin light chain kinases by SDS-PAGE and kinase activities.

Perfusion of Permeable Fibroblasts with Cy3-labeled Myosin Light Chain Kinases—Fibroblasts were made permeable with Triton X-100 according to a modification of a low salt-extracted cell model (4). Swiss 3T3 fibroblasts were seeded onto 22-mm square coverslips in 35-mm Petri dishes. Cells were cooled rapidly from 37 to 4°C, washed briefly with ice-cold phosphate-buffered saline, and extracted in ice-cold 10 mM Tris-Cl, 60 mM KCl, 125 mM sucrose, and 0.05% Triton X-100 at pH 7.0 for 10 min at 4 °C. Cells were then briefly washed three times in ice-cold wash buffer (10 mM Tris, 30 mM KCl, 5 mM MgCl₂, 1 μM CaCl₂, at pH 7.0) and incubated with 100 μl of a mixture of fluorescein phalloidin (1500-unit stock, Molecular Probes) and 3 μl Cy3-labeled myosin light chain kinase in wash buffer containing 1 mg/ml bovine serum albumin. After incubation at room temperature for 2 min, cells were briefly washed three times in wash buffer to remove the unbound fluorescein probes and subsequently imaged by fluorescence microscopy.

Preparation of Primary Bovine Tracheal Smooth Muscle Cells in Culture and Microinjection—Bovine tracheal smooth muscle cells were prepared as described previously (30). Isolated smooth muscle cells were either frozen in liquid nitrogen with 10% dimethyl sulfoxide or maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Thawed or freshly isolated primary cells were passed at confluence by trypsin-EDTA (0.05% and 0.53 mM, respectively) and seeded onto 40-mm round coverslips (Fisher, 40 Circles-1D) in 60-mm Petri dishes. Cells were used after first passage and within 24–48 hr after reaching 80–90% confluence and subsequently serum-deprived for 48 h. Cultured long and spindle-shaped smooth muscle cells were chosen for microinjection. Cy3-labeled myosin light chain kinase (2–8 mg/ml) was mixed with fluorescein-labeled 10-kDa dextran (2–4 mg/ml, Molecular Probes) and injected buffer (10 mM MOPS, 30 mM NaCl, and 1% sucrose at pH 7.1) containing 10 mg/ml bovine serum albumin and clarified using an Airfuge (Beckman) at 24 p.s.i. for 15 min to remove any aggregated protein. Microinjection needles were made as follows: capillary glass was siliconized with Sigma (Sigma) by capillarity, air-dried by nitrogen gas, and pulled into needles by a vertical pitpole puller (David Kopf Instruments). The clarified fluorescein sample was loaded directly into the tip of a needle by a thin, elongated glass pipette. To remove air bubbles in the tip of the needle before microinjection, the tip needle was severed. Microinjection was performed as described previously (21), and culture dishes were kept at 37 °C in a thermal chamber during microinjection. Before fluorescence imaging, microinjected cells were incubated at 37 °C for at least 2 h to allow cells to provide time for equilibration of the microinjected protein. Coverslips with microinjected cells were sealed in a temperature-controlled chamber at 37 °C for fluorescence imaging.

Fluorescence Imaging—Fluorescence imaging was performed as described previously (31). Fourteen-bit fluorescence images were acquired by a cooled CCD camera (Photometrics CCD200, Tucson, AZ) and BIDS-Image software (Oncor, Gaithersburg, MD). Narrow band pass interference filters (Omega) were used to select either fluorescein (excitation at 490 nm and emission at 520 nm) or Cy3 (excitation at 550 nm and emission at 575 nm) fluorescence.

RESULTS

Properties of the Full-length and Truncated Smooth Muscle Myosin Light Chain Kinases Purified from SF9 Cells—To define the domain of smooth muscle myosin light chain kinase responsible for the high affinity binding to contractile proteins in cells, we constructed full-length rabbit smooth muscle myosin light chain kinase (32) and truncations at the C-terminal amino acid 1004–1147 (ΔC), the N-terminal amino acids 2–655 (ΔN), and both the N and C termini (ΔNC) (Fig. 1). Truncated myosin light chain kinases contain the kinase catalytic core and calmodulin-binding sequence but lack C and/or N termini, which are proposed as the binding sites for myosin and actin, respectively (18, 19). Purified kinases appeared as one band on SDS-PAGE (see Fig. 4, A–D, lane 1, left panel). Kinetic properties of purified full-length and truncated myosin light chain kinases were determined (Table I). Full-length myosin light chain kinase purified from infected SF9 cells had similar kinetic properties compared with the parent kinase purified from chicken gizzard (10) or expressed in COS cells (33). ΔC myosin light chain kinase had similar Kₘ, Vₘₐₓ, and Kᵥₐₘ values compared with the full-length myosin light chain kinase (Table I), indicating that the deletion at the C terminus of smooth muscle myosin light chain kinase had no significant effect on kinase activation or catalytic properties. Deletion at the N terminus or both N and C termini increased Kᵥₐₘ and Kᵥₐ₈ values. ΔN myosin light chain kinase (deleted at the N-terminal 2–655 amino acids) had a similar Kᵥₐₘ value compared with a mutant construct SM
Table I

| MLCK    | $K_m$ (μM) | $V_{max}$ (pmol/min/pmol) | $K_{CM}$ (nM) |
|---------|------------|---------------------------|---------------|
| FL      | 7.9 ± 1.8  | 2947 ± 161                | 0.7 ± 0.2     |
| ΔC      | 10.2 ± 2.6 | 2991 ± 683                | 1.4 ± 0.5     |
| ΔN      | 43.6 ± 11.7| 2357 ± 445                | 27.7 ± 6.1*   |
| ΔNC     | 58.5 ± 12.9| 1900 ± 383*               | 8.6 ± 0.7*    |

*a p < 0.05 by Student's t test compared with the values of full-length smooth muscle myosin light chain kinase.

Δ2–653 expressed in COS cells (41 ± 4 μM) in previous studies (33). The increases of $K_{CM}$ values for both ΔN and ΔNC myosin light chain kinases were unexpected, because the deleted N terminus is separated from the calmodulin-binding sequence by the catalytic core (Fig. 1). Further investigations are needed to identify the reason for the increased $K_{CM}$ values. Although there are changes in some of the kinetic properties for ΔN and ΔNC myosin light chain kinases, all myosin light chain kinases purified from recombinant baculovirus-infected SF9 cells showed Ca$^{2+}$/calmodulin-dependent activity and were not active in the presence of EGTA (data not shown).

Properties of Extracted Smooth Muscle Myofilaments—Smooth muscle myofilament proteins with or without MgCl$_2$ pretreatment are shown in Fig. 2A. Both smooth muscle myofilaments (lanes 2 and 3) had the same myosin:actin molar ratio (1.6:8) and a similar tropomyosin:actin molar ratio (1.6:5 and 1.7:2 for myofilaments without and with MgCl$_2$ pretreatment, respectively). The Coomassie Blue-stained patterns for smooth muscle myofilament proteins are similar with or without MgCl$_2$ pretreatment (Fig. 2A). Purified smooth muscle F-actin is also shown on SDS-PAGE (Fig. 2A, lane 1). Although telokin is an abundant protein in gizzard smooth muscle tissue as shown by SDS-PAGE analysis, little was present in washed myofilaments, and it decreased further with MgCl$_2$ treatment (Fig. 2B). In addition, the amounts of caldesmon in both smooth muscle myofilaments (with or without MgCl$_2$ pretreatment) were similar as measured by immunoblotting with an anti-caldesmon antibody (data not shown). In a previous report, 50 μM MgCl$_2$ was used to release myosin light chain kinase from smooth muscle myofilaments (20). Consistent with this observation, we found that smooth muscle myofilaments pretreated with 50 μM MgCl$_2$, unlike myofilaments without MgCl$_2$ pretreatment, did not have significant endogenous myosin light chain kinase as analyzed by kinase activity assays and immunoblots (data not shown).

Effect of MgCl$_2$ Pretreatment and Ca$^{2+}$/Calmodulin on the Binding of Full-length Smooth Muscle Myosin Light Chain Kinases to Smooth Muscle Myofilaments—The binding of full-length myosin light chain kinase to smooth muscle myofilaments was measured by a cosedimentation assay. Recombinant full-length myosin light chain kinase bound to smooth muscle myofilaments (96 ± 2% and 80 ± 5% bound, respectively) either with or without MgCl$_2$ pretreatment (Fig. 3A). The bound fraction was slightly less with smooth muscle myofilaments lacking MgCl$_2$ pretreatment compared with treated smooth muscle myofilaments. These data indicate that full-length smooth muscle myosin light chain kinase added to smooth muscle myofilaments binds either in the presence or absence of endogenous myosin light chain kinase and MgCl$_2$-extractable proteins. Additionally, smooth muscle myosin light chain kinase bound to smooth muscle myofilaments in the presence of EGTA (94 ± 5%). In the presence of Ca$^{2+}$/calmodulin, the extent of binding was decreased 22% (Fig. 3B). The binding of the exogenously added kinase showed properties similar to the endogenous kinase. It remained bound with washing in low MgCl$_2$-containing buffer and was extracted with 50 mM MgCl$_2$ (data not shown).

Binding of Myosin Light Chain Kinases to Smooth Muscle Myofilaments, Skeletal Muscle Myofibrils, and Smooth Muscle F-actin in Vitro—The binding of full-length and truncated smooth muscle myosin light chain kinases to smooth muscle myofilaments in vitro was measured by co-sedimentation assay (Fig. 4A). The fractions bound to smooth muscle myofilaments for the purified full-length myosin light chain kinase, ΔC myosin light chain kinase, ΔN myosin light chain kinase, and ΔNC myosin light chain kinase were 74 ± 2%, 80 ± 3%, 13 ± 6%, and 6 ± 2%, respectively (Fig. 4A). Thus, truncations at the N terminus alone or both N and C termini of smooth muscle myosin light chain kinase resulted in the loss of myosin light chain kinase binding to smooth muscle myofilaments in vitro. However, truncation at the C terminus did not affect binding to smooth muscle myofilaments. Not surprisingly, skeletal muscle myosin light chain kinase did not bind to smooth muscle myofilaments (Fig. 4A), consistent with previous results indicating that this myosin light chain kinase does not bind to skeletal muscle myofibrils (13).

An N-terminal peptide containing 1–114 amino acids of smooth muscle myosin light chain kinase has been proposed as an actin-binding site (19). To see whether this accounts for the binding to myofilaments, we compared the binding properties of full-length smooth muscle myosin light chain kinase with...
FIG. 3. Effect of MgCl₂-pretreatment and Ca²⁺/calmodulin on the binding of full-length myosin light chain kinases to smooth muscle myofilaments. A, MgCl₂ pretreatment. Smooth muscle myofilaments extracted from gizzard tissues were washed with 1% Triton X-100 followed by incubation in 50 mM MgCl₂ buffer to release endogenous myosin light chain kinase (20). Smooth muscle myofilaments (4 mg/ml) without (open bar) and with (closed bar) MgCl₂ pretreatment were incubated in 10 mM MOPS, 50 mM NaCl, 0.1 mM EGTA, 2 mM dithiothreitol, 1 mM MgCl₂, 1 mg/ml bovine serum albumin, and 0.2 μM myosin light chain kinase at pH 7.0. The amount of myosin light chain kinase bound was determined as described for B. B, effect of Ca²⁺/calmodulin binding of myosin light chain kinase to myofilaments pretreated with 50 mM MgCl₂. Binding reactions were same as in A, except that either 1 mM EGTA (Ca²⁺/CaM, open bar) or 0.2 mM CaCl₂ and 0.4 μM calmodulin (+Ca²⁺/CaM, closed bar) were preincubated with myosin light chain kinases at room temperature for 5 min before adding myofilaments. Binding of myosin light chain kinases to myofilaments in vitro was measured by co-sedimentation. After incubation at room temperature for 10 min, the mixture was centrifuged at 15,000 × g for 20 min to obtain the myofilament pellet. The supernatant fractions were assayed by SDS-PAGE followed by immunoblotting with an antibody against the smooth muscle myosin light chain kinase catalytic core. Control reactions without myofilaments, which contained the same concentration (0.2 μM) of myosin light chain kinase, were included in each experiment for determination of the total amount of kinase. The amount of full-length myosin light chain kinase was quantitated by a densitometric scanner (Molecular Dynamics). The values of the fraction bound (percentage) were calculated by dividing the relative intensity from the fractions. The data represent the means ± S.D. for eight (A) or six (B) samples in three individual experiments.

Myosin Light Chain Kinase Binding

FIG. 4. Binding of myosin light chain kinases to smooth muscle myofilaments, skeletal muscle myofilaments, and F-actin in vitro. A, binding of myosin light chain kinases to smooth muscle myofilaments. Binding of full-length and truncated myosin light chain kinases to smooth muscle myofilaments in vitro was measured by a co-sedimentation procedure described in the legend of Fig. 2. Binding reactions contained 10 mM MOPS, 50 mM NaCl, 0.1 mM EGTA, 2 mM dithiothreitol, 1 mM MgCl₂, 1 mg/ml bovine serum albumin, 40 mM myosin light chain kinase, and 0.7 mg/ml MgCl₂-pretreated smooth muscle myofila-
ments at pH 7.0. The MgCl₂-pretreated smooth muscle myofilaments had no significant endogenous myosin light chain kinase activity. After incubation at room temperature for 10 min, the reaction mixtures were centrifuged at 15,000 × g for 20 min. The supernatant and pellet fractions were assayed for myosin light chain kinase activity. The values of the fraction bound (percentages) were calculated by dividing the amount of myosin light chain kinase activity in the pellet fraction by the amount in the supernatant plus pellet fractions. Skeletal muscle myosin light chain kinase (SK), which does not bind to myofilaments (13), was also included as a negative control. FL, full-length smooth muscle myosin light chain kinase. B, comparison of binding for full-length smooth muscle myosin light chain kinase to smooth muscle myofilaments (closed circles), purified smooth muscle F-actin (open circles), and skeletal muscle myofilaments (closed triangle). The concentra-
tions of actin in smooth muscle myofilaments and skeletal muscle myofilaments were estimated by densitometric scanning after SDS-PAGE. Binding reactions to smooth and skeletal muscle myofilaments, as well as to purified smooth muscle F-actin, were the same as described in Fig. 3A, except that the concentrations of smooth muscle myofilaments and smooth muscle F-actin were varied, and the centrifugation for purified smooth muscle F-actin filaments was performed by an Airfuge at 30 p.s.i. for 30 min. Control reactions without the myofilaments or actin filaments, which contained the same concentration (40 mM) of myosin light chain kinase, were included in each experiment for determination of the total amount of kinase. The data represent means ± S.E. for three individual experiments.
other binding protein needs to be investigated.

Fluorescently Labeled Smooth Muscle Myosin Light Chain Kinases—Purified myosin light chain kinases were fluorescently labeled with Cy3 to measure binding of the truncated smooth muscle myosin light chain kinases to cytoskeletal filaments in permeable and living cells. The unlabeled and Cy3-labeled full-length myosin light chain kinase, ΔC myosin light chain kinase, ΔN myosin light chain kinase, and ΔNC myosin light chain kinase were analyzed by 10% SDS-PAGE (Fig. 5). The proteins were stained by Coomassie Blue or exposed for fluorescence under UV light, respectively. Unlike Cy3-labeled myosin light chain kinases after dialysis, Cy3-labeled myosin light chain kinases after dialysis had no significant free fluorescent dye, which appeared at the bottom of the gels in lanes 2 (Fig. 5, right panels). The molar ratio for incorporation of Cy3 into myosin light chain kinase ranged from 1.5 to 2.5 under optimal conditions. The Ca\(^{2+}\)/calmodulin-dependent kinase activities of the fluorescent derivatives were 70–100% compared with unlabeled kinases (data not shown). In addition, Cy3-labeled full-length myosin light chain kinase binds similarly to smooth muscle myofilaments in vitro as the unlabeled kinase (data not shown). Thus, the labeled kinases provide suitable probes to examine association and localization in cells.

Association of Myosin Light Chain Kinases to Cytoskeleton in Triton X-100-solubilized Fibroblasts—To test whether truncated smooth muscle myosin light chain kinases associated with cytoskeletal filaments, fibroblasts made permeable with Triton X-100 were perfused with a mixture of Cy3-labeled myosin light chain kinases and fluorescein-labeled phalloidin. The latter binds to actin filaments, thereby providing a marker for the actin-containing cytoskeleton. Fig. 6 shows the localization of actin-containing stress fibers (left panels, a–d) and Cy3-myosin light chain kinase in the same cell (right panels, e–h). Cy3-labeled full-length and ΔC myosin light chain kinases were associated with the actin-containing cytoskeleton in these permeable cells. In contrast, Cy3-labeled ΔN or ΔNC myosin light chain kinases showed weak fluorescent signals in the nucleus and no significant binding to the actin-containing cytoskeleton. These results are consistent with binding of myosin light chain kinase to smooth muscle myofilaments in vitro and demonstrate that truncation at the N terminus, not the C terminus, significantly affects myosin light chain kinase binding.

Association of Myosin Light Chain Kinases to Filaments in Living Smooth Muscle Cells—To further verify the region of smooth muscle myosin light chain kinase responsible for binding to myofilaments in living cells, Cy3-labeled kinases were co-microinjected with 10-kDa fluorescein-labeled dextran into smooth muscle cells in primary culture (Fig. 7). The 10-kDa fluorescein-labeled dextran distributes diffusely in the nucleus and cytoplasm. Microinjected Cy3-labeled full-length and ΔC myosin light chain kinases were localized to the myofilament bundles and cytoplasm but were excluded from the nucleus. On the other hand, microinjected Cy3-labeled ΔN or ΔNC kinases were distributed evenly in the cytoplasm and nucleus with no significant localization on filaments. This evidence further establishes that the N terminus, not the C terminus, of smooth muscle myosin light chain kinase is responsible for the binding to the contractile apparatus in cells.

DISCUSSION

The region of smooth muscle myosin light chain kinase required for the binding to the contractile apparatus in cells was identified in this study. A co-sedimentation binding assay showed that purified smooth muscle myosin light chain kinase truncated at the C terminus was capable of binding to myofilament proteins from gizzard smooth muscle, to the actin-containing cytoskeleton in fibroblasts and to myofilament bundles in living smooth muscle cells. In contrast, myosin light chain kinase truncated at the N terminus (ΔN or ΔNC myosin light chain kinases) showed no significant binding. Thus, results from three different approaches in vitro and in vivo demonstrate that the N terminus alone, and not the C terminus of smooth muscle myosin light chain kinase, is required for binding to filaments in cells.

Ca\(^{2+}\)/calmodulin decreased the amount of smooth muscle myosin light chain kinase bound to smooth muscle myofilaments in vitro. This result is similar with previous reports on myosin light chain kinase binding to myosin or actin. The apparent Kₐ values of smooth muscle myosin light chain kinase in the presence of Ca\(^{2+}\)/calmodulin for myosin (2.4 μM) and actin (14 μM) are 5-fold lower than those in the absence of Ca\(^{2+}\)/calmodulin (0.8 μM and 4 μM for myosin and actin, respectively) (14). In addition, actin binding of smooth muscle myosin light chain kinase causes the assembly of actin filaments into thick bundles in vitro; the bundling ability of myosin light chain kinase is inhibited in the presence of Ca\(^{2+}\)/calmodulin (34). Interestingly, the actin-binding sequence of smooth muscle myosin light chain kinase is at the N terminus, which is removed from the calmodulin-binding sequence at the C terminus of the catalytic core. It is not clear why Ca\(^{2+}\)/calmodulin has an inhibitory effect on smooth muscle myosin light chain kinase binding to filaments. It has been speculated that smooth muscle myosin light chain kinase might associate and dissociate with thin and thick filaments alternatively during a contraction cycle (19).
The contractile filaments in cells consist of actin and myosin with their associated proteins. Previous studies showed that the N and C termini of smooth muscle myosin light chain kinase were responsible for binding of myosin light chain kinase to purified actin and myosin in vitro, respectively (18, 19), suggesting that the N or C terminus or both might be sufficient for myosin light chain kinase binding to filaments in cells. However, our data indicate that the N terminus, but not the C terminus, is necessary for association and localization to filaments in living cells, permeable cells, and gizzard actin-myosin containing filaments. This conclusion is distinct from an earlier report on the possible importance of the C terminus of smooth muscle myosin light chain kinase for binding to myosin (18). One possible explanation is that myosin-binding proteins might compete with myosin light chain kinase for binding to myosin filaments in these more complex systems used herein. For instance, telokin, an independent protein that is identical to the C terminus of smooth muscle myosin light chain kinase, may associate with unphosphorylated myosin (18). However, significant amounts of telokin were not present in the gizzard myofilaments used to characterize the binding. Additionally, telokin is not present in fibroblasts (35). Although the myosin binding affinity for telokin is similar to that for smooth muscle myosin light chain kinase, the concentration of telokin (80–90 μM) in some smooth muscle cells is much higher than that of myosin light chain kinase (3–4 μM) (18, 21). It appears that the measured binding affinity of myosin light chain kinase for myosin may be much lower than the affinity of the kinase for cellular filaments.

The binding affinity of the full-length smooth muscle myosin light chain kinase to smooth muscle myofilaments is greater than the binding affinity to purified smooth muscle F-actin filaments and skeletal muscle myofibrils. Therefore, it seems unlikely that myosin light chain kinase binds more strongly to smooth muscle F-actin than to skeletal muscle F-actin. A possible explanation is that another protein in the smooth muscle and nonmuscle contractile filaments is involved in binding smooth muscle myosin light chain kinase, i.e., the kinase is not simply binding to F-actin. This protein(s) may act to target smooth muscle myosin light chain kinase to myofilaments.

Anchoring proteins serve to translocate protein kinases to specific subcellular locations in response to extracellular stimuli and presumably localize the target enzymes close to their physiological substrates (36, 37). For example, several A kinase anchoring proteins associate with type II regulatory domain of cAMP-dependent protein kinase and translocate it to the cell cytoskeleton, where the kinase substrates are localized. Another example for targeting enzymes to a specific site is the trimeric phosphatase PP-1M (smooth muscle protein phosphatase) (38, 39), also referred to as MBP (myosin-bound phospho-
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FIG. 7. Fluorescent images after microinjection with Cy3-labeled myosin light chain kinases into living smooth muscle cells. Cy3-labeled myosin light chain kinases were co-microinjected with a fluorescein-labeled 10-kDa dextran into primary bovine tracheal smooth muscle cells in culture at 37 °C. Cells were imaged by fluorescence microscopy. Left panels (a–d), F-Dextran, fluorescein-labeled 10-kDa dextran. Right panels (e–h), Cy3-MLCK, Cy3-labeled smooth muscle myosin light chain kinase; e, full-length myosin light chain kinase; f, ΔN myosin light chain kinase; g, ΔNC myosin light chain kinase. The fibers labeled with kinases are identified with arrowheads in panels e and f.

tase) (40). Two regulatory subunits act as anchoring proteins to localize the catalytic subunit to the smooth muscle myofilaments (41). Additional possibilities may also be considered, and investigations are needed to identify the mechanism involved in myosin light chain kinase localized to myofilaments via its N-terminal sequence.

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